

Laboratory techniques in rabies

Fifth edition
Volume 1

Edited by

Charles E. Rupprecht
LYSSA LLC
Atlanta, Georgia, USA

Anthony R. Fooks
Animal and Plant Health Agency
Addlestone, Surrey, United Kingdom

Bernadette Abela-Ridder
Department of Control of Neglected
Tropical Diseases
World Health Organization
Geneva, Switzerland



World Health
Organization

Laboratory techniques in rabies

Fifth edition

Volume 1

Edited by

Charles E. Rupprecht

LYSSA LLC

Atlanta, Georgia, USA

Anthony R. Fooks

Animal and Plant Health Agency

Addlestone, Surrey, United Kingdom

Bernadette Abela-Ridder

Department of Control of Neglected Tropical Diseases

World Health Organization

Geneva, Switzerland



Laboratory techniques in rabies, fifth edition. Volume 1/Charles E Rupprecht, Anthony R Fooks, Bernadette Abela-Ridder, editors.

ISBN 978-92-4-151515-3

© World Health Organization 2018

Some rights reserved. This work is available under the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 IGO licence (CC BY-NC-SA 3.0 IGO; <https://creativecommons.org/licenses/by-nc-sa/3.0/igo>).

Under the terms of this licence, you may copy, redistribute and adapt the work for non-commercial purposes, provided the work is appropriately cited, as indicated below. In any use of this work, there should be no suggestion that WHO endorses any specific organization, products or services. The use of the WHO logo is not permitted. If you adapt the work, then you must license your work under the same or equivalent Creative Commons licence. If you create a translation of this work, you should add the following disclaimer along with the suggested citation: "This translation was not created by the World Health Organization (WHO). WHO is not responsible for the content or accuracy of this translation. The original English edition shall be the binding and authentic edition".

Any mediation relating to disputes arising under the licence shall be conducted in accordance with the mediation rules of the World Intellectual Property Organization.

Suggested citation. Rupprecht CE, Fooks AR, Abela-Ridder B, editors. Laboratory techniques in rabies, fifth edition. Volume 1. Geneva: World Health Organization; 2018. Licence: [CC BY-NC-SA 3.0 IGO](https://creativecommons.org/licenses/by-nc-sa/3.0/igo).

Cataloguing-in-Publication (CIP) data. CIP data are available at <http://apps.who.int/iris>.

Sales, rights and licensing. To purchase WHO publications, see <http://apps.who.int/bookorders>. To submit requests for commercial use and queries on rights and licensing, see <http://www.who.int/about/licensing>.

Third-party materials. If you wish to reuse material from this work that is attributed to a third party, such as tables, figures or images, it is your responsibility to determine whether permission is needed for that reuse and to obtain permission from the copyright holder. The risk of claims resulting from infringement of any third-party-owned component in the work rests solely with the user.

General disclaimers. The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of WHO concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted and dashed lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by WHO in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

All reasonable precautions have been taken by WHO to verify the information contained in this publication. However, the published material is being distributed without warranty of any kind, either expressed or implied. The responsibility for the interpretation and use of the material lies with the reader. In no event shall WHO be liable for damages arising from its use.

The named editors alone are responsible for the views expressed in this publication.

Contents

Foreword	iv
Preface	v
List of abbreviations and acronyms used in this manual	vi
Part 1. General considerations	
Chapter 1. Introduction	1
Chapter 2. Lyssaviruses	7
Chapter 3. Biosafety	26
Chapter 4. The role of diagnostics in surveillance	35
Chapter 5. An overview of antemortem and postmortem tests for diagnosis of human rabies	43
Chapter 6. Histopathological techniques in the laboratory diagnosis of human rabies	55
Chapter 7. Brain removal	67
Part 2. Detection of virus	
Chapter 8. Virus isolation in animals: the mouse inoculation test	74
Chapter 9. Virus isolation in cell culture: the rabies tissue culture infection test	85
Chapter 10. Transmission electron microscopy in rabies diagnosis, ultrastructural studies and research	96
Part 3. Demonstration of antigens	
Chapter 11. The direct fluorescent antibody test	108
Chapter 12. The direct rapid immunohistochemistry test for the detection of lyssavirus antigens	130
Chapter 13. Immunohistochemistry	136
Chapter 14. Antigenic typing of lyssaviruses by monoclonal antibodies	142
Chapter 15. Use of a rapid skin biopsy technique for human rabies antemortem diagnosis	155
Chapter 16. Demonstration of lyssavirus antigens by flow cytometry	169
Chapter 17. Rapid immunochromatographic tests for the detection of rabies virus antigens in brain material	176
Chapter 18. Mass spectrometry-based proteomic approaches for the detection of rabies virus peptides	183
Part 4. Demonstration of viral antibodies	
Chapter 19. The rapid fluorescent focus inhibition test	196
Chapter 20. The fluorescent antibody virus neutralization test	219
Chapter 21. An indirect fluorescent antibody test for the detection of rabies virus immunoglobulin G and immunoglobulin M antibodies	232
Chapter 22. The mouse neutralization test	246
Chapter 23. Demonstration of lyssavirus antibodies by pseudotype virus micro-neutralization assays	252
Chapter 24. A simplified fluorescence inhibition microtest for the determination of rabies virus neutralizing antibodies	259
Chapter 25. The immunoperoxidase inhibition assay	266
Chapter 26. Demonstration of rabies virus antibodies by the counter immunoelectrophoresis test	271
Appendix 1 List of contributors	283
Appendix 2 WHO collaborating centres on rabies, neurovirology, viral zoonoses and zoonoses control	288

Laboratory techniques in rabies

Foreword

For more than 5000 years, humans have lived in fear of a bite from a rabid animal, so much so that the first written account of rabies, in the 23rd century BC, set the penalty for an owner's dog biting another individual at "two-thirds of a mine of silver", or about a half-day's work. Today, our focus is more on preventing rabies and advocating for its elimination, rather than imposing penalties, and our understanding of the virus has greatly improved since the 23rd century BC.

The Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE) and the World Health Organization (WHO) have prioritized action against rabies and, together with Member countries, have set a goal of zero rabies deaths by 2030. Diagnostics are crucial in attaining this goal.

New laboratory techniques and advancements in science have yielded better diagnostic techniques and control strategies to aid the more than 3 billion people, mainly children, in Asia and Africa who are threatened by the virus every day. Rabies is a preventable disease, yet despite the availability of efficacious and affordable vaccines, more than 60 000 people worldwide die agonizing deaths every year from the disease.

No diagnostic tests are available to detect the rabies virus before the onset of clinical disease, and further research on diagnostic techniques in the field of rabies is therefore paramount. The impact of suitable laboratory capacity on surveillance and elimination of the disease worldwide is evident.

The OIE's Manual of diagnostic tests and vaccines for terrestrial animals provides internationally agreed standards for the production and control of validated veterinary diagnostic methods and vaccines for use in animals. The fourth edition of WHO's Laboratory techniques in rabies has been a guiding reference for many rabies laboratories. The first edition (1954) stated that "rabies research is far from static" and, since its publication more than 60 years ago, OIE and WHO have worked to evaluate subsequent advancements in laboratory techniques in rabies. This fifth edition provides insight into validated methods recommended for use in diagnostic laboratories, but it also includes research. While not currently applicable to all settings, these research methods may stimulate the development of improved techniques for diagnosis of rabies in the future. Improved diagnostics will strengthen surveillance of the disease, leading to enhanced control of rabies where it is most needed.



Laboratory techniques in rabies

Preface

Rabies has an enormous impact on both agriculture and conservation biology, but its greatest burden is undeniably on public health. As such, routine methods for rapid risk assessment after human exposures to rabies as well as applications for laboratory-based surveillance, production of biologicals and management of this infectious disease are critical. Given its mandate to improve human health and control disease among its Member States, WHO has led the production of this fifth edition of *Laboratory techniques in rabies*.

During the more than 60 years that have elapsed since the first edition was published, methods of viral diagnosis, characterization of pathogens and production of biologicals have advanced. At that time, only a single etiological agent was recognized as causing rabies. Detection of Negri bodies was the standard for diagnosis. Nerve tissue-based vaccines were the norm. Combination use of vaccines and rabies immunoglobulins in human prophylaxis was not standard. Global elimination of canine rabies was merely a dream. Rabies in wildlife was managed via population reduction. All of that has changed for the better.

In the ensuing decades, further advancements in detection, prevention and control of lyssaviruses have been monitored by regular meetings of WHO experts, international research groups and countries in which rabies is endemic. The second edition of the manual was published in 1966, the third in 1973 and the fourth in 1996. The late Martin Kaplan and Hilary Koprowski were instrumental in editing the previous editions, as was input on the fourth edition by François-Xavier Meslin, now retired from WHO. Initial plans for preparation of this edition were made in 2016 and its contents were discussed at the WHO Expert meeting on rabies (Bangkok, Thailand) and modified in response.

This fifth edition of *Laboratory techniques in rabies* contains 44 detailed chapters written by more than 85 authors from Africa, the Americas and Eurasia. The text was peer reviewed by Dr Matthias Schnell, Head of the WHO Collaborating Centre for Neurovirology; Professor Thiravat Hemachudha, Head of the WHO Collaborating Center for Research and Training on Viral Zoonoses; and Dr Asefa Deressa, Team Leader of Zoonoses Research at the Ethiopian Public Health Institute. The manual focuses on the basic methods for detection of lyssavirus antigens, antibodies and nucleic acids and the relevance of their use under different operating conditions, from the basic to the advanced. The chapters on older, less sensitive techniques used to detect Negri bodies have been removed, as have those chapters on methods of vaccine production given the progress made in the commercial use of tissue culture products in human and veterinary medicine. Recommendations for the preparations of antibodies by homologous or heterologous production have been replaced by newer methods in an effort to promote a next generation of less expensive and more readily available immunoglobulins in the future. Other basic chapters have been retained and updated and more than a dozen added. Each of the protocols described are prescriptive and should be followed point by point in the laboratory.

We gratefully acknowledge the collaboration of the many eminent scholars who contributed to the current volume, and look forward to the publication of the next edition as continued advances in the field are made.

List of abbreviations and acronyms used in this manual

3Rs	“Replacement, Reduction and Refinement” of laboratory animal testing
AALAS	American Association for Laboratory Animal Science
Ab	antibody
ABLV	Australian bat lyssavirus
ACD	acid citrate dextrose
ACIP	Advisory Committee on Immunization Practices
ACS	American Chemical Society
AEC	3-Amino-9-ethylcarbazole
Ag	antigen
ANSM	Agence Nationale de Sécurité du Médicament et des produits de santé
AMA	African Medicines Agency
AP	alkaline phosphatase
APS	ammonium persulphate
ARAV	Aravan virus
ATCC	American Type Culture Collection
AVMA	American Veterinary Medical Association
BBLV	Bokeloh bat lyssavirus
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BEEM	better equipment for electron microscopy
BHK	baby hamster kidney
bnAbs	broadly neutralizing antibodies
bp	base pair
BP	British Pharmacopeia
BPL	β-propiolactone
BRP	Biological Reference Preparation
BSA	bovine serum albumin
BSC	biosafety cabinet
BSL	biosafety level
CCID	cell culture infectious dose
CDC	United States Centers for Disease Control and Prevention
cDNA	complementary deoxyribonucleic acid
CER	chicken embryo-related
CFIA	Canadian Food Inspection Agency

CHAPS	3-(3-cholamidopropyl) dimethylammonium 1-propanesulfonate
CHO	Chinese Hamster Ovary cells
CIE	counter immunoelectrophoresis
CLRW	clinical laboratory reagent water
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
Ct	Cycle threshold
CVS	challenge virus standard strain
ddNTP	dideoxynucleotide
DDSA	dodecanyl succinic anhydride
dNTP	deoxynucleosidetriphosphate
DEAE	diethylaminoethyl
Defra	Department for Environment, Food and Rural Affairs
DEPC	diethylpyrocarbonate
DFAT	direct fluorescent antibody test
DH2O	distilled water
DIG	digoxigenin
DMEM10	Dulbecco's minimum essential medium with 10% fetal calf serum
DMP30	tris dimethylaminomethyl phenol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide-tri phosphate
DPX	mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate) and xylene
DRIT	direct rapid immunohistochemistry test
dsDNA	double stranded DNA
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	dithiothreitol
DUVV	Duvenhage virus
EBLV-1	European bat lyssavirus, type 1
EBLV-2	European bat lyssavirus, type 2
ED50	50% end-point
EDQM	European Directorate for the Quality of Medicines
EDTA	ethylenediaminetetraacetic acid
EIU	equivalent international units
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EMEM	Eagle's minimum essential medium
EPAA	European Partnership for Alternatives to Animal Testing
ERA	Evelyn Rokitniki Abelseth strain
ERIG	equine rabies immunoglobulin

ESI	electrospray ionization
EtBr	ethidium bromide
EVAg	European Virus Archive Global
Fabs	antigen-binding fragments
FACS	fluorescence-activated cell sorting
FAVN	fluorescent antibody virus neutralization test
FBS	fetal bovine serum
FCA	Freund's Complete Adjuvant
FCS	fetal calf serum
FFID	fluorescent focus infectious dose
FFPE	formalin-fixed, paraffin-embedded
FIA	Freund's Incomplete Adjuvant
FIMT	fluorescence inhibition microtest
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
FRET	Fluorescence Resonance Energy Transfer
FTA	Flinders Technology Associates
G	glycoprotein
GBLV	Gannoruwa bat lyssavirus
GFP	green fluorescent protein
GM	genetically modified
GMEM	Glasgow Minimum Essential Medium
GMP	Good Manufacturing Practices
gRNA	genomic RNA
HBO	mercury luminance unforced cooling lamp
HDCV	human diploid cell vaccine
H&E	hematoxylin and eosin
HEK	human embryonic kidney
HEP	high egg passage strain
HEPES	hydroxyethyl piperazine ethane sulfonic acid
HIV	human immunodeficiency virus
hn	hemi-nested
HPLC	high-performance liquid chromatography
HRIG	human rabies immunoglobulin
HRP	horse radish peroxidase
IAA	iodoacetamide
IACUC	Institutional Animal Care and Use Program
IBCMP	integrated bite case management program
IC (i.c.)	intracerebral
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods

ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICTV	International Committee on Taxonomy of Viruses
IEF	isoelectric focusing
IFA	indirect fluorescent antibody test
IgG	immunoglobulin G
IgY	immunoglobulin Y
IMAC	immobilized metal affinity chromatography
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
ISH	in situ hybridization
IIA	immunoperoxidase inhibition assay
ICTV	International Committee on the Taxonomy of Viruses
IHC	immunohistochemistry
IFA	indirect fluorescent antibody
IIF	indirect immunofluorescence
IKOV	Ikoma lyssavirus
i.m.	intramuscular
i.p.	intraperitoneal
IPC	in-process control
IRIT	indirect rapid immunohistochemistry test
IRKV	Irkut virus
IS	indicator serum
ISH	in situ hybridization
IU	international unit
KHUV	Khujand virus
L	"large" protein (i.e. the viral RNA-dependent polymerase)
lacZ	structural gene for β -galactosidase
LAMP	loop-mediated isothermal amplification
LB	Luria-Bertani broth
LBV	Lagos bat virus
LC	liquid chromatography
LD ₅₀	50% lethal dose
LED	light-emitting diode
LEP	low egg passage strain
LFA	lateral flow assay
LFD	lateral flow devices
LIMC	low and middle-income countries
LLEBV	Lleida bat lyssavirus
M	matrix protein
MAb	monoclonal antibody
MALDI	matrix-assisted laser desorption/ionization
MCIE	modified CIE

MEM	modified Eagle's medium
MES	2-(N-morpholino) ethanesulfonic acid buffer
MIT	mouse inoculation test
MLV	murine leukaemia virus
MNA	murine neuroblastoma cell
MNT	mouse neutralization test
MOI	multiplicity of infection
MOKV	Mokola virus
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
M/Z	mass to charge ratio
N	nucleoprotein
NA	numerical aperture
NAA	nucleic acid amplification
nAb	neutralizing antibody
nAChR	nicotinic acetylcholine receptor
NASBA	nucleic acid sequence based amplification
NBT	nitro blue tetrazolium
NC	negative control
NC	nucleocapsid
NCBI	National Center for Biotechnology Information
NDDR	National Donor Referral Registry
NGS	next generation sequencing
NIBSC	National Institute for Biological Standards and Control
NIH	National Institutes of Health (USA)
NMDA	N-Methyl-D-aspartic acid
NMRI	Naval Medical Research Institute
NS	negative serum
NSS	non-specific staining
NTC	no template control
OCT	optimal cutting temperature
OD	optical density
OMCL	Official Medicine Control Laboratories
OIE	World Organisation for Animal Health
ORF	open reading frame
P	phosphoprotein
PAHO	Pan American Health Organization
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PC	positive control

PCEC	purified chick embryo cell vaccine
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
PEP	post-exposure prophylaxis
Ph Eur	European Pharmacopeia
pl	isoelectric point
PNA	pseudotype neutralization assay
PPE	personal protective equipment
PPHS	passive public health surveillance
PMF	peptide mass fingerprinting
PT	proficiency test or testing
PTFE	polytetrafluoroethylene
PTV	pseudotyped viruses
PV	Pasteur virus
PVRV	purified vero cell rabies vaccine
QA	quality assurance
QC	quality control
RABV	rabies virus
RER	rough endoplasmic reticulum
RF	rheumatoid factors
RFFIT	rapid fluorescent focus inhibition test
rG-F	recombinant G truncated protein
RIDT	rapid immunochromatographic diagnostic test
RITM	Research Institute of Tropical Medicine
RLU	relative light unit
RIG	rabies immunoglobulin
rN	recombinant nucleoprotein
RNA	ribonucleic acid
RNP	ribonucleoprotein
rP	recombinant P protein
RP	relative potency
RPMI	Roswell Park Memorial Institute medium
rRNA	ribosomal RNA
RS	reference serum
RTCIT	rabies tissue culture infection test
RT-LAMP	reverse transcriptase loop-mediated isothermal amplification
RT-PCR	reverse transcriptase polymerase chain reaction
RVNA	rabies virus neutralizing antibodies
SAD	Street Alabama Dufferin strain
scFv	single-chain antibody fragment
SD	standard deviation

SDS	sodium dodecyl sulfate
SE-HPLC	size exclusion high-pressure liquid chromatography
SFIMT	simplified fluorescence inhibition microtest
SHIBV	Shimoni bat virus
SMB	suckling mouse brain
SNP	single nucleotide polymorphism
SPA	serological potency assay
SPRI	solid phase reversible immobilization [beads]
SRIG	standard rabies immunoglobulin
SSC	saline-sodium citrate
ssRNA	single stranded RNA
SYBR	Synergy Brand
Taq	thermostable DNA polymerase via bacterium (<i>Thermus aquaticus</i>)
TBS	tris-buffered saline
T-DNA	transfer DNA
TC	tissue culture
TCID	tissue culture infectious dose
Thyb	probe hybridization temperature
TPBS	Tween phosphate buffered saline
TBE	tris borate EDTA buffer
TE	tris EDTA buffer
TEA	triethanolamine
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylenediamine
TLR3	Toll-like receptor-3
TOF	time-of-flight
TRIS	tris(Hydroxymethyl)aminomethane
TS	samples such as sera or plasma to be tested
TST	Tris-buffered saline and Tween 20
USP	United States Pharmacopeia
UTR	untranslated region
UV	ultraviolet
VEP	viral envelope protein
VH	heavy-chain variable regions
VHH	variable llama-derived heavy-chain antibody fragments
VL	light chain variable regions
VNA	virus-neutralizing antibody
WCBV	West Caucasian bat virus
WHO	World Health Organization
WRS	street rabies virus
WTA	whole-transcription amplification
YM	yeast minimal

Part 1. General considerations

Chapter 1

Introduction

A simplified, early 20th century view of the historical rabies laboratory may have been predisposed towards a linear, passive role of sample receipt and diagnosis only. Today, the ideal role of the modern rabies laboratory is more complex and multi-dimensional (Table 1.1). While some laboratories may perform one or only a few core functions, others conduct much more diverse activities, especially the reference laboratories of the World Organisation for Animal Health (OIE) and the World Health Organization (WHO). Moreover, because rabies is a fundamental disease of nature, the laboratory should be engaged not only in diagnostics per se but also in actively maintaining a central role in the assessment, planning and investigation of cases of rabies in humans, domestic animals and wildlife locally and abroad as part of routine prevention and control using a One Health approach (Fig. 1.1). This critical responsibility for involvement in human, veterinary and environmental health has guided the production of this fifth edition of *Laboratory techniques in rabies* and informed the practical approach requested by countries in which canine rabies is endemic to support the goal of eliminating human deaths from dog-mediated rabies by 2030.

Table 1.1. Multiplicity of roles and responsibilities of the rabies laboratory in the 21st century

Basic actions	Examples
Confirmation	Replication of results produced in another local or reference rabies laboratory
Diagnosis	Primary analytical antemortem and postmortem testing of specimens from suspect animals and humans
Discovery	Identification of new lyssavirus species, reservoirs, variants, etc.
Education	Pre-analytical definition of critical elements to providers of routine sample collection, storage, shipping, etc.
Monitoring	Routine creation of local biosafety standards, adherence to QA/QC, proficiency testing, staff response to vaccination, etc.
Production	Preparation of animal and human biologicals for detection or prophylaxis
Reference	Inauguration of a centralized national laboratory or global OIE/WHO network
Regulation	Validation of proposed label claims related to product performance, potency, purity, efficacy, safety, etc. and adherence to national and global norms
Research	Development and evaluation of new biologicals, techniques, etc.
Surveys	Utilization of active outreach for enhanced data and sample acquisition, such as sero-surveillance of suspect animal reservoirs
Training	Tutorials and updates of current or novel methods to local, national, regional and global laboratorians
Typing	Utilization of methods to characterize biodiversity of viral variants, phylogroups, etc.

QA, quality assurance; QC, quality control; OIE, World Organisation for Animal Health; WHO, World Health Organization

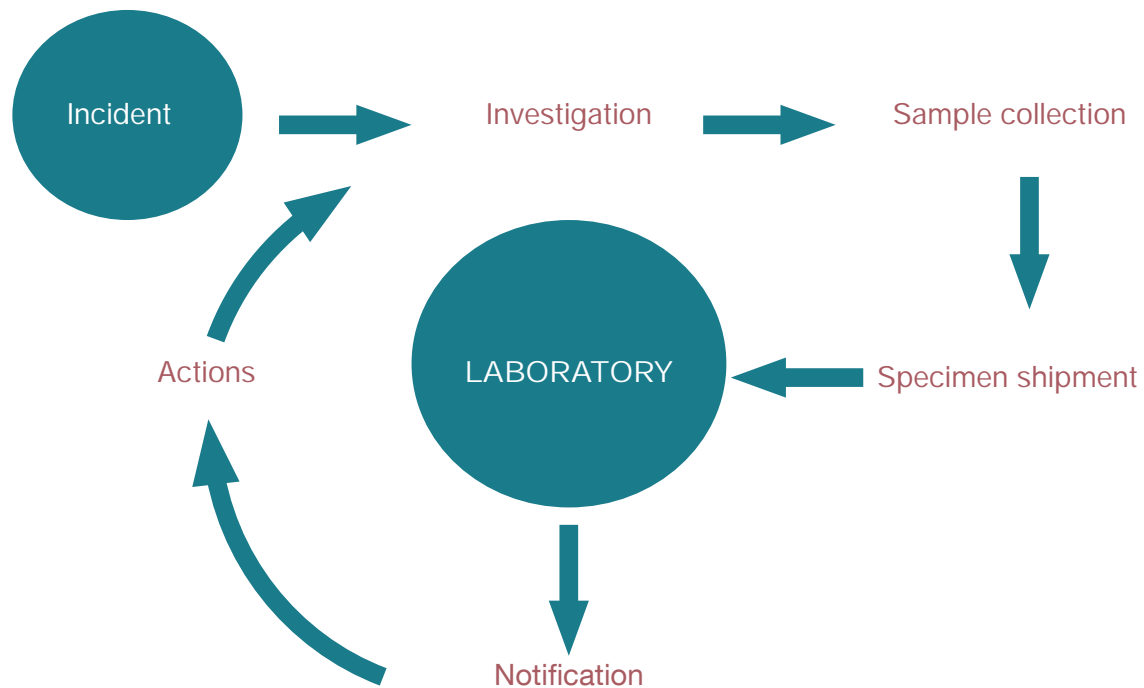


Fig. 1.1. Simplified dynamic of a modern rabies laboratory in basic diagnosis, risk assessments, enhanced surveillance and disease response

At a minimum, all rabies laboratories should be capacitated to perform diagnosis using the “gold standard” direct fluorescent antibody test (DFAT). This highly sensitive and specific method has been in routine use for more than 60 years. In retrospect, this manual is a culmination not only of reliable laboratory techniques that have stood the test of time but also of newer protocols for detection of viral antigens, antibodies and amplicons, production of relevant biologicals, where applicable, and applied research.

The current design relies on the successful outline of previous editions, starting with general considerations about the disease. This fifth edition is separated into two volumes to reflect operational and research focused content, and is available online only to facilitate rapid electronic updates as further advances in laboratory techniques become available. As rabies is caused by numerous lyssaviruses, an introduction ([Chapter 2](#)) to the genus is an appropriate reminder that rabies virus is only one viral species of special concern and to promote further pathogen discovery from a global perspective. Biosafety ([Chapter 3](#)) is of paramount importance to all laboratorians and therefore warrants a separate chapter. Notably, human and animal diagnostics alike must operate within a logical sphere for laboratory-based surveillance to operate ([Chapter 4](#)). Human rabies is underreported on all continents, and the current guidance seeks to address this shortcoming ([Chapters 5 and 6](#)). Clearly, a base suspicion of human encephalitis is not straightforward and should be approached within a structured framework of differential diagnoses (1). Advanced neuroimaging may be useful to consider an infectious etiology, including rabies (2). Antemortem detection of specific immune complexes can also be useful in cases of paralytic rabies (3).

As rabies is an acute progressive encephalitis, the central nervous system (CNS) is the main tissue of interest. Whereas in the past the entire brain was removed typically for diagnosis in animals, this cumbersome and sometimes dangerous exercise is now largely unnecessary when a full section of the brainstem is available (Chapter 7). Safe and secure methods are also suggested for obtaining and shipping samples from the field for use in basic diagnosis and characterization of pathogens (Chapter 15). Beyond routine needs for enhanced laboratory-based surveillance, laboratorians should also appreciate current regulatory norms for evaluation of diagnostic products available nationally or imported, human or animal vaccines, reagents used in applied research, and expectations of other future human and animal biologicals (Chapters 35 and 36).

A wide variety of methods are available for use in rabies diagnosis. Lyssavirus virions may be visualized directly via electron microscopy (Chapter 10). Infectious viruses are used in a diversity of protocols from pathobiological studies to tests of vaccine efficacy, and can be isolated in animals (Chapter 8) or, preferably, in tissue culture (Chapter 9). Beyond traditional demonstration of viral antigens in the CNS by the DFAT (Chapter 11), an alternative is the direct rapid immunohistochemistry test (DRIT) using light microscopy (Chapter 12), as a simplification of its formal predecessor, traditional immunohistochemistry (Chapter 13). Antigenic typing of viral variants (Chapter 14) may be performed using fluorescent or light microscopy to discriminate laboratory strains from field isolates and to differentiate infected animals and humans by variant type. The DFAT may also be performed upon frozen skin biopsies for diagnosis of human rabies, with results available in a few hours (Chapter 15). Several other tests (Chapters 16–18) have also been suggested for the detection of viral antigens, including latex agglutination protocols (4).

Ideally, a simple point-of-care test would greatly assist in surveillance and risk assessments. Unfortunately, currently available linear flow tests vary from lot to lot and are not recommended for making decisions on post-exposure prophylaxis (PEP) in humans.

The detection of viral antibodies (Chapters 19–22) is useful for several purposes, including basic diagnosis, risk assessments, immunogenicity studies and epidemiological investigations. Antibody detection may be discerned functionally by neutralization or through binding studies, such as enzyme-linked immunosorbent assays (ELISAs), as detailed in earlier editions of this manual and in a recent publication (5), are not repeated herein as protocols have not changed considerably. Additionally, over the past few years a number of commercial ELISA kits have become available. Recent international testing has shown good reproducibility among laboratories as particularly useful for immunological insights for oral vaccination programmes against rabies in wildlife (6). Given the opportunity for cell culture or ELISA formats, antibody detection in animals is no longer recommended as state of the art and is included only as a courtesy for those countries in which it has been retained historically until newer options may be adopted.

Beyond demonstration of viral antigens and antibodies, a wide variety of molecular techniques are available today for nucleic acid amplification and genetic comparisons (Chapters 27–34). While in the past many of these molecular protocols were used in a confirmatory basis, today many are used as a standalone for primary diagnosis. Both conventional and real-time reverse transcriptase

polymerase chain reaction (RT-PCR) protocols have been validated to cover the spectrum of known lyssaviruses. Strict adherence to quality control limits the risk of cross-contamination. Protocols other than those given in this chapter have also been validated, such as oligonucleotide microarrays for viral speciation (7). Sangar sequencing of PCR products is still a useful method to use for differentiation of variants if the costs of equipment and reagents are prohibitive. Next-generation sequencing may be inaccessible for many laboratories in the developing world, but is a field that is expected to grow in refinement, simplicity and cost.

Several topics were not carried forward from the earlier editions, either because they are obsolete or not considered necessary given modern alternatives. For example, the dependence upon detection of Negri bodies is grounded in the 19th century, and replaced by many more sensitive and specific tests today. Other protocols were not included in this edition if commercial kits, conjugates or reagents were produced. For example, multiple commercial conjugates are available for the DFAT; however, in localities where access to them is more limited, lyssavirus proteins may be purified and conjugates can be produced in-house (8). Hence, one such protocol was retained for production of polyclonal anti-sera in goats, for use in diagnostic applications (Chapter 37). Other smaller species such as rabbits can also be used for production and ad hoc conjugation of anti-serum (9).

Similarly, fewer protocols dealing with generation of other biologicals were retained than in previous editions; most are now absent from the fifth edition. Information on nerve tissue-based vaccines is clearly obsolete, as they have been replaced by much safer tissue culture vaccine alternatives such as avian or Vero cell vaccines for humans. Similarly, multiple modified-live attenuated and recombinant vaccines for use in domestic animals and wildlife have been licensed in Eurasia and North America and are no longer considered novel given their routine use (10–17). Readers interested in the local national production of such 20th century human or veterinary biologicals should refer to the listed citations for review or contact commercial entities for transfer of technology.

In contrast to rabies vaccines, there are even more comparative needs today beyond human and equine rabies immunoglobulins for PEP in humans (18). Thus, contributions on generation of monoclonal antibodies, immunoglobulin Y and plant-based antibodies are intended to spur further research and development on more cost-effective use of PEP in the future (Chapters 38–41). Similarly, alternatives to the standard National Institutes of Health test (Chapter 42) for measurement of vaccine potency continue to be sought (19). To date, progress has been made for application to both veterinary (Chapter 43) and human (Chapter 44) biologicals.

This fifth edition of *Laboratory techniques in rabies* focuses on the needs of laboratories for surveillance of rabies in humans and animals as well as that of prevention and control programmes. Rabies continues to carry the highest case fatality rate of any infectious disease. As understanding of viral pathobiology improves and relevant models for investigation become available, future editions may include suitable antiviral approaches for PEP and therapy, as are being developed for other negative-stranded RNA viruses (20). In the interim, early economical detection, characterization and response (Fig. 1.1) remain the hallmarks for reducing the burden of this ancient zoonosis.

References

1. Levin SN, Lyons JL. Infections of the nervous system. *Am J Med.* 2018; 131:25–32. doi:10.1016/j.amjmed.2017.08.020.
2. Laothamatas J, Sungkarat W, Hemachudha T. Rabies diagnosis: MR imaging. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 1. New York (NY): Academic Press; 2014:221–34.
3. Madhusudana SN, Muhamuda K, Mani RS. Demonstration of immune complexes by capture enzyme-linked immunosorbent assay. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 1. New York (NY): Academic Press; 2014:119–25.
4. Kasempimolporn S, Chaiyabutr N, Sitprija. Demonstration of rabies virus antigens by a latex agglutination test. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 1. New York (NY): Academic Press; 2014:37–42.
5. Fehlner-Gardiner C, Wandeler AI. Detection of rabies virus antibodies by competitive enzyme-linked immunosorbent assay. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 2. New York (NY): Academic Press; 2014:145–55.
6. Wasniewski M, Almeida I, Baur A, Bedekovic T, Boncea D, Chaves LB, et al. First international collaborative study to evaluate rabies antibody detection method for use in monitoring the effectiveness of oral vaccination programmes in fox and raccoon dog in Europe. *J Virol Methods.* 2016; 238:77–85.
7. Dastjerdi A, Fooks AR, Johnson N. Oligonucleotide microarray: applications for lyssavirus speciation. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 2. New York (NY): Academic Press; 2014:293–303.
8. Dietzschold B. Purification of rabies virus and its subunits. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 2. New York (NY): Academic Press; 2014:47–56.
9. Caporale G, Silva S, Peixoto Z. Preparation of fluorescent antibody conjugate in rabbits. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 2. New York (NY): Academic Press; 2014:57–68.
10. Vos A, Neumann G, Hundt B, Neubert A. Attenuated vaccines for veterinary use. In: Rupprecht CE, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research and prevention.* Volume 1. New York (NY): Academic Press; 2014:237–44.
11. Mähl P, Cliquet F, Guiot AL, Niin E, Fournials E, Saint-Jean N, et al. Twenty year experience of the oral rabies vaccine SAG2 in wildlife: a global review. *Vet Res.* 2014;10; 45:77. doi:10.1186/s13567-014-0077-8.

12. Weyer J, Nel LH. Pox viral vectored vaccines for rabies. In: Rupprecht CE, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention. Volume 1. New York (NY): Academic Press; 2014:245–54.
13. Maki J, Guiot AL, Aubert M, Brochier B, Cliquet F, Hanlon CA, et al. Oral vaccination of wildlife using a vaccinia-rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): a global review. *Vet Res.* 2017; 48:57. doi:10.1186/s13567-017-0459-9.
14. Faber M. Recombinant rabies virus vaccines. In: Rupprecht CE, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention. Volume 1. New York (NY): Academic Press; 2014:255–64.
15. Astray RM, Jorge SA, Pereira CA. Rabies vaccine development by expression of recombinant viral glycoprotein. *Arch Virol.* 2017; 162:323–32. doi:10.1007/s00705-016-3128-9.
16. Ertl HCJ. Adenoviral vectors as vaccine carriers for prevention of rabies. In: Rupprecht CE, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention. Volume 1. New York (NY): Academic Press; 2014:265–74.
17. Slate D, Chipman RB, Algeo TP, Mills SA, Nelson KM, Croson CK, et al. Safety and immunogenicity of Ontario Rabies Vaccine Bait (ONRAB) in the first US field trial in raccoons (*Procyon lotor*). *J Wildl Dis.* 2014; 50:582–95. doi:10.7589/2013-08-207.
18. Khomvilai S, Daviratanasilpa S, Pormuttakun D, Sakolpap L, Akesowan S, Pakmanee N, et al. Production of equine rabies immune globulin of high purity, potency and safety. In: Rupprecht CE, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention. Volume 2. New York (NY): Academic Press; 2014:265–74.
19. Morgeaux S, Poirier B, Ragan CI, Wilkinson D, Arabin U, Guinet-Morlot F, et al. Replacement of in vivo human rabies vaccine potency testing by in vitro glycoprotein quantification using ELISA – Results of an international collaborative study. *Vaccine.* 2017; 35:966–71. doi:10.1016/j.vaccine.2016.12.039.
20. Castel G, Mechlia MB, Jallet C, Tordo N. Rabies therapeutics: development of anti-viral approaches. In: Rupprecht CE, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention. Volume 2. New York (NY): Academic Press; 2014:331–51.

Chapter 2

Lyssaviruses

Introduction

Rabies is an acute viral encephalitis with a case fatality rate approximating 100%, which is the highest among known infectious diseases. This zoonosis, known to humankind for millennia, is associated primarily with the bites of “mad” dogs (1). The English name likely originates from the Sanskrit word “rabhas”, meaning “violence”. A similar etymology exists in other languages; the name of the disease typifies uncontrolled violence, aggression, madness or frenzy.

Besides “classical” rabies virus (RABV), a number of other phylogenetically-related viruses segregate into the genus *Lyssavirus* as described during recent decades (2). Some of these viruses are antigenically and serologically related to RABV, whereas others are more divergent and as such are not covered fully by commercial biologicals for rabies prophylaxis (3–5). Still, more than 99% of human rabies cases are caused by infection with RABV mediated by dogs. Post-exposure prophylaxis (PEP) virtually assures complete prevention of the disease if administered promptly and properly, and has been available for over a century. Nevertheless, rabies still causes tens of thousands of human deaths every year due to poor availability or affordability of appropriate biologicals, particularly in those areas where the demand for them is highest, such as developing countries of tropical Asia and Africa (6).

As with any infectious disease, effective programmes of rabies management rely on rigorous laboratory-based surveillance, robust diagnostic tools and highly efficacious biologicals. This laboratory manual covers the most essential modern methods of rabies diagnosis, prevention and control. This chapter reviews salient features of lyssavirus structure, function, pathobiology and phylogeny.

Virion structure and life cycle

Lyssavirus virions are bullet-shaped, 130–250 nm long and 60–100 nm in diameter. They are composed of two functional units: the internal nucleocapsid (NC) core that includes the genomic RNA tightly bound to the nucleoprotein (N), phosphoprotein (P) and viral polymerase (L, from “large protein”); and the external unit represented by a bi-layer lipid envelope (acquired from host cell membranes during budding) with protruding spikes of the viral glycoprotein (G). The matrix protein (M) present between these two units condenses the NC and interacts with the G protein endodomain (7).

The genome is represented by a non-segmented linear, negative-sense, single-stranded RNA (ssRNA), about 12 kb long. It includes five major genes that are arranged in the conserved linear order 3'-N-P-M-G-L-5'. Each of the individual

genes is flanked by transcription initiation and termination or polyadenylation signals that are largely conserved among members of the same viral species. Transcription units are separated by short untranscribed intergenic regions. The exception is the G–L intergenic region, which consists of 400–700 nucleotides and may represent a remnant gene that lost its functionality during lyssavirus evolution (8–9). The ends of the genomic RNA (leader and trailer sequences) exhibit terminal complementarity and contain promoter sequences that initiate replication of the genome and antigenome, respectively.

Viral proteins are multi-functional. The N protein, the major component of the NC, protects the viral genome from cellular RNase activity and interacts with the L and P proteins during transcription and replication. The P protein plays multiple roles during the same processes as a non-catalytic cofactor of the polymerase. It mediates the physical link and proper positioning of the L protein on the N-RNA template, and acts as a chaperone during synthesis of the N protein by forming N–P complexes that prevent the N protein from self-aggregation and binding to cellular RNA (7). The P protein also interacts with the host cellular transport systems such as the dynein motor complex, nucleo-cytoplasmic transporters and microtubules (10–12) to facilitate intracellular movement of viral components. Furthermore, the P protein interferes with innate immunity by inhibiting different steps of the host cell interferon response. The M protein binds to NCs and the cytoplasmic domain of the G protein, thereby facilitating the budding process, and also mediates such pathobiological effects as cell rounding, intracellular membrane redistribution and apoptosis. The G protein is the only viral component present on the virion surface. The viral G protein is organized in trimers protruding through the virion envelope and consists of an endo- (cytoplasmic) domain, transmembrane domain and ectodomain. The latter mediates binding to host cell receptors, and induces endocytosis and fusion of viral and endosomal membranes, whereas the internal domains of the G protein interact with the M protein, facilitating virion morphogenesis and budding. As the only external component, the G protein induces production of virus-neutralizing antibodies (VNA) and elicits cell-mediated immune responses. The L protein has multiple domains and performs the functions required for genome transcription and replication, including RNA-dependent RNA polymerase, mRNA 5' capping enzyme, cap methyltransferase, 3' poly (A) polymerase and protein kinase activities (13).

Lyssaviruses may replicate at the inoculation site, as has been shown for skeletal muscle fibres, and typically penetrate neurons via the neuromuscular junction (13, 14). After virions bind to cellular receptors and endocytosis, the endosomal vesicle is transported by retrograde axonal flow dependent on the microtubule network, using dynein motors. The decrease of pH within the endosome provokes conformational changes of the G protein, which mediates fusion between the endosomal and viral membranes, and the (–) strand RNA genome encapsidated by the N protein is liberated into the cytoplasm (11, 12). Transcription and replication occur at the neuronal perikaryon.

The N-RNA template is transcribed by the L viral polymerase and its cofactor, the P protein. This generates in cascades the monocistronic (+) strand RNAs, corresponding to the leader RNA (Le+) and its five mRNAs [messenger RNAs] (12). All but one of the monocistronic mRNAs produce a single protein from a single open reading frame (ORF), initiated at the first AUG codon. The P mRNA produces three or four proteins, initiated from downstream in-frame AUG codons

(15). The replication process yields NCs containing full-length antigenome-sense RNA, which in turn serve as templates for the synthesis of genome-sense RNA. During their synthesis, both the nascent antigenome and the genome are encapsidated by the N protein.

Cytoplasmic inclusions, known historically as Negri bodies (16), can be frequently observed by light microscopy in Seller-stained brain impressions. These inclusions and viral antigens are detected by the direct rapid immunohistochemistry test (DRIT) and the direct fluorescent antibody test (DFAT), and are typical for lyssavirus infection. Such inclusions are functional structures in which viral transcription and replication take place and where neo-assembled NCs accumulate (17). Simultaneously, the viral M and G proteins localize to the plasma membrane. The M protein accumulates on the cytoplasmic side of G-enriched microdomains on the plasma membrane as NCs condense into tightly coiled structures by interacting with the M protein. The microdomains containing high levels of G protein, along with the continued condensation of M-RNP structures, facilitate outward membrane curvature and eventual virion budding (18). Viral progeny are released at the synaptic junctions and propagate to the central nervous system (CNS) by using motor or sensory neurons, following neuronal connections.

Viral pathogenesis

Generally, virions are delivered into a wound via a bite or, rarely, contamination of mucous surfaces such as highly-concentrated aerosols generated in laboratory settings or in the unique environment of certain caves inhabited by colonies of multi-million individual bats including *Tadarida brasiliensis* (19). Using either sensory or motor neurons, viruses move centripetally to the CNS. Neurotropism avoids or limits surveillance by the host immune system, resulting in the absence of an early antibody response. The duration of a non-clinical incubation period averages 1–2 months but may vary from a week to several months, or even years (20, 21). During the incubation period, rabies diagnosis cannot be performed on animal or human tissues due to the very limited viral load, uncertain location in peripheral nerves and the absence of a detectable immune response.

Upon delivery to the CNS, lyssaviruses disseminate rapidly and may affect nearly all regions of the CNS. The medulla oblongata is the main area from which virus is transported to the salivary glands along innervation pathways, allowing further excretion with saliva and transmission to other susceptible hosts via a bite (22). Therefore, the most reliable diagnosis of rabies can be done during the clinical period of the disease (or after death) on CNS tissues; the brainstem is the most important region for diagnostic tests. Virus can be spread centrifugally from the CNS to peripheral tissues also via innervation pathways, allowing antemortem detection of antigens and nucleic acids in hair follicles by a skin biopsy at the nape of the neck. Such deposition of peripheral tissue may pose a hazard for scavenging mammals when they consume carcasses of rabid animals (23) and in human recipients of tissue or organ transplantation (24, 25).

The clinical period of rabies is usually limited to 1–2 weeks. The prodromal period is lacking in specific clinical signs, when patients develop fever, malaise and sometimes flu-like symptoms. Affected subjects may feel paraesthesia and tingling at the inoculation site. With progression to encephalitis, cerebral signs

manifest. Humans often develop hallucinations and delirium. Some symptoms, which have been considered as “classical” but are observed in only $\leq 50\%$ of patients, are hydrophobia, aerophobia, photophobia and phonophobia. Immune response can usually be detected after several days of disease onset. As the N protein is present in infected cells in large amounts, anti-N antibodies (non-neutralizing) can be detected by such methods as the indirect fluorescent antibody (IFA) test or the enzyme-linked immunosorbent assay (ELISA). The virus-neutralizing anti-G antibodies can be usually detected a few days later in both serum and cerebrospinal fluid (CSF). Anti-lyssavirus antibodies may be present in the serum of vaccinated patients, whereas their presence in the CSF is indicative of CNS infection (rabies). In most cases, however, the immune response cannot clear lyssavirus infection. Once clinical symptoms appear, death occurs usually within 1–10 days due to cardiac or respiratory failure, although intensive care may prolong the disease beyond a month (26). A few cases of recovery from clinical rabies have been documented in humans and animals (6, 27).

Phylogeny and taxonomy

Until the 1950s, it was believed that RABV was unique as the sole cause of rabies. However, further discoveries of Lagos bat virus (LBV) and Mokola virus (MOKV) in Africa demonstrated the existence of other rhabdoviruses antigenically related to RABV, and the term “rabies serogroup” was introduced (28). Simultaneously, another rabies-related agent, Duvenhage virus (DUVV), was described from South Africa (29). Based on serological and antigenic cross-reactivity, the rabies-related viruses were classified into serotypes. Later, with the advent of monoclonal antibodies for diagnostic and typing purposes, it was demonstrated that “rabies viruses” circulating in European bats were related to DUVV, and they were included in the same serotype (29, 30). Further rigorous studies with extended panels of monoclonal antibodies helped to distinguish between these viruses as well as between European bat viruses themselves. Such terms as “biotypes” appeared in the taxonomic literature for a limited period of time (31). However, the most comprehensive differentiation of rabies-related viruses was achieved via sequencing and comparison of their genes (32). These latter efforts in general confirmed and clarified findings from monoclonal antibody studies, but classification switched from viral serotypes to genotypes, along with the establishment of the genus *Lyssavirus*, where all rabies-related viruses have been included. The existing “genotypes” served as a basis for taxonomy of lyssaviruses for almost two decades, but were refined to satisfy the official rules established by the International Committee on Taxonomy of Viruses (ICTV), which operates with more complex definitions such as viral species.

Increased surveillance has led to the discovery of several novel lyssaviruses during recent decades. At present, the genus includes at least 16 established species and one virus awaiting ICTV assessment (Table 2.1). During 2017, the ICTV introduced a variant of binomial virus nomenclature (33). This will likely evolve in the future, as in the present form it may cause confusion (e.g. rabies virus [a virus] but *Rabies lyssavirus* [a viral species]). Of note, only virus names, but not species names, can be abbreviated (Fig. 2.1). This manual uses virus names when in reference to the viruses, whereas species names are used solely in the

specific taxonomic context. In contrast to other areas of biology, virus names and viral species names cannot be used as synonyms.

Inter-species genetic distances between lyssaviruses are shorter than inter-species genetic distances in other genera of Rhabdoviridae. To accomplish more comprehensive demarcation of lyssavirus species, four major criteria are used:

- (i) Genetic distances, with the threshold of 80–82% nucleotide identity for the complete N gene or 80–81% nucleotide identity for the concatenated coding regions of N+P+M+G+L genes. Globally, all isolates belonging to the same species have higher identity values than the threshold, except the viruses currently included into the Lagos bat lyssavirus species. Based on these observations and on a poor cross-neutralization between isolates from different Lagos bat virus lineages, several authors have suggested subdividing Lagos bat lyssavirus into several separate species (34, 35).
- (ii) Topology and consistency of phylogenetic trees, obtained with various evolutionary models.
- (iii) Antigenic patterns in reactions with anti-NC monoclonal antibodies and serological cross-reactivity using polyclonal antisera.
- (iv) Additional characteristics such as ecological properties, host and geographical range; pathological features are considered, whenever available (7).

More globally, based on genetic distances and serological cross-reactivity, the *Lyssavirus* genus has been subdivided into two main phylogroups (36). The demarcation was based on phylogenetic relationships: $\geq 74\%$ amino acid sequence identity within the G ectodomain between viruses of one phylogroup and $< 64\%$ sequence identity between viruses from different phylogroups; cross-neutralization within phylogroups, but absence of cross-neutralization between members of different phylogroups; the presence of K/R333 in the G of phylogroup I viruses and of D333 in the G of phylogroup II viruses, with the following implication for a reduced pathogenicity for the latter. Generally, serological cross-reactivity correlated somewhat with genetic distances, and sequences that shared more than 72% amino acid sequence identity within their G ectodomains cross-neutralized each other (36).

Several lyssaviruses characterized in the 21st century (e.g. WCBV [West Caucasian bat virus], IKOV [Ikoma lyssavirus] and LLEBV [Lleida bat lyssavirus]) could not be included into either of these groups. Phylogenetically, all three viruses are related, but long genetic distances and apparent absence of cross-neutralization between WCBV, LLEBV and IKOV do not allow placing them into one phylogroup, based on the existing demarcation criteria (Fig. 2.1).

The limited serological cross-reactivity between lyssaviruses from different phylogroups is important from a public health and veterinary perspective. All commercially available rabies biologicals are based on RABV and elicit efficient protection against phylogroup I lyssaviruses but not against other members of the genus, particularly those lyssaviruses in phylogroup 2, WCBV, IKOV and LLEBV (3, 36–38). Moreover, antigenic and genetic distinctions should be considered in application of any diagnostic tests to ensure their ability to detect all divergent lyssaviruses, as described in the relevant chapters of this manual.

Table 2.1. Viruses currently included in the genus *Lyssavirus*

Recognized and proposed species	Phylogroup	Natural host	Geographical range	Comments
<i>Rabies lyssavirus</i> (type species)	I	Bats (Chiroptera) of multiple species, terrestrial mammals (predominantly Carnivora)	Terrestrial mammals – worldwide (except Australia, Antarctica and several insular territories); bats – New World only	Responsible for most cases of human rabies in the world. All currently available human and veterinary vaccine strains originate from viruses of this species.
<i>Australian bat lyssavirus</i>	I	Pteropodid bats (at least four species of <i>Pteropus</i> genus) and insectivorous bats <i>Saccolaimus albiventris</i>)	Australia (perhaps, with several surrounding islands)	Given limited surveillance, host range among insectivorous bats may be greater. Three human cases documented.
<i>European bat lyssavirus, type 1</i>	I	Insectivorous bats (predominantly <i>Eptesicus serotinus</i>)	The major part of Europe, from Spain to the Ukraine	Given the limited surveillance in eastern Europe and Asia, may be distributed more broadly, along the reservoir species range. Spill-over infections in wild and companion animals, as well as a human case have been documented.
<i>European bat lyssavirus, type 2</i>	I	Insectivorous bats (predominantly <i>Myotis daubentonii</i> and <i>Myotis dasycneme</i>)	North-western Europe	Two human cases have been documented.
<i>Khujand lyssavirus</i>	I	Insectivorous bat <i>Myotis mystacinus</i>	Central Asia	Known by a single isolate. Given the limited surveillance in eastern Europe and Asia, may be distributed more broadly. No human cases have been documented.
<i>Aravan lyssavirus</i>	I	Insectivorous bat <i>Myotis blythii</i>	Central Asia	Known by a single isolate. Given the limited surveillance in eastern Europe and Asia, may be distributed more broadly. No human cases have been documented.
<i>Bokeloh bat lyssavirus</i>	I	Insectivorous bat <i>Myotis nattereri</i>	Europe	Known by three isolates from Western Europe. No human cases have been documented.
<i>Irkut lyssavirus</i>	I	Insectivorous bats from <i>Murina</i> genus	Eastern Asia	Known by three isolates, from bats and from a human who developed rabies after a bat bite.
<i>Duvenhage lyssavirus</i>	I	Insectivorous bats	Sub-Saharan Africa	Known by four isolates, three of which came from humans, bitten by bats, and one from a bat, of the presumably <i>Miniopterus</i> species.

Table 2.1. Continued

Recognized and proposed species	Phylogroup	Natural host	Geographical range	Comments
<i>Lagos bat lyssavirus</i>	II	Pteropodid bats of several genera (<i>Eidolon helvum</i> , <i>Rousettus aegyptiacus</i> , <i>Epomophorus</i> spp. etc.)	Sub-Saharan Africa	Includes several lineages with long genetic distances. Potentially, in the future may be subdivided into 2–3 separate species. Spill-over infections reported in wild and companion animals. No human cases documented.
<i>Mokola lyssavirus</i>	II	Unknown	Sub-Saharan Africa	Twice isolated from shrews, once from a rodent. The majority of other isolates were obtained from companion animals, such as cats and dogs, as the result of spill-over infections. Two human cases have been reported.
<i>Shimoni bat lyssavirus</i>	II	Insectivorous bat <i>Hipposideros commersoni</i> (<i>H. vittatus</i>)	Kenya	Known by a single isolate. Serological surveys suggest that <i>Hipposideros commersoni</i> (<i>H. vittatus</i>) is the likely reservoir. No human cases have been documented.
<i>West Caucasian bat lyssavirus</i>		Insectivorous bats from genus <i>Miniopterus</i>	South-eastern Europe, probably Africa	Known by a single isolate from the Caucasian region. However, serological surveys suggest that this or other serologically-related virus may be present in <i>Miniopterus</i> bats in Africa (Kenya). No human cases have been documented.
<i>Ikoma lyssavirus</i>		African civet <i>Civettictis civetta</i>	United Republic of Tanzania	Known by a single isolate. The natural host is questionable. Given phylogenetic relatedness to the West Caucasian bat virus, the index case in African civet may result from a spill-over infection of bat origin. No human cases have been documented.
<i>Lleida bat lyssavirus</i>		Insectivorous bat <i>Miniopterus schreibersii</i>	Spain and France (2017) [unpublished data]	Known by a single isolate. No human cases have been documented.
<i>Gannoruwa bat lyssavirus</i>	I	Indian flying fox <i>Pteropus medius</i> (<i>P. giganteus</i>)	Sri Lanka	Several isolates obtained from the single bat species in Sri Lanka. Given the host range, may be present in the Indian subcontinent and surrounding areas. No human cases have been documented.
<i>Taiwan bat lyssavirus</i>	I	Insectivorous bat <i>Pipistrellus abramus</i>	Taiwan, China	Known by two isolates. Given the host range, may be distributed broadly in eastern Asia. No human cases have been documented.

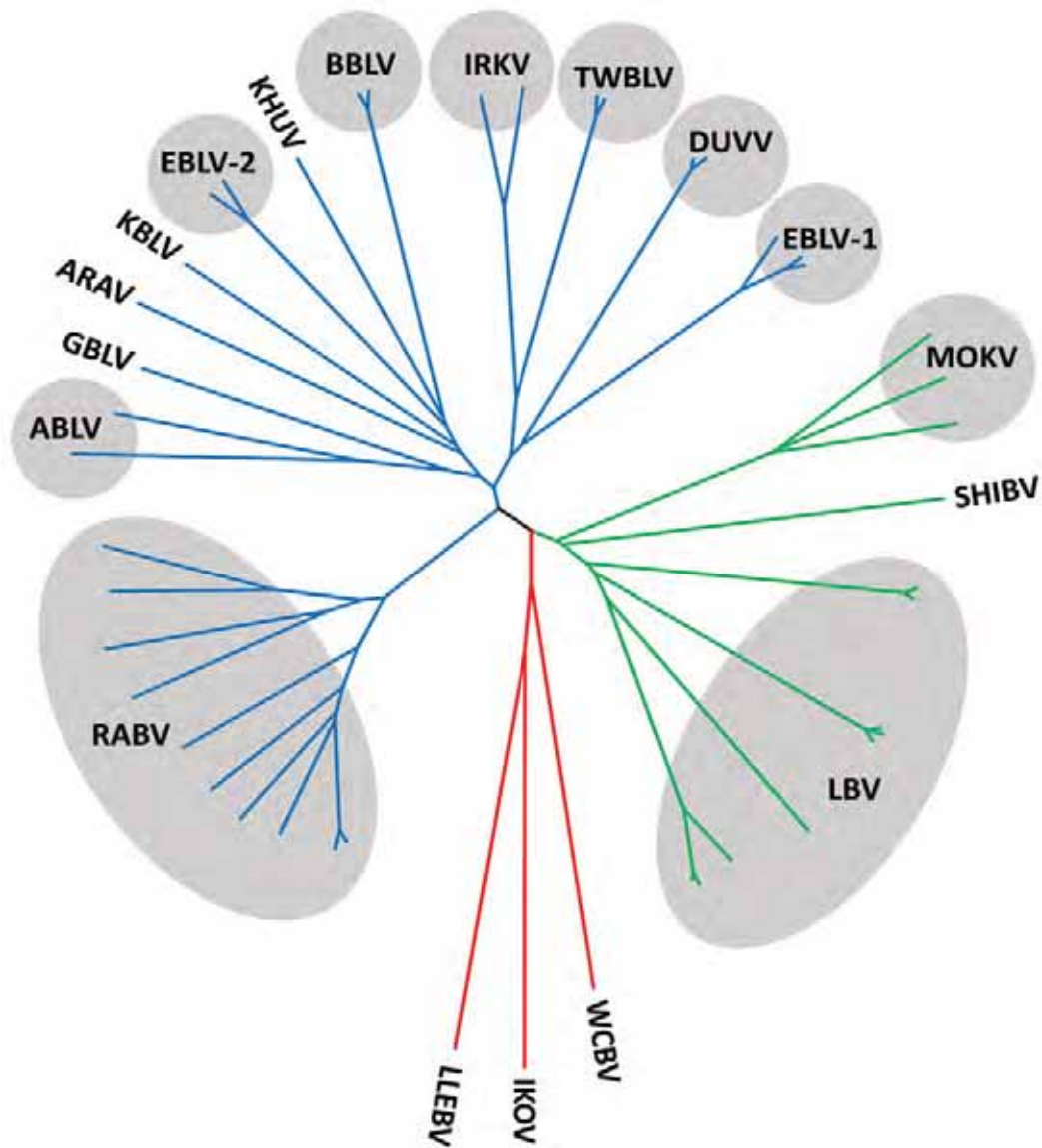


Fig. 2.1. Unrooted phylogenetic tree of currently recognized and putative lyssaviruses (neighbour-joining method, p-distances matrix). Lineage colours correspond to Phylogroup I (blue), Phylogroup II (green) or ungrouped lyssaviruses Phylogroup III (red).

ABLV, Australian bat lyssavirus; ARAV, Aravan virus; BBLV, Bokeloh bat lyssavirus; DUVV, Duvenhage virus; EBLV-1, EBLV-2, European bat lyssaviruses, type 1 and 2; GBLV, Gannoruwa bat lyssavirus; IKOV, Ikoma lyssavirus; IRKV, Irkut virus; KHUV, Khujand virus; LBV, Lagos bat virus; LLEBV, Lleida bat lyssavirus; MOKV, Mokola virus; RABV, rabies virus; SHIBV, Shimoni bat virus; WCBV, West Caucasian bat virus

Distribution, evolution and host range

Lyssaviruses are distributed globally except in Antarctica and several insular islands. In the latter, introduction of the infection is still possible with migratory bats, or via human activity as happened with the introduction of rabid dogs followed by host switching to populations of mongooses (introduced by humans as well) in Grenada (39) and Puerto Rico (40), or the introduction of rabid ferret badgers to Taiwan (41).

Although all warm-blooded animals, including birds (42), are susceptible, mammals from the orders Chiroptera (bats) and Carnivora are the major natural reservoirs in which lyssaviruses perpetuate and evolve (43). The major diversity of lyssaviruses is in bats, whereas RABV, as the most evolutionary successful representative of the genus, is highly adapted to circulation in carnivores. Possible exceptions include IKOV, which was isolated only once from a carnivore (e.g. an African civet), but given the phylogenetic relatedness of IKOV to such viruses as WCBV and LLEBV, it is possible that IKOV is a bat virus (5). Another exception is MOKV, which was isolated several times from shrews, domestic dogs and cats, but it is not known whether any of these animals serve as reservoirs or accidental hosts of the virus (44).

In addition, all lyssavirus diversity has been documented in the Old World but only RABV was detected in the New World. It is remarkable that in the New World, bats of different species maintain circulation of multiple RABV lineages, whereas in the Old World, bats harbour a variety of divergent lyssaviruses but not RABV. Such discrepancies compromise the commonly accepted theory that lyssaviruses evolved in bats and that at least the initial evolution took place in the Old World, likely in Africa where the majority of divergent lyssaviruses are present (43). In particular, if we accept the estimations of lyssavirus age based on molecular clock techniques, which indicate that most RABV lineages emerged tens to hundreds of years ago, then the entire genus is no older than 7000–11 000 years (45–47).

An increasing amount of evidence suggests that timescale estimation performed on a limited set of recently sampled sequences cannot provide realistic inferences for viruses evolving under constraints of purifying selection (48). This is exactly the case for lyssaviruses that evolve neutrally under strong purifying selection constraints (49). In general, evolutionary rates observed in a set of sequences exhibit time dependency and increase towards the present because of the transient mutations yet to be removed by purifying selection. Substitution rates estimated during long time frames will therefore be lower systematically than those obtained during short time frames. This effect is as strong as, or stronger than, purifying selection, which shapes virus evolution (50, 51). In other words, timescale estimations based on substitution rates are useful only for the time frame encompassed by the sampling period, but cannot be extrapolated for longer periods of time. For example, an alternative approach, with improved substitution saturation, increased the age of coronaviruses by several orders of magnitude compared with previous molecular clock estimates, and the resulting timescale was comparable to that of the coronavirus reservoir hosts (52). Perhaps lyssaviruses should be considered more ancient pathogens that may have evolved in bats during the Cretaceous period in the territory of modern Africa (the region from which the bats originated), and that an ancient RABV followed bat dispersion

to South America 50–80 million years ago via island hopping and intercontinental flight (53). This hypothesis (54) might explain an independent evolution of RABV in New World bats and the absence of the virus in related taxa of Old World bats (e.g. from genera *Eptesicus*, *Myotis*). Such New World bat viruses further shifted to carnivores including skunks and raccoons, which are parts of the “indigenous American RABV lineage” and are well distinguished from all RABV lineages circulating in the Old World, and from the “cosmopolitan” canine RABV, which is dispersed globally, with rabid dogs accompanying human migrations during recent centuries (55, 56).

Usually, each lyssavirus phylogenetic lineage is associated with a specific host. These relationships are best studied for RABV, which is distributed globally. The number of phylogenetic lineages of RABV documented in New World bats corresponds roughly to the number of bat species tested. Moreover, some broadly distributed bat species, such as *Eptesicus fuscus* (the big brown bat) and *Tadarida brasiliensis* (the Mexican free-tailed bat), harbour several lineages of RABV (47, 57).

Among wild carnivores, skunks of several different species appear highly permissive to various RABV variants. In addition to the south-central and Mexican skunk RABVs, which are associated phylogenetically with bat viruses, there are several other lineages (i.e. north-central skunk, California skunk and other skunk lineages from Mexico) which belong to a “cosmopolitan” RABV lineage. Skunks in eastern Canada have acquired the Arctic RABV variant (55, 56). Additionally, skunks along the east coast of North America are frequently infected by the raccoon RABV (58, 59).

Conversely, *Procyon lotor* (the North American raccoon) seems well adapted and constrained only to one specific RABV variant, which is believed to have emerged during the 1940s in Florida, USA and thereafter has rapidly spread along the eastern coast of North America (60, 61).

Other American lineages of RABV, circulating in dogs, coyotes (*Canis latrans*) and several species of foxes, belong to the “cosmopolitan” group. Management of canine rabies resulted in elimination of dog-mediated rabies in the USA and Canada, although RABV still circulates in some parts of Latin America. Mongooses in several Caribbean islands acquired the “cosmopolitan” RABV from dogs, and maintain its circulation (56). Oral rabies vaccination of wildlife significantly reduced RABV circulation in their populations in North America. In addition to the representatives of “cosmopolitan” RABV lineage, Arctic RABV circulates among Arctic foxes (*Vulpes lagopus*), red foxes (*V. vulpes*) and striped skunks (*Mephitis mephitis*) in northern territories of the continent (62, 63).

In the Old World, RABV has been documented in carnivores only, with the exception of epizootics in greater kudu antelopes (*Tragelaphus strepsiceros*) in southern Africa (64), predominantly in canids and herpestides (65). The “cosmopolitan” lineage of RABV circulates in dogs and wild canids in vast territories of Eurasia and Africa (55, 56). In addition, distinct “autochthonous” lineages of RABV circulate in dogs in several areas of Africa and south-east Asia. Arctic RABV circulates circumpolarly among Arctic and red foxes. Phylogenetic inferences suggest that the Arctic RABV lineage originated from so-called Arctic-like lineages, which are present in the Indian subcontinent and in central and eastern Asia (63, 66). Raccoon dogs (*Nyctereutes procyonoides*), besides maintaining the circulation of Arctic-like RABV in their native habitats in eastern Asia, readily participate in circu-

lation of “cosmopolitan” RABV in areas enzootic for fox rabies, where this species was successfully introduced. The wolf (*Canis lupus*) was believed historically to be a major wildlife rabies host in Eurasia; however, reductions in its population have diminished the significance of this species in true circulation of RABV (67,68).

Following the rational use of oral vaccination programmes, rabies was eliminated from the red fox populations in Western Europe by the end of the 20th century (69); nevertheless, the disease is still present in red foxes, corsac foxes, jackals and raccoon dogs in Eastern Europe and Asia, which offer a risk of re-introduction of the disease to the countries that are considered “rabies free” (65).

Lyssaviruses of other species have more limited geographical and host ranges than RABV (Table 2.1). For example, several lineages of LBV have been documented in pteropodid bats in sub-Saharan Africa. The elusive DUUV, known by several isolates from humans bitten by insectivorous bats and from unidentified insectivorous bats, circulates in the same geographical region (70, 71). The reservoir host for MOKV is still unknown. Sporadic isolations from dogs and cats are considered spill-over events, and the initial isolations from shrews reported during the 1960s have not been repeated since that time. Serologically, MOKV cross-reacts with LBV, making difficult major inferences from serological surveys alone (44). WCBV has been isolated only once in the Caucasus area from a *Miniopterus schreibersii* bat, but antibodies neutralizing this virus (and not neutralizing a panel of other lyssaviruses) have been detected in *Miniopterus* spp. bats from Kenya (72). The LLEBV nucleic acid has been detected in a *M. schreibersii* bat in Spain (73). IKOV has been isolated once from an African civet in the United Republic of Tanzania, which could be a spill-over infection, given its phylogenetic relatedness to WCBV and LLEBV (5).

European bat lyssaviruses types 1 (EBLV-1) and 2 (EBLV-2) have been documented in several countries of Europe. The principal reservoir of EBLV-1 is the serotine bat (*Eptesicus serotinus*) whereas that of EBLV-2 appears to be bats from the *Myotis* genus, particularly *M. daubentonii* and *M. dasycneme* (74). As all of these bat species are distributed quite broadly in Eurasia, circulation of EBLV-1 and -2 could correspond to their broader host ranges but has gone undetected to date due to limited surveillance (75). Despite extensive (albeit passive) surveillance, another European bat lyssavirus, BBLV (Bokeloh bat lyssavirus), was discovered only during 2011 (76). A few BBLV isolates from Natterer’s bats (*Myotis nattereri*) have been documented in Germany, France, and also more recently in Poland (77).

Two bat lyssaviruses, ARAV (Aravan virus) and KHUV (Khujand virus), one isolate of each, have been obtained in Central Asia, from bats of the *Myotis* genus (78). Another bat lyssavirus, Irkut virus (IRKV), has been isolated twice from bats of the *Murina* genus in Eastern Siberia and China (79, 80) and from a human bitten by an unidentified bat in the eastern Russian Federation (81). Although several reports on serological surveillance have suggested circulation of lyssaviruses among bats in South Eastern Asia (82–84), and at least two papers have described detection of “rabies virus” from bats in India (85) and Thailand (86), no isolates were available for characterization until 2014–2015, when several dead and moribund Indian flying foxes (*Pteropus medius*, also known as *P. giganteus*) in Sri Lanka were diagnosed with a novel virus, Gannoruwa bat lyssavirus (GBLV), which is phylogenetically related to ABLV, the Australian bat lyssavirus (87). Finally, recent reports account for isolation of a lyssavirus from an insectivorous bat (*Pipistrellus abramus*)

in Taiwan, China during 2016–2017 (88, 89). In Australia, ABLV has been isolated or serologically detected in several species of pteropodid bats and viruses from a phylogenetically distinct ABLV lineage isolated from the insectivorous bat, *Sacco-laimus albiventris* (90, 91). Serological surveillance has suggested that lyssaviruses related to ABLV may circulate in South-East Asia more extensively (92).

Applications to disease management

The design of surveillance systems for detection and applicable testing of lyssaviruses should be based in part upon the primary intent of each programme, the available resources and personnel and the staging of disease prevention and control from an evidence-based perspective (93). For example, in countries at the early stage of canine rabies elimination, an essential goal should focus upon relevant risk assessments and the rapid diagnosis of animals that expose humans, preferably by postmortem detection of antigens in brain tissue. Antemortem methods would be appropriate for humans with a suspect encephalitis, especially after a known recent exposure to a suspect animal. Viral antibodies may be detected in sera and CSF, viral antigens in skin biopsy samples and viral amplicons in saliva and related infectious fluids. In cases of acute human death from suspect viral encephalitis, electron microscopy and immunohistochemistry at a national reference centre may be beneficial (94). For localities further advanced towards zero human fatalities from rabies mediated by dogs and thus concerned with broader pathogen discovery and detection of novel lyssaviruses, serological screening may be useful as a preliminary tool to detect viral antibodies associated with various mammalian taxa, such as bats, recognizing that successful introspection is dependent upon the correct choice of viruses or viral antigens as targets (95). Thereafter, a focus on clinically ill mammals (i.e. based on local reports), moribund (i.e. at bat roosts) or those found freshly dead (i.e. road-killed animals) is more productive and efficient than collection of normal subjects (87).

Characterization of lyssaviruses circulating in a particular country or region should utilize appropriate panels of monoclonal antibodies for antigenic typing or relevant protocols of molecular techniques, such as real-time polymerase chain reaction (PCR) and full genomic sequencing (96). From a global perspective, assistance from WHO Collaborating Centres, twinning exercises with OIE reference laboratories and engagement with regional academic or government centres of excellence, skilled in appropriate technical approaches to lyssavirus surveillance, detection and characterization, is readily available, regardless of the stage in a path towards 2030 human rabies elimination goals.

References

1. Tarantola A. Four thousand years of concepts relating to rabies in animals and humans, its prevention and its cure. *Trop Med Infect Dis.* 2017; 2:4. doi:10.3390/tropicalmed2020005.
2. Amarasinghe GK, Bào Y, Basler CF, Bavari S, Beer M, Bejerman N, et al. Taxonomy of the order *Mononegavirales*: update 2017. *Arch Virol.* 2017; 162:2493–2504. doi:10.1007/s00705-017-3311-7.
3. Hanlon CA, Kuzmin IV, Blanton JD, Weldon WC, Manangan JS, Rupprecht CE. Efficacy of rabies biologics against new lyssaviruses from Eurasia. *Virus Res.* 2005; 111:44–54.
4. Horton DL, McElhinney LM, Marston DA, Wood JL, Russell CA, Lewis N, et al. Quantifying antigenic relationships among the lyssaviruses. *J Virol.* 2010; 84:11841–8. doi:10.1128/JVI.01153-10.
5. Horton DL, Banyard AC, Marston DA, Wise E, Selden D, Nunez A, et al. Antigenic and genetic characterization of a divergent African virus, Ikoma lyssavirus. *J Gen Virol.* 2014; 95:1025–32. doi:10.1099/vir.0.061952-0.
6. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 8 May 2018).
7. Dietzgen RG, Calisher CH, Kurath G, Kuzmin IV, Rodriguez LL, Stone DM, et al. Family Rhabdoviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EL, editors. *Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses.* Oxford (UK): Elsevier; 2011:686–714.
8. Tordo N, Badrane H, Bourhy H, Sacramento D. Molecular epidemiology of lyssaviruses: focus on the glycoprotein and pseudogenes. *Onderstepoort J Vet Res.* 1993; 60:315–23.
9. Kuzmin IV, Wu X, Tordo N, Rupprecht CE. Complete genomes of Aravan, Khujand, Irkut and West Caucasian bat viruses, with special attention to the polymerase gene and non-coding regions. *Virus Res.* 2008; 136:81–90. doi:10.1016/j.virusres.2008.04.021.
10. Min BE, Martin K, Wang R, Tafelmeyer P, Bridges M, Goodin M. A host-factor interaction and localization map for a plant-adapted rhabdovirus implicates cytoplasm-tethered transcription activators in cell-to-cell movement. *Mol Plant Microbe Interact.* 2010; 23:1420–32. doi:10.1094/MPMI-04-10-0097.
11. Albertini A, Bressanelli S, Lepault J, Gaudin Y. Structure and working of viral fusion machinery. *Curr Top Membr.* 2011; 68:49–80. doi:10.1016/B978-0-12-385891-7.00003-9.
12. Albertini AA, Ruigrok RW, Blondel D. Rabies virus transcription and replication. *Adv Virus Res.* 2011;79:1–22. doi:10.1016/B978-0-12-387040-7.00001-9.

13. Lyles DS, Kuzmin IV, Rupprecht CE. Rhabdoviridae. In: Knipe DM, Howley PM, editors. *Fields virology*, 6th edition. Philadelphia (PA): Lippincott Williams & Wilkins; 2013:885–922.
14. Charlton KM, Nadin-Davis S, Casey GA, Wandeler AI. The long incubation period in rabies: delayed progression of infection in muscle at the site of exposure. *Acta Neuropathol.* 1997; 94:73–7.
15. Chenik M, Chebli K, Blondel D. Translation initiation at alternate in-frame AUG codons in the rabies virus phosphoprotein mRNA is mediated by a ribosomal leaky scanning mechanism. *J Virol.* 1995; 69:707–12.
16. Negri A. Contributo allo studio dell eziologia della rabia [Contribution to the study of the etiology of rabies]. *Bol Soc Med Chir.* 1903; 2:88–114 (in Italian).
17. Lahaye X, Vidy A, Pomier C, Obiang L, Harper F, Gaudin Y, et al. Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication. *J Virol.* 2009; 83:7948–58. doi:10.1128/JVI.00554-09.
18. Okumura A, Harty RN. Rabies virus assembly and budding. *Adv Virus Res.* 2011;79:23–32. doi:10.1016/B978-0-12-387040-7.00002-0.
19. Constantine DG. Rabies transmission by air in bat caves. Washington (DC): Public Health Service Publication; 1967.
20. Smith JS, Fishbein DB, Rupprecht CE, Clark K. Unexplained rabies in three immigrants in the United States: a virologic investigation. *N Engl J Med.* 1991; 324:205–11.
21. Boland TA, McGuone D, Jindal J, Rocha M, Cumming M, Rupprecht CE, et al. Phylogenetic and epidemiologic evidence of multiyear incubation in human rabies. *Ann Neurol.* 2014; 75:155–60. doi:10.1002/ana.24016.
22. Hanlon CA, Smith JS, Anderson GR. Recommendations of a national working group on prevention and control of rabies in the United States. Article II: Laboratory diagnosis of rabies. The National Working Group on Rabies Prevention and Control. *J Am Vet Med Assoc.* 1999; 215:1444–6.
23. Baer GM. Vampire bat and bovine paralytic rabies. In: Baer GM, editor. *The natural history of rabies*, 2nd edition. Boca Raton (FL): CRC Press; 1991:390–406.
24. Bronnert J, Wilde H, Tepsumethanon V, Lumlertdacha B, Hemachudha T. Organ transplantations and rabies transmission. *J Travel Med.* 2007;14:177–80.
25. Gong C, Li X, Luo M, Zhang Z, Wang Q, Wang Q, et al. Rabies transmission following organ transplantation in China. *J Infect.* 2017; 74:427–31. doi:10.1016/j.jinf.2017.01.008.
26. Jackson AC, Fu ZF. Pathogenesis. In: Jackson AC, editor. *Rabies*, 3rd edition. Elsevier (Academic Press); 2013:299–349.
27. Hamir AN, Niezgodna M, Rupprecht CE. Recovery from and clearance of rabies virus in a domestic ferret. *J Am Assoc Lab Anim Sci.* 2011; 50:248–51.

28. Shope RE, Murphy FA, Harrison AK, Causey OR, Kemp GE, Simpson DI, et al. Two African viruses serologically and morphologically related to rabies virus. *J Virol.* 1970; 6:690–2.
29. Schneider LG. Antigenic variants of rabies virus. *Comp Immunol Microbiol Infect Dis.* 1982; 5:101–7.
30. Schneider LG, Barnard BJH, Schneider HP. Application of monoclonal antibodies for epidemiological investigations and oral vaccination studies: I. African viruses. In: Kuwert E, Merieux C, Koprowski H, Bogel K, editors. *Rabies in the tropics.* Berlin, Germany: Springer-Verlag; 1985:49–53.
31. King A, Davis P, Lawrie A. The rabies viruses of bats. *Vet Microbiol.* 1990;23:165–74.
32. Bourhy H, Kissi B, Tordo N. Molecular diversity of the Lyssavirus genus. *Virology.* 1993;194:70–81.
33. Afonso CL, Amarasinghe GK, Bányai K, Bào Y, Basler CF, Bavari S, et al. Taxonomy of the order *Mononegavirales*: update 2016. *Arch Virol.* 2016; 161:2351–60.
34. Markotter W, Kuzmin I, Rupprecht CE, Nel LH. Phylogeny of Lagos bat virus: challenges for lyssavirus taxonomy. *Virus Res.* 2008;135:10–21. doi:10.1016/j.virusres.2008.02.001.
35. Delmas O, Holmes EC, Talbi C, Larrous F, Dacheux L, Bouchier C, et al. Genomic diversity and evolution of the lyssaviruses. *PLoS One.* 2008; 3:e2057. doi:10.1371/journal.pone.0002057.
36. Badrane H, Bahloul C, Perrin P, Tordo N. Evidence of two lyssavirus phylogroups with distinct pathogenicity and immunogenicity. *J Virol.* 2001; 75:3268–76.
37. Bahloul C, Jacob Y, Tordo N, Perrin P. DNA-based immunization for exploring the enlargement of immunological cross-reactivity against the lyssaviruses. *Vaccine.* 1998; 16:417–25.
38. Jallet C, Jacob Y, Bahloul C, Drings A, Desmezieres E, Tordo N, et al. Chimeric lyssavirus glycoproteins with increased immunological potential. *J Virol.* 1999; 73:225–33.
39. Zieger U, Marston DA, Sharma R, Chikweto A, Tiwari K, Sayyid M, et al. The phylogeography of rabies in Grenada, West Indies, and implications for control. *PLoS Negl Trop Dis.* 2014; 8:e3251. doi:10.1371/journal.pntd.0003251.
40. Everard CO, Everard JD. Mongoose rabies in the Caribbean. *Ann N Y Acad Sci.* 1992; 653:356–66.
41. Lin YC, Chu PY, Chang MY, Hsiao KL, Lin JH, Liu HF. Spatial temporal dynamics and molecular evolution of re-emerging rabies virus in Taiwan. *Int J Mol Sci.* 2016; 17:392. doi:10.3390/ijms17030392.
42. Baby J, Mani RS, Abraham SS, Thankappan AT, Pillai PM, Anand AM, et al. Natural rabies infection in a domestic fowl (*Gallus domesticus*): a report from India. *PLoS Negl Trop Dis.* 2015; 9:e0003942. doi:10.1371/journal.pntd.0003942.

43. Rupprecht CE, Turmelle A, Kuzmin IV. A perspective on lyssavirus emergence and perpetuation. *Curr Opin Virol.* 2011; 1:662–70. doi:10.1016/j.coviro.2011.10.014.
44. Coertse J, Markotter W, le Roux K, Stewart D, Sabeta CT, Nel LH. New isolations of the rabies-related Mokola virus from South Africa. *BMC Vet Res.* 2017; 13:37. doi:10.1186/s12917-017-0948-0.
45. Badrane H, Tordo N. Host switching in Lyssavirus history from the Chiroptera to the Carnivora orders. *J Virol.* 2001; 75:8096–104.
46. Davis PL, Bourhy H, Holmes EC. The evolutionary history and dynamics of bat rabies virus. *Infect Genet Evol.* 2006;6:464–73.
47. Kuzmina NA, Kuzmin IV, Ellison JA, Taylor ST, Bergman DL, Dew B, et al. A reassessment of the evolutionary timescale of bat rabies viruses based upon glycoprotein gene sequences. *Virus Genes.* 2013; 47:305–10. doi:10.1007/s11262-013-0952-9.
48. Wertheim JO, Kosakovsky Pond SL. Purifying selection can obscure the ancient age of viral lineages. *Mol Biol Evol.* 2011; 28:3355–65. doi:10.1093/molbev/msr170.
49. Holmes EC, Woelk CH, Kassis R, Bourhy H. Genetic constraints and the adaptive evolution of rabies virus in nature. *Virology.* 2002; 292:247–57.
50. Duchêne S, Duchêne D, Holmes EC, Ho SY. The performance of the date-randomization test in phylogenetic analyses of time-structured virus data. *Mol Biol Evol.* 2015; 32:1895–906. doi:10.1093/molbev/msv056.
51. Holmes EC. Complexities of estimating evolutionary rates in viruses. *J Virol.* 2016; 90:2155. doi:10.1128/JVI.02570-15.
52. Wertheim JO, Chu DK, Peiris JS, Kosakovsky Pond SL, Poon LL. A case for the ancient origin of coronaviruses. *J Virol.* 2013; 87:7039–45. doi:10.1128/JVI.03273-12.
53. Eick GN, Jacobs DS, Matthee CA: A nuclear DNA phylogenetic perspective on the evolution of echolocation and historical biogeography of extant bats (Chiroptera). *Mol Biol Evol.* 2005; 22:1869–86.
54. Rupprecht C, Kuzmin I, Meslin F. Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies. *F1000Res.* 2017; 23:184. doi:10.12688/f1000research.10416.1.
55. Smith JS, Orciari LA, Yager PA, Seidel HD, Warner CK. Epidemiologic and historical relationships among 87 rabies virus isolates as determined by limited sequence analysis. *J Infect Dis.* 1992; 166:296–307.
56. Nadin-Davis SA, Real LA. Molecular phylogenetics of the lyssaviruses: insights from a coalescent approach. *Adv Virus Res.* 2011; 79:203–38. doi:10.1016/B978-0-12-387040-7.00011-1.
57. Streicker DG, Altizer SM, Velasco-Villa A, Rupprecht CE. Variable evolutionary routes to host establishment across repeated rabies virus host shifts among bats. *Proc Natl Acad Sci U S A.* 2012; 109:19715–20. doi:10.1073/pnas.1203456109.

58. Blanton JD, Palmer D, Rupprecht CE. Rabies surveillance in the United States during 2009. *J Am Vet Med Assoc.* 2010; 237:646–57. doi:10.2460/javma.237.6.646.
59. Wallace RM, Gilbert A, Slate D, Chipman R, Singh A, Cassie Wedd, et al. Right place, wrong species: a 20-year review of rabies virus cross species transmission among terrestrial mammals in the United States. *PLoS One.* 2014; 9:e107539. doi:10.1371/journal.pone.0107539.
60. Biek R, Henderson JC, Waller LA, Rupprecht CE, Real LA. A high-resolution genetic signature of demographic and spatial expansion in epizootic rabies virus. *Proc Natl Acad Sci U S A.* 2007; 104:7993–8. doi:10.1073/pnas.0700741104.
61. Nadin-Davis SA, Colville A, Trewby H, Biek R, Real L. Application of high-throughput sequencing to whole rabies viral genome characterisation and its use for phylogenetic re-evaluation of a raccoon strain incursion into the province of Ontario. *Virus Res.* 2017; 232:123–33. doi:10.1016/j.virusres.2017.02.007.
62. Nadin-Davis SA, Muldoon F, Wandeler AI. Persistence of genetic variants of the arctic fox strain of *Rabies virus* in southern Ontario. *Can J Vet Res.* 2006; 70:11–9. PMID:16548327.
63. Kuzmin IV, Hughes GJ, Botvinkin AD, Gribencha SG, Rupprecht CE. Arctic and Arctic-like rabies viruses: distribution, phylogeny and evolutionary history. *Epidemiol Infect.* 2008; 136:509–19. doi:10.1017/S095026880700903X.
64. Scott TP, Fischer M, Khaiseb S, Freuling C, Höper D, Hoffmann B, et al. Complete genome and molecular epidemiological data infer the maintenance of rabies among kudu (*Tragelaphus strepsiceros*) in Namibia. *PLoS One.* 2013; 8:e58739. doi:10.1371/journal.pone.0058739.
65. Rupprecht CE, Slate D. Rabies prevention and control: advances and challenges. In: Dietzgen RG, Kuzmin IV, editors. *Rhabdoviruses: molecular taxonomy, evolution, genomics, ecology, host–vector interactions, cytopathology and control.* London (UK): Caister Academic Press; 2012:215–52.
66. Nadin-Davis SA, Turner G, Paul JP, Madhusudana SN, Wandeler AI. Emergence of Arctic-like rabies lineage in India. *Emerg Infect Dis.* 2007; 13:111–6. doi:10.3201/eid1301.060702.
67. Bourhy H, Kissi B, Audry L, Smreczak M, Sadkowska-Todys M, Kulonen K, et al. Ecology and evolution of rabies virus in Europe. *J Gen Virol.* 1999; 80:2545–57. doi:10.1099/0022-1317-80-10-2545.
68. Kuzmin IV, Botvinkin AD, McElhinney LM, Smith JS, Orciari LA, Hughes GJ, et al. Molecular epidemiology of terrestrial rabies in the former Soviet Union. *J Wildl Dis.* 2004; 40:617–31.
69. Pastoret PP, Brochier B. Epidemiology and control of fox rabies in Europe. *Vaccine.* 1999; 17:1750–4. doi:10.1016/S0264-410X(98)00446-0.
70. Markotter W, Van Eeden C, Kuzmin IV, Rupprecht CE, Paweska JT, Swanepoel R, et al. Epidemiology and pathogenicity of African bat lyssaviruses. *Dev Biol (Basel).* 2008; 131:317–25.

71. van Thiel PP, de Bie RM, Eftimov F, Tepaske R, Zaaijer HL, van Doornum GJ, et al. Fatal human rabies due to Duvenhage virus from a bat in Kenya: failure of treatment with coma-induction, ketamine, and antiviral drugs. *PLoS Negl Trop Dis.* 2009; 3:e428. doi:10.1371/journal.pntd.0000428.
72. Kuzmin IV, Niezgodá M, Franka R, Agwanda B, Markotter W, Beagley JC, et al. Possible emergence of West Caucasian bat virus in Africa. *Emerg Infect Dis.* 2008; 14:1887–9. doi:10.3201/eid1412.080750.
73. Aréchéga Ceballos N, Vázquez Morón S, Berciano JM, Nicolás O, Aznar López C, Juste J, et al. Novel lyssavirus in bat, Spain. *Emerg Infect Dis.* 2013; 19:793–5. doi:10.3201/eid1905.121071.
74. Banyard AC, Hayman D, Johnson N, McElhinney L, Fooks AR. Bats and lyssaviruses. *Adv Virus Res.* 2011; 79:239–89. doi:10.1016/B978-0-12-387040-7.00012-3.
75. Kuzmin IV, Botvinkin AD, Poleschuk EM, Orciari LA, Rupprecht, CE. Bat rabies surveillance in the former Soviet Union. *Dev Biol (Basel).* 2006; 125:273–82. PMID:16878485.
76. Freuling CM, Beer M, Conraths FJ, Finke S, Hoffmann B, Keller B, et al. Novel lyssavirus in Natterer's bat, Germany. *Emerg Infect Dis.* 2011; 17:1519–22. doi:10.3201/eid1708.110201.
77. Picard-Meyer E1, Servat A, Robardet E, Moinet M, Borel C, Cliquet F. Isolation of Bokeloh bat lyssavirus in *Myotis nattereri* in France. *Arch Virol.* 2013; 158:2333–40. doi:10.1007/s00705-013-1747.
78. Kuzmin IV, Orciari LA, Arai YT, Smith JS, Hanlon CA, Kameoka Y, et al. Bat lyssaviruses (Aravan and Khujand) from Central Asia: phylogenetic relationships according to N, P and G gene sequences. *Virus Res.* 2003; 97:65–79. PMID:14602198.
79. Botvinkin AD, Poleschuk EM, Kuzmin IV, Borisova TI, Gazaryan SV, Yager P, et al. Novel lyssaviruses isolated from bats in Russia. *Emerg Infect Dis.* 2003; 9:1623–5. doi:10.3201/eid0912.030374.
80. Liu Y, Zhang S, Zhao J, Zhang F, Hu R. Isolation of Irkut virus from a *Murina leucogaster* bat in China. *PLoS Negl Trop Dis.* 2013; 7:e2097. doi:10.1371/journal.pntd.0002097.
81. Leonova GN, Belikov SI, Kondratov IG, Krylova NV, Romanova EV, Pavlenko EV. A fatal case of bat lyssavirus infection in Primorye Territory of the Russian Far East. *Rabies Bull Europe.* 2009; 4:5–8.
82. Reynes JM, Molia S, Audry L, Hout S, Ngin S, Walston J, et al. Serologic evidence of lyssavirus infection in bats, Cambodia. *Emerg Infect Dis.* 2004; 10:2231–4. doi:10.3201/eid1012.040459.
83. Kuzmin IV, Niezgodá M, Carroll D S, Keeler N, Hossain MJ, Breiman RF, et al. Lyssavirus surveillance in bats, Bangladesh. *Emerg Infect Dis.* 2006;12:486–8. doi:10.3201/eid1203.050333.
84. Lumlertdacha B, Boongird K, Wanghongsa S, Wacharapluesadee S, Chanhome L, Khawplod P, et al. Survey for bat lyssaviruses, Thailand. *Emerg Infect Dis.* 2005;11:232–6. doi:10.3201/eid1102.040691.

85. Pal SR, Arora BM, Chuttani PN, Broer S, Choudhury S, Joshi RM. Rabies virus infection of a flying fox bat, *Pteropus poliocephalus*, in Chandigarh, Northern India. *Trop Geogr Med*. 1980; 32:265–7. PMID:7210163.
86. Smith PC, Lawhaswasd, K, Vick WE, Stanton JS. Isolation of rabies virus from fruit bats in Thailand. *Nature*. 1968; 216:384. doi:10.1038/216384a0.
87. Gunawardena PS, Marston DA, Ellis RJ, Wise EL, Karawita AC, Breed AC, et al. Lyssavirus in Indian flying foxes, Sri Lanka. *Emerg Infect Dis*. 2016; 22:1456–9. doi:10.3201/eid2208.151986.
88. Novel bat lyssavirus – Taiwan. ProMed Mail (<http://www.promedmail.org/post/20161114.4627752>, accessed 23 April 2018). [[published manuscript to be inserted](#)].
89. Bat lyssavirus – Taiwan. ProMed Mail (<https://www.promedmail.org/post/4978769>, accessed 23 April 2018). [[published manuscript to be inserted](#)].
90. Gould AR, Hyatt AD, Lunt R, Kattenbelt JA, Hengstberger S, Blacksell SD. Characterization of a novel lyssavirus isolated from Pteropid bats in Australia. *Virus Res*. 1998; 54:165–87. PMID:9696125.
91. Gould AR, Kattenbelt JA, Gumley SG, Lunt RA. Characterisation of an Australian bat lyssavirus variant isolated from an insectivorous bat. *Virus Res*. 2002; 89:1–28. PMID:12367747.
92. Arguin PM, Murray-Lillibridge K, Miranda ME, Smith JS, Calaor AB, Rupprecht CE. Serologic evidence of lyssavirus infections among bats, the Philippines. *Emerg Infect Dis*. 2002; 8:258–62. doi:10.3201/eid0803.010330.
93. Human rabies: 2016 updates and call for data. *Wkly Epidemiol Rec*. 2017; 92:77–86.
94. Wohlsein P, Baumgärtner W, Kreipe HH, Haverich A, Hori A, Stan AC. [Rabies transmission through organ transplantation]. *Pathologe*. 2011; 32:406–10 (in German). doi:10.1007/s00292-011-1452-0.
95. Mani RS, Dovich DP, Ashwini MA, Chattopadhyay B, Harsha PK, Garg KM, et al. Serological evidence of lyssavirus infection among bats in Nagaland, a north-eastern state in India. *Epidemiol Infect*. 2017; 145:1635–41. doi:10.1017/S0950268817000310.
96. Brunker K, Marston DA, Horton DL, Cleaveland S, Fooks AR, Kazwala R, et al. Elucidating the phylodynamics of endemic rabies virus in eastern Africa using whole-genome sequencing. *Virus Evol*. 2015; 1:vev011.

Chapter 3

Biosafety

Introduction

Lyssaviruses pose occupational risks for staff tasked with their detection, characterization and production. Multiple publications describe the precautions for staff in laboratories working with such infectious agents (1–5). This chapter discusses relevant properties of lyssaviruses and precautions to protect laboratory personnel, by a combination of facility design, equipment, supplies, techniques and immunizations.

Laboratory incidents

Despite potential occupational hazards, relatively few laboratory-acquired cases of rabies have been reported. In the 21st century, no deaths in personnel at risk have been documented, despite multiple punctures or other exposures while handling virus in hundreds of diagnostic, research and vaccine production laboratories. Appropriate wound cleansing and availability of efficacious rabies vaccine booster doses are crucial to the prevention of infections after exposure. During the 20th century, one death was reported during 1972 in a laboratory worker from the USA, who was engaged in the preparation of vaccine from infected animal brains and was exposed to laboratory-adapted rabies virus via an aerosol from a blender (6). Another laboratory infection from a laboratory-adapted rabies virus aerosol occurred in the USA during 1977; the victim survived, albeit with severe residual mental impairment, for approximately 40 years (7). These laboratory aerosol exposures occurred when higher titred virus samples were handled outside of the recommended laboratory containment requirements for these manipulations.

The general recommendations in this chapter provide guidance on logical steps to prevent untoward laboratory incidents and recommendations on actions to take if they do occur.

Agents

Lyssaviruses belong to the genus, family Rhabdoviridae. They are enveloped, bullet-shaped virions containing proteins, lipid and single-stranded RNA (see [Chapter 2](#)). Such viruses are sensitive to detergents and lipid solvents (e.g. soap solutions, ether, chloroform, acetone), alcohols, iodine preparations and quaternary ammonium compounds (8). However, caution should be exercised when handling acetone-fixed brain impression slides, since acetone may not completely inactivate virus during routine tissue fixation times (9). Other relevant proper-

ties include: resistance to drying and repeated freezing and thawing; relative stability at pH 5–10; and sensitivity to pasteurization temperatures, ultraviolet light and formalin-fixation. The viruses are inactivated by β -propiolactone (BPL) and gamma irradiation (10), but are more resistant to 0.25–0.5% phenol (i.e. as used historically in the production of Semple-type vaccines), where several days are required to obtain complete inactivation (11).

Exposure

Lyssavirus exposure usually occurs by a bite wound, although penetration can occur through intact mucous membranes and the gastrointestinal tract, but not through intact skin (12). As a model of temporal dynamics, in tissue-culture systems virus penetrates the cell within ~10–15 min, after which neutralization by specific antiserum is ineffective (13). Airborne infection is possible under exceptional circumstances, for example, in nature within maternity caves inhabited by millions of bats (14). Marked differences are apparent between different strains of virus in their ability to infect, spread within the body and produce disease (15, 16). Laboratory strains of “fixed” virus, used to produce vaccines or in diagnostic and research procedures, have lower pathogenicity when inoculated peripherally in low doses. Fermi-type vaccines, as well as the original Pasteur vaccine, contained residual rabies virus. Such vaccines were used for many decades but are no longer recommended by the WHO Expert Consultation on Rabies. Fatal incidents occurred in humans when such fixed laboratory virus vaccines were not properly inactivated to reduce their viral content, and large amounts of material of a high titre were injected (17). Laboratory incidents involving tiny puncture wounds may be less dangerous when laboratory-adapted or modified attenuated virus is handled by vaccinated personnel. Such incidents need not cause undue concern if appropriate wound treatment and booster vaccine doses are administered after exposure. This includes all fixed or modified rabies viruses, such as SAD (Street Alabama Dufferin), ERA (Evelyn Rokitnicki Abelseth), CVS (Challenge Virus Standard), LEP (low egg passage) and HEP (high egg passage) strains and their derivatives. Non-laboratory-adapted, wild-type rabies virus (“street virus”), whether in tissue, saliva or infected cell cultures, should always be handled with extreme caution, despite apparent differences in the ability of different variants of street virus to infect through peripheral inoculation. This ability to infect and cause disease is mainly a function of virus route and dosage, in that there appears to be a threshold below which disease is not produced. For example, the susceptibility of humans to small amounts of street rabies virus is apparently not as great as that of other species, such as foxes and livestock, but since human infection has been known to occur even after relatively minor bite wounds (e.g. on the fingers), it is prudent to consider all wounds contaminated with street virus as potentially dangerous. In any event, all laboratory personnel should receive pre-exposure vaccination to minimize risk from any unknown exposures and prompt booster doses of vaccine administered after recognized exposures, to allow for rapid anamnestic responses (18).

Laboratory precautions

All laboratories should have their own dedicated standard operating procedures for handling infective substances based upon national or international guidelines (1–5). These institutional and lyssavirus-specific safety procedures should be accessible to all personnel in an annually reviewed biosafety manual. General recommendations in this chapter are given for procedures in consideration of the major laboratory operations employed with lyssaviruses.

Facility

Laboratory facilities should be designed with quality standards, biosafety and biosecurity in mind. Lyssaviruses are defined as risk group 2 or 3 depending on the jurisdiction; depending on local risk assessments, they may be handled in biosafety level (BSL) 2 or 3 facilities (1, 19, 20). Practices that require production of large volumes of infectious material, procedures that may generate an aerosol or work with non-phylogroup I lyssaviruses divergent from human biologicals used for prophylaxis may require consideration of BSL 3 practices. Use of a class II biosafety cabinet (BSC) is recommended for any procedures that may produce an aerosol or for techniques employed to produce lyssaviruses within cell culture. Access to such facilities should be restricted to those personnel vaccinated against rabies.

Personal protective equipment

Any necropsy operations requiring the opening of skulls or situations in which sharp material could be encountered (broken glassware, bone) should be performed by experienced personnel wearing thick protective gloves, sleeved gowns, safety glasses or goggles and a face shield (Fig. 3.1). Wrap-around safety glasses or goggles and a face shield provide adequate protection to prevent splashes of fluid from contacting mucous membranes. Use of a N95 mask minimizes the potential for aerosol exposure, when applicable work must be conducted outside of a BSC. Rubber or plastic aprons that can easily be disinfected or discarded may also be worn. Closed-front laboratory coats, gowns or coveralls are recommended. Close-fitting heavy rubber gloves or double gloves should be worn when suspect animals are being necropsied (Fig. 3.2). Shoe covers or rubber boots should be worn in the necropsy area. In the laboratory, the minimum personal protective equipment (PPE) should include a laboratory coat or gown, gloves and eye protection (Fig. 3.3A). Long hair should be secured. Open-toed shoes are prohibited. Piercings should be covered with medical tape.



Fig. 3.1. Personal protective equipment recommended for necropsy areas: safety glasses, face shield, closed-front gown, dedicated footwear (rubber boots) and heavy rubber gloves



Fig. 3.2. Protective gloves recommended for necropsy areas: (A) heavy rubber glove, (B) cut-resistant glove, (C) laboratory glove

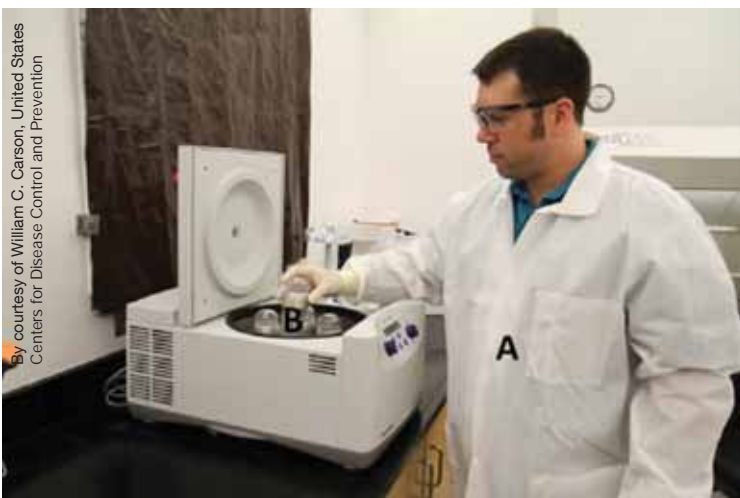


Fig. 3.3. Personal protective equipment recommended for laboratory work: (A) laboratory coat or gown, gloves and eye protection; (B) aerosol containment carriers required for centrifugation of infectious materials

Aerosols

Since airborne rabies virus infection has been demonstrated in nature and in the laboratory, all operations which have a potential to cause aerosols should be performed within a class II BSC that is certified annually. High-speed mixing procedures should be conducted in tightly closed containers with gaskets within an appropriate BSC, and high-speed centrifugation should be conducted using aerosol containment carriers that are only opened and manipulated under the BSC (Fig. 3.3B). Other operations that might generate aerosols, such as pipetting and tissue homogenization, should also be conducted within a BSC. Pipetting by mouth should be prohibited.

Disinfectants

Quaternary ammonium disinfectants in 1:256 dilution, 70% alcohol (ethanol or isopropanol), 1% soap solution, 5–7% iodine solutions and oxidizing agents inactivate lyssaviruses within minutes (8, 21). Additional contact times are required to inactivate virus on materials, instruments or surfaces heavily contaminated with proteins found in cell culture media and tissues (1, 22, 23). Commercial quaternary ammonium disinfectants, iodophors and oxidizing agents should be diluted according to the registered label for hard surface decontamination (1, 21). For pipette receptacles, a 1:1000 dilution of a quaternary ammonium compound, any iodine disinfectant with residual available iodine of at least 1:10000 or a 1% concentration of soapy water or detergent can be used before autoclaving. The solution should be autoclaved and discarded after each use. Hot soapy water, detergents or diluted bleach solutions can be used to clean floors and laboratory tables. Standard operating procedures for containing and cleanup of spills should be included in the site-specific lyssavirus biosafety manual and training provided on these procedures to all personnel. Incident records for spills and other hazardous occurrences should be maintained.

Glassware, plasticware and instruments

Proper supplies should be selected to minimize the opportunity for injury by sharps. When possible, alternatives to glassware such as plastics should be substituted. Sharps should be discarded into proper containers or appropriate receptacles containing one of the disinfectants mentioned above, and autoclaved before reuse or disposal.

Carcasses and animal tissues

Carcasses and animal tissues are best disposed of within biohazard bags and then incinerated. Rabies virus may be transmitted orally in laboratory animals when raw brain tissue containing virus is consumed (12). Although thorough cooking will inactivate virus, consumption of meat from animals, such as livestock, submitted to the diagnostic laboratory, should be prohibited.

Treatment of wounds

All wounds should be washed immediately and thoroughly for at least 10–15 min with soap and water. Washing should be gentle to avoid further traumatization of the tissues. Suturing of wounds should be delayed for as long as possible (from several hours up to 3 days). Puncture wounds should be probed gently for debris, taking care to minimize further trauma. Local infiltration of wounds should be treated with rabies immunoglobulin in the naive patient (although all staff working in the rabies laboratory should have received pre-exposure vaccination). Additional measures, such as administration of antimicrobials or antitetanus procedures, when indicated, should follow local medical guidance.

Pre-exposure vaccination

Pre-exposure vaccination should be required for all persons at risk of exposure, such as laboratory staff working with lyssaviruses. Such immunization should preferably consist of at least three intramuscular or intradermal doses of tissue-culture rabies vaccine of a potency of at least 2.5 international units (IU) per dose. Vaccination should occur according to the product label or by a schedule as recommended by the current WHO Expert Consultation on Rabies (18). The presence of virus-neutralizing antibodies (VNA) in vaccinated individuals should be ascertained where feasible, using serum samples collected 1–3 weeks after the last vaccine dose.

Based on serological surveillance, periodic booster injections of vaccine are recommended for persons at continuing risk of exposure to virus. The following guidelines are recommended for determining when boosters should be administered:

All persons who work with live virus in a diagnostic, research or vaccine production laboratory should have a serum sample tested for VNA every 6 months and a booster vaccination administered when the titre falls below 0.5 IU/mL; this is the minimum recommended level indicative of adequate immunization. Responsible authorities should ensure that all staff are properly immunized. All other persons at continuing risk of exposure to lyssaviruses should have a serum sample tested for VNA about every 2 years; a booster vaccination should be administered when the titre falls below 0.5.

Post-exposure prophylaxis

All previously vaccinated staff exposed to a lyssavirus should receive local wound treatment and two booster doses of vaccine on days 0 and 3.

Other considerations

Biosafety in the lyssavirus laboratory has been discussed in this chapter. Other safety issues, such as processing CNS tissues which may be infected by other pathogens such as arboviruses or prions, the storage and use of hazardous chemicals, and potential mechanical, electrical and steam hazards, are beyond the focus of this chapter. A dynamic laboratory safety programme will be inclusive and adaptable to include such other generic considerations, irrespective of the biohazard under consideration. As new laboratory molecular techniques are incorporated into routine diagnosis, the choice of appropriate disinfectant will depend upon more than effectiveness for inactivating virus during routine decontamination of surfaces, necropsy instruments and reusable material, but will also require consideration of the ability to destroy nucleic acids to avoid cross-contamination and false-positive results (22). Other biosafety challenges will undoubtedly occur in the future.

References

1. Chosewood LC, Wilson DE, eds. Biosafety in microbiological and biomedical laboratories, 5th edition. Washington (DC): Government Printing Office; 2009 (United States Department of Health and Human Services Publication No. (CDC) 21–111; <http://www.cdc.gov/biosafety/publications/bmbl5/index.htm/>, accessed 25 April 2018).
2. Canadian biosafety handbook, 2nd edition. Government of Canada; 2016 (<https://www.canada.ca/en/public-health/services/canadian-biosafety-standards-guidelines/handbook-second-edition.html>, accessed 25 April 2018).
3. Laboratory biosafety manual, 3rd edition. Geneva: World Health Organization; 2004 (<http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf>, accessed 25 April 2018).
4. Biosafety and biosecurity: standard for managing biological risk in the veterinary laboratory and animal facilities. In: OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris: World Organisation for Animal Health; 2015:1–16 [Chapter 1.1.4] (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.04_BIOSAFETY_BIOSECURITY.pdf, accessed 25 April 2018).
5. Biological agents: managing the risks in laboratories and healthcare premises. Advisory Committee on Dangerous Pathogens. United Kingdom Health and Safety Executive; 2005 (<http://www.hse.gov.uk/biosafety/biologagents.pdf>, accessed 25 April 2018).
6. Winkler WG, Fashinell TR, Leffingwell L, Howard P, Conomy P. Airborne rabies transmission in a laboratory worker. JAMA. 1973; 226:1219–21. PMID:4800498.
7. Rabies in a laboratory worker – New York. MMWR. 1977;26:183–4.
8. Kaplan MM, Wiktor T, Koprowski H. An intracerebral assay procedure in mice for chemical inactivation of rabies virus. Bull WHO. 1966;34:293–7. PMID:5296135.
9. Jarvis JA, Franke MA, Davis AD. Rabies direct fluorescent antibody test does not inactivate rabies or eastern equine encephalitis viruses. J Virol Methods. 2016; 234:52–3. doi:10.1016/j.jviromet.2016.04.005.
10. Clavijo A, Freire de Carvalho MH, Orciari LA, Velasco-Villa A, Ellison JA, Greenberg L, et al. An inter-laboratory proficiency testing exercise for rabies diagnosis in Latin America and the Caribbean. PLoS Negl Trop Dis. 2017; 11:e0005427. doi:10.1371/journal.pntd.0005427.
11. Seligmann EB. Semple-type vaccine. In: Atanasiu P, editor. Laboratory techniques in rabies, 2nd edition. Geneva: World Health Organization; 1966:110–6.
12. Fischman H Ward FE. Oral transmission of rabies virus in experimental animals. Am J Epidem. 1968; 88:132–8.
13. Kaplan MM, Wiktor TJ, Maes RF, Campbell JB, Koprowski H. Effect of polyions on the infectivity of rabies virus in tissue culture: construction of a single-cycle growth curve. J Virol. 1967; 1:145–51.

14. Constantine DG. Rabies transmission by nonbite route. *Public Health Rep.* 1962; 77:287–89. PMID:13880956.
15. Morimoto K, Patel M, Corisdeo S, Hooper DC, Fu ZF, Rupprecht CE, et al. Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America. *Proc Natl Acad Sci U S A.* 1996; 28:5653–8. PMID:PMC39303.
16. Jackson AC. Virus infection: an update. *J Neurovirol.* 2003;9:253–8. doi:10.1080/13550280390193975.
17. Pará M. An outbreak of post-vaccinal rabies (rage de laboratoire) in Fortaleza, Brazil, in 1960: residual fixed virus as the etiological agent. *Bull WHO.* 1965; 33:177–82. PMID:PMC2475823.
18. WHO Expert Consultation on Rabies: third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 25 April 2018).
19. Canadian Biosafety Standard, 2nd edition, 2015. Government of Canada. (<https://www.canada.ca/en/public-health/services/canadian-biosafety-standards-guidelines/second-edition.html>; accessed 25 April 2018).
20. The Approved List of biological agents. Advisory Committee on Dangerous Pathogens. United Kingdom Health and Safety Executive; 2013 (www.hse.gov.uk/pubns/misc208.pdf, accessed 25 April 2018).
21. The Antimicrobial Testing Program: hospital disinfectant and tuberculocidal products tested or pending testing. United States Environmental Protection Agency; 2017 (<https://www.epa.gov/sites/production/files/2017-07/documents/atp-july-2017.pdf>, accessed 25 April 2018).
22. Aiello R, Zecchin B, Caenazzo ST, Giovanni C, De Benedictis P. Disinfection protocols for necropsy equipment in rabies laboratories: Safety of personnel and diagnostic outcome. *J Virol Methods.* 2016; 234:75–9. doi:10.1016/j.jviromet.2016.03.017.
23. Wu G, Selden D, Fooks AR, Banyard A. Inactivation of rabies virus. *J Virol Methods.* 2017; 243:109–12. doi:10.1016/j.jviromet.2017.02.002.

Chapter 4

The role of diagnostics in surveillance

Introduction

Comprehensive rabies prevention and control programmes are built on a framework of effective surveillance capacity. Public health surveillance consists of continuous systematic collection, analysis, interpretation and dissemination of information on health events (1, 2). There are many possible objectives of surveillance: to demonstrate the presence and distribution of disease in humans and animals; to aid in control efforts; to determine the course of medical care for exposed individuals; or ultimately, to document absence of disease during elimination programmes (3). By definition, surveillance is always linked to specific actions for monitoring interventions and/or immediate responses. Furthermore, the capacity to detect, assess, notify and report on health events is a critical component of the International Health Regulations (2005) (2). Additional guidance on rabies surveillance is available from WHO and OIE and in the rabies blueprint (3–5).

Case definitions

To promote awareness and vigilance, human and animal rabies must be notifiable diseases based on standard case definitions (Table 4.1) that are published and widely disseminated by national health and veterinary services. Surveillance data should be reportable, based on these definitions, through appropriate channels by published protocols to facilitate timely data sharing and analysis. Case definitions should be formulated to allow classification based on clinical signs or symptoms (suspect or probable) or diagnostic tests (confirmed or non-case).

Methods of surveillance

Measures of incidence are essential for rabies control and prevention programmes to ensure appropriate management of outbreaks, to monitor trends, to evaluate the effectiveness of interventions and to estimate the burden of disease. Data can be derived from numerous surveillance methods, ranging from public health systems to road-kill surveillance to direct sampling from animal populations. Each method has unique applications, interpretations and laboratory testing requirements that must be considered. Of note is that testing healthy animals is likely to yield negative results and is therefore of limited value, as the presence of rabies virus (RABV) can only be confirmed in the late or final stages of the disease and there is no “carrier state” (subclinical infection).

Table 4.1. Case definitions for animal and human rabies

	Animal case definition	Human case definition
Suspect case	<p>An animal that presents with any of the following clinical signs:</p> <ul style="list-style-type: none"> • hypersalivation • paralysis • lethargy • unprovoked abnormal aggression (biting \geq 2 people or animals, and/or inanimate objects) • abnormal vocalization • diurnal activity of nocturnal species 	<p>A person presenting with an acute neurological syndrome (i.e. encephalitis) that progressively worsens towards coma and death, typically within 7–10 days of onset, if no intensive care is instituted. May include any of the following signs and symptoms:</p> <ul style="list-style-type: none"> • aerophobia • hydrophobia • paraesthesia or localized pain • dysphagia • localized weakness • nausea or vomiting
Probable case	<p>A suspect animal with a history of a bite by another (suspect/probable/confirmed) animal</p> <p><i>and/or</i></p> <p>A suspect animal that is killed, dies or disappears within 4–5 days of observing illness</p>	<p>A suspect human with a reliable history of contact with a (suspect/probable/confirmed) rabid animal</p>
Confirmed case	<p>A suspect or probable animal confirmed using a standard diagnostic test as defined by WHO or OIE</p>	<p>A suspect or probable human that is laboratory-confirmed using a standard diagnostic test</p>
Not a case	<p>A suspect or probable animal in which rabies is ruled out by appropriate laboratory testing</p>	<p>A suspect or probable human that is ruled out by appropriate laboratory testing</p> <p><i>or</i></p> <p>A suspect or probable human with no reasonable risk of animal contact in the past 6 months</p>

OIE, World Organisation for Animal Health; WHO, World Health Organization

Passive public health surveillance

Passive public health surveillance (PPHS) measures the incidence of disease at the human–animal interface, rather than the incidence within an animal population (more accurately captured through active surveillance). PPHS is primarily a public health intervention, of which the outcome of the intervention provides pertinent surveillance data for monitoring and evaluating rabies control programmes. A comprehensive PPHS programme consists of three core activities: (i) surveillance for suspected RABV exposures (i.e. bite events); (ii) surveillance of rabies-suspect animals involved in human or domestic animal exposures; and (iii) monitoring of the utilization of post-exposure prophylaxis (PEP). When these three systems are formally linked with reporting and feedback mechanisms, it is referred to as an integrated bite case management programme. Such programmes have been shown to reduce the unnecessary use of rabies PEP and increase adherence to rabies PEP schedules, emphasizing the importance of timely and accurate diagnosis (6).

Test results from rabies suspect animals identified under a PPHS programme are often used to determine the medical care provided to exposed humans and domestic animals. A false–negative under these conditions may result in the lack of provision of PEP, and a potential fatal outcome for the exposed. Under this rubric, tests with high specificity are critical. Infrequent false–positives are not likely to affect PPHS programmes negatively, and over application of PEP will rarely result in a negative clinical outcome. Currently, WHO and OIE recognize the DFAT (direct fluorescent antibody test), the DRIT (direct rapid immunohistochemistry test) and reverse transcriptase polymerase chain reaction (RT-PCR) as primary diagnostic tests when decisions on PEP may be affected (Table 4.2). Alternative tests, such as lateral flow assays, should not be used as a sole diagnostic assay in the context of a passive surveillance programme until they have been fully evaluated and recognized by the relevant international organizations. Routine monitoring of diagnostic proficiency of laboratorians should be conducted, and, in situations where proficiency cannot be guaranteed or confirmation of primary diagnostic test results is needed, confirmatory testing by another method (e.g. DFAT confirmed by PCR).

Consideration of sample quality is more important within the context of a PPHS programme than in active surveillance programmes. The neurotropic pathways traversed by RABV may lead to infection in only a portion of the brainstem; therefore, testing a full cross-section is required to definitively rule out RABV infection (4). When a full cross-section of brainstem cannot be obtained or the sample quality is insufficient to enable identification of anatomic structures, and RABV is not identified by the DFAT, the DRIT or RT-PCR, and confirmatory tests are not available, the sample should be declared indeterminate. In this situation, public health officials should formulate PEP decisions based on a complete evaluation of the circumstances of the bite event, local rabies epidemiology and an assessment of the offending animal.

Reporting and notification protocols are important considerations in the context of a PPHS programme. Laboratories should be familiar with mechanisms for transportation and submission of samples and be able to rapidly test and report results. Samples should be tested rapidly and reported no less than within 5–7 days of the exposure event to ensure that the results can be used to inform PEP decisions, if

Table 4.2. Selective comparison of various rabies tests and their utility in different surveillance programmes

	Test	Passive surveillance	Active surveillance	Clinical diagnosis	Sample types	Notes
Molecular	Reverse transcriptase polymerase chain reaction (RT-PCR)	++	++	+++	Brain Skin biopsy Saliva	Used as a primary diagnostic assay with sequencing and as a confirmatory test
	Hemi-nested RT-PCR	++	++	+++	Brain Skin biopsy Saliva	Used as a primary diagnostic assay with sequencing and as a confirmatory test
	Real-time RT-PCR	+	+++	+++	Brain Skin biopsy Saliva	Used as a primary diagnostic assay
	Direct fluorescent antibody test (DFAT)	+++	+++	+++	Brain Skin biopsy	<ul style="list-style-type: none"> • Primary “gold standard” test • Recognized by OIE and WHO
Antigen	Direct rapid immuno-histochemistry test (DRIT)	+++	+++	+++	Brain Skin biopsy	<ul style="list-style-type: none"> • Primary test • Recognized by OIE and WHO
	Indirect rapid immuno-histochemistry test (IRIT)	-	++	++	Brain Skin biopsy	Can also be used for antigenic typing studies
	Immuno-chromatographic test (i.e. lateral flow devices)	-	+	+	Brain	<ul style="list-style-type: none"> • Potentially low-cost • Transportable • Variable sensitivity and specificity
Antibody	Rapid fluorescent focus inhibition test (RFFIT) Fluorescent antibody virus neutralization test (FAVN)	-	-	+++	Serum CSF	Can be useful in defining disease activity as part of surveys in new areas and species (such as bats)
	Indirect fluorescent antibody test (IFA)	-	-	++	Serum CSF	Requires suitable control materials
	Indirect enzyme-linked immunosorbent assay (ELISA)	-	-	++	Serum	
	Competitive ELISA	-	-	++	Serum	May be useful in immunogenicity studies, such as evaluation of wildlife vaccination programmes

CSF, cerebrospinal fluid; OIE, World Organisation for Animal Health; WHO, World Health Organization

–, not recommended; +, low recommendation, useful for confirmatory testing; ++, moderate recommendation, useful for primary or confirmatory testing; +++, high recommendation, useful for primary or confirmatory testing

they have not already begun. Standard reporting protocols should be in place so that results are integrated with regional and national reporting structures to ensure results are also included in epidemiological analysis and reports. The decision to initiate PEP should be determined by public health officials with knowledge of the circumstances of the exposure event, in accordance with WHO recommendations, and should not be solely dependent on laboratory testing results.

Active or enhanced surveillance

Active surveillance (sometimes referred to as enhanced surveillance) is the targeted sampling of animals in a population for determining disease incidence. This method of surveillance is a more accurate measure of the true occurrence of rabies in the targeted population, but may still be biased depending on the choice of sampling technique. Active surveillance does not include the investigation of rabies-suspect animals involved in human or animal exposure events (passive public health surveillance). Active surveillance is typically enacted to answer specific questions related to an intervention or to improve situational awareness for making rabies control decisions.

Studies have shown that RABV has a relatively low reproductive number and a long incubation period (7, 8). Therefore, in an endemic state of transmission, indiscriminate sampling of a population is likely to result in a low rate of detection. While active surveillance is a better reflection of the true transmission dynamics in a population, indiscriminate testing of healthy subjects requires high levels of terminal sampling that may not be economically or ethically feasible. Targeted active surveillance (e.g. nuisance animals, road kill) may provide a higher rate of detection; however, the resulting rates will be less representative of the entire population of interest (9). Active surveillance is often implemented in conjunction with a PPHS programme to provide a comprehensive understanding of the rabies epidemiology in the target area (10).

Laboratory diagnostics are an essential component of an active surveillance programme. The importance of increased sensitivity and specificity of the tests used for active surveillance should be balanced based on programme objectives. While clinical outcomes of persons involved in an exposure should not be a concern for these programmes, misdiagnosis may have substantial impacts on management practices and determination of a disease-free status. Therefore, primary diagnostic methods (e.g. DFAT, DRIT, RT-PCR), recognized by OIE or WHO should be prioritized (Table 4.2). However, in the context of an active surveillance programme, sensitivity and specificity may be sacrificed at times when primary methods are not available or economically feasible.

Non-primary diagnostic methods such as lateral flow assays (LFA) have shown variable reliability between manufacturers and manufacturing lots; however, these products are easily transportable and provide a field-ready diagnostic option (11, 12). Also, numerous molecular assays for detection of RABV have been described as outlined in this manual; however, their sensitivity and specificity can differ by technique, and cross-contamination may result in false-positive results when used under field conditions (13, 14). Active surveillance programmes that choose to use non-primary diagnostic assays should consider periodic validation of their results before making substantial programmatic changes. A validation subset of

10% of negative and 100% of positive samples has been previously suggested (that is, in the fourth edition of this book). Measurement of RABV-specific antibodies is not recommended for routine monitoring of the success of a vaccination programme but may be relevant to disease detection when attempting to gauge basic viral activity in certain wildlife populations, such as bats.

Human surveillance

Detection of human rabies cases can have direct implications on the PEP recommendations for the victim's friends, family and medical providers who may have had exposure to infectious materials. Therefore, human surveillance systems are most analogous to PPHS programmes. Investigations should be undertaken promptly and primary diagnostics should be sought (e.g. DFAT, DRIT, RT-PCR) when available.

Unfortunately, the capacity to conduct autopsies and the ability to perform primary rabies diagnostics are lacking in many countries, the result of which is under-detection of cases and propagation of a cycle of neglect. Non-standard methods of case detection may therefore prove beneficial in the context of an active surveillance programme (Table 4.2). It should be noted that non-primary diagnostic tests should be considered as "rule-in" tests, where the detection of antigen by most any means may be interpreted as a positive result but the lack of detection by a single test cannot rule out the presence of RABV infection. When antigen is not detected by non-primary tests, the WHO clinical case definition for rabies should be applied, and appropriate follow-up determined by public health officials (4). Antemortem diagnostic tests have been described but are rarely conducted outside of international reference laboratories (15). Availability of alternate tests as described in this manual should be adapted to local circumstances to improve confirmation of human rabies cases.

Virus characterization

Routine characterization of lyssavirus isolates from cases and outbreaks using molecular (partial or full genome sequencing) or antigenic (sets of anti-N or anti-G monoclonal antibodies) techniques is helpful to identify sources of infection as well as animal host and geographical origins (16–19). Characterization thus provides valuable information on the epidemiology of the disease in terms of the presence of dog-, and/or wildlife- and/or bat-mediated rabies as well as circulating virus variants in a given area. However, contrary to popular opinion, routine characterization of all RABV and non-rabies lyssavirus isolates is not a prerequisite for successful rabies control programmes in endemic areas once the epidemiology has been enlightened and the target species identified. A notable exception is the detection of rabies cases in "free" or previously rabies-free areas. Here, characterization of the virus isolates is helpful in identifying the source of infection (autochthonous vs imported or introduced), particularly in view of increased international travel and animal movement (20).

As part of the global 2030 programme to eliminate human rabies mediated by dogs, canine RABV variants may be readily differentiated from those viruses main-

tained by wildlife. During the course of such enhanced surveillance to monitor programme success, undoubtedly new reservoirs, variants and viral species are expected to be identified using the methods recommended in the ensuing chapters of this manual.

References

1. German RR, Lee LM, Horan JM, Milstein RL, Pertowski CA, Waller MN; Guidelines Working Group; Centers for Disease Control and Prevention (CDC). Updated guidelines for evaluating public health surveillance systems: recommendations from the Guidelines Working Group. *MMWR Recomm Rep*. 2001; 50(RR-13):1–35; quiz CE1–7. PMID:18634202.
2. Kasolo F, Yoti Z, Bakayita N, Gaturuku P, Katz R, Fischer JE, et al. IDSR as a platform for implementing IHR in African countries. *Biosecur Bioterror*. 2013;11:163–9. doi:10.1089/bsp.2013.0032.
3. Manual of diagnostic tests and vaccines for terrestrial animals. Paris: World Organisation for Animal Health; 2012.
4. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 8 May 2018).
5. Lembo T, Partners for Rabies Prevention. The blueprint for rabies prevention and control: a novel operational toolkit for rabies elimination. *PLoS Negl Trop Dis*. 2012; 6:e1388. doi:10.1371/journal.pntd.0001388.
6. Etheart M, Kligerman M, Augustin PD, Blanton JD, Monroe B, Fleurinord L, Millien M, et al. Effect of counselling on health-care-seeking behaviours and rabies vaccination adherence after dog bites in Haiti, 2014–15: a retrospective follow-up survey. *Lancet Glob Health*. 2017; 5:1017–25. doi:10.1016/S2214-109X(17)30321-2.
7. Fooks AR, Banyard AC, Horton DL, Johnson N, McElhinney LM, Jackson AC. Current status of rabies and prospects for elimination. *Lancet*. 2014; 384:1389–99. doi:10.1016/S0140-6736(13)62707-5.
8. Zinsstag J, Durr S, Penny MA, Mindekem R, Roth F, Menendez Gonzalez S, et al. Transmission dynamics and economics of rabies control in dogs and humans in an African city. *Proc Natl Acad Sci U S A*. 2009; 106:14996–5001. doi:10.1073/pnas.0904740106.
9. Slate DK, Morgan, DP, Algeo TP, Trimarchi CV, Nelson KM, Rudd RJ, et al. Cost and relative value of road kill surveys for enhanced rabies surveillance in raccoon rabies management. *Trop Med Infect Dis*. 2017; 2:13. doi:10.3390/tropicalmed2020013.
10. Monroe BP, Yager P, Blanton J, Birhane MG, Wadhwa A, Orciari L, et al. Rabies surveillance in the United States during 2014. *J Am Vet Med Assoc*. 2016; 248:777–88. doi:10.2460/javma.248.7.777.

11. Eggerbauer E, de Benedictis P, Hoffmann B, Mettenleiter TC, Schlottau K, Ngoepe EC, et al. Evaluation of six commercially available rapid immunochromatographic tests for the diagnosis of rabies in brain material. *PLoS Negl Trop Dis.* 2016; 10:e0004776. doi:10.1371/journal.pntd.0004776.
12. Lechenne M, Naissengar K, Lepelletier A, Alfaroukh IO, Bourhy H, Zinsstag J, et al. Validation of a rapid rabies diagnostic tool for field surveillance in developing countries. *PLoS Negl Trop Dis.* 2016; 10:e0005010. doi:10.1371/journal.pntd.0005010.
13. Wadhwa A, Wilkins K, Gao J, Condori Condori RE, Gigante CM, Zhao H, et al. A Pan-lyssavirus Taqman real-time RT-PCR assay for the detection of highly variable rabies virus and other lyssaviruses. *PLoS Negl Trop Dis.* 2017; 11:e0005258. doi:10.1371/journal.pntd.0005258. PMID: PMC5230753 following competing interests: patents have been filed for assays described in this manuscript through CDC Technology Transfer Office/NIH Office.
14. Hoffmann B, Freuling CM, Wakeley PR, Rasmussen TB, Leech S, Fooks AR, et al. Improved safety for molecular diagnosis of classical rabies viruses by use of a TaqMan real-time reverse transcription-PCR “double check” strategy. *J Clin Microbiol.* 2010; 48:3970–8. doi:10.1128/JCM.00612-10.
15. Mani RS, Madhusudana SN. Laboratory diagnosis of human rabies: recent advances. *ScientificWorldJournal.* 2013;2013:569712. doi:10.1155/2013/569712.
16. David D, Hughes GJ, Yakobson BA, Davidson I, Un H, Aylan O, et al. Identification of novel canine rabies virus clades in the Middle East and North Africa. *J Gen Virol.* 2007; 88:967–80. doi:10.1099/vir.0.82352-0.
17. Kuzmin IV, Hughes GJ, Botvinkin AD, Gribencha SG, Rupprecht CE. Arctic and Arctic-like rabies viruses: distribution, phylogeny and evolutionary history. *Epidemiol Infect.* 2008; 136:509–19. doi:10.1017/S095026880700903.
18. Kuzmin IV, Shi M, Orciari LA, Yager PA, Velasco-Villa A, Kuzmina NA, et al. Molecular inferences suggest multiple host shifts of rabies viruses from bats to mesocarnivores in Arizona during 2001–2009. *PLoS Pathog.* 2012; 8:e1002786. doi:10.1371/journal.ppat.1002786.
19. Streicker DG, Turmelle AS, Vonhof MJ, Kuzmin IV, McCracken GF, Rupprecht CE. Host phylogeny constrains cross-species emergence and establishment of rabies virus in bats. *Science.* 2010; 329:676–9. doi:10.1126/science.1188836.
20. Sinclair JR, Wallace RM, Gruszynski K, Freeman MB, Campbell C, Semple S, et al. Rabies in a dog imported from Egypt with a falsified rabies vaccination certificate – Virginia, 2015. *MMWR.* 2015; 64:1359–62. doi:10.15585.mmwr.mm6449a2.

Chapter 5

An overview of antemortem and postmortem tests for diagnosis of human rabies

Introduction

Rabies, one of the most neglected zoonotic diseases worldwide, disproportionately affects children and impoverished rural communities. The true burden of the disease and its public health impact remain underestimated and can be attributed to inadequate or non-existent surveillance of animal and human rabies, lack of laboratory diagnostic facilities, misdiagnosis of rabies, underreporting of cases by local and central authorities, and inadequate legislation for compulsory notification of cases. The lack of accurate data on disease burden, upon which regional and national priorities for research and control are based, further perpetuates a cycle of indifference and neglect (1). It is essential therefore to improve epidemiological surveillance using diagnostic approaches that are based on validated protocols and specimens and evaluated under field conditions. Clinical diagnosis of human rabies is challenging and often unreliable. Laboratory confirmation must therefore be done in all suspected cases wherever feasible (2).

Significant progress has been made in rabies laboratory techniques during the past few decades, especially for antemortem diagnosis. Several validated techniques are now available to detect specific viral antigens, nucleic acids or antibodies in selected biological samples. The selection, collection and shipment of biological specimens are crucial steps, particularly for antemortem diagnosis, since several factors such as the duration of illness, prior vaccination, single/multiple/serial clinical samples tested, quality of samples and type of assay used influence the test results. Furthermore, the choice of diagnostic tests also largely depends on the availability of infrastructure and technical expertise, and may vary in settings in which rabies is endemic or non-endemic (3).

This chapter describes the various types of assays and clinical samples that can be used for antemortem and postmortem diagnosis of human rabies. The aim is to serve as an introductory guide before reference to specific diagnostic assays detailed elsewhere in this manual. Clearly, the performance of each laboratory is highly dependent on quality assurance and quality control practices. All laboratories are encouraged to develop and implement stringent quality management standards, including periodic participation in internal and external proficiency testing programmes (2).

Laboratory confirmation to rule out clinical mimics

Two major and distinct clinical forms of rabies have been recognized in humans: classical encephalitic (furious) rabies and paralytic rabies (4). Based on these descriptions, the standard clinical case definition corresponds to a patient presenting with an acute neurological syndrome (i.e. encephalitis) dominated by forms of hyperactivity or paralytic syndromes progressing towards coma and death, usually by cardiac or respiratory failure, and typically within 7–10 days after the first sign if no intensive care is instituted (2).

However, a diagnosis of rabies based solely on clinical features, especially in the absence of a history of exposure, is difficult and often unreliable (2). Laboratory confirmation must be done wherever feasible. Indeed, the clinical presentation of rabies is often variable and may represent a continuum of signs and symptoms (5). In addition, atypical and non-classical clinical forms of rabies are increasingly being recognized (2, 4–7). Furthermore, rabies can be confused clinically with Guillain–Barré syndrome (often clinically indistinguishable from the paralytic form of rabies) or even with other infectious etiologies, such as cerebral malaria or herpes simplex encephalitis. Psychiatric disorders, post-vaccinal encephalitis, anti-NMDA receptor encephalitis, scorpion and snake envenomations, illicit drug use and organophosphate poisoning all can also cause a clinical syndrome suggestive of rabies (5). Reports of rabies being transmitted through tissue and organ transplants (8, 9) also emphasize the need for scrupulous organ donor screening for rabies, especially in donors with a neurological illness of unexplained etiology and the presence of other risk factors for rabies.

Clinical management and public health measures

Early diagnosis can obviate the need for unwarranted medical tests and treatment, and help also in prognostication, case closure and grief counselling with family members. Antemortem confirmation of diagnosis is crucial to aid physicians contemplating imminent treatment (critical care or experimental therapeutic options) or palliation. Laboratory tests such as viral load and neutralizing antibody titres may also be useful to monitor the clinical course of illness and the patient's response to therapy (2, 5).

Laboratory confirmation of rabies in a hospitalized patient also aids in instituting appropriate infection control measures, including barrier nursing and administration of post-exposure prophylaxis (PEP) to healthcare workers and family members exposed to the patient's infectious secretions (2). Laboratory confirmation is imperative for clinical management as well as to guide public health measures especially in non-endemic settings, to detect importation or emergence of rabies and to institute PEP to others who may have been potentially exposed to the same source. Laboratory tests also aid in documenting control and research (e.g. seroprevalence studies, PEP failures).

Disease surveillance

The true human burden of this neglected zoonotic disease remains underestimated, particularly in rabies endemic countries in Asia and Africa due to limited reporting of laboratory-confirmed cases. Regular reporting of diagnostic data is crucial for estimation of disease burden and helps prioritize resources towards prevention of human rabies (10). Continual laboratory-based surveillance is essential even in previously “rabies-free” countries. As long as foci of wildlife or canine rabies exist anywhere and transborder migration of infected animals from enzootic areas or international travel and global trade of livestock, pets and wildlife continue, the threat of reintroduction of rabies always exists. Additionally, some laboratory tests can aid in characterization of the virus as well as in molecular epidemiological studies to monitor the disease dynamics at regional, national and international levels, and identify the source of emergence of the disease in previously rabies-free areas (1, 11).

Antemortem diagnostic tests

Several laboratory techniques are available for antemortem confirmation of a suspected or probable case of rabies (Table 5.1). Techniques for viral nucleic acid detection, such as reverse transcriptase and polymerase chain reaction (RT-PCR) or other nucleic acid amplification and detection methods (e.g. NASBA [nucleic acid sequence based amplification]), using clinical samples such as saliva, nuchal skin, extracted hair follicles, tears, urine and cerebrospinal fluid (CSF) have achieved a rapid reduction in turnaround times. These tests are in routine use in several centralized and reference laboratories globally, especially for antemortem diagnosis, since traditional methods are fraught with several limitations (10, 12). Such testing permits antemortem confirmation of rabies in clinical samples obtained by less invasive techniques and can therefore alleviate the requirement for autopsy or postmortem brain biopsy, and the accompanying logistics and safety procedures (13–15).

The sensitivity of these techniques for antemortem diagnosis varies according to the clinical form of rabies, stage of the disease, immunological status of host, intermittent viral excretion and the technical expertise of the laboratory. However, studies have demonstrated that testing at least three samples of saliva taken at 3–6 h intervals, together with a nuchal skin biopsy, approaches 100% sensitivity in the encephalitic form of rabies (15). Other samples, such as CSF and urine, have more limited sensitivity. Tears have been proposed, but have not been validated on a large scale. As with any other diagnostic assays, procedures for stringent validation and quality control are imperative. While a positive validated result is indicative of rabies, a negative result does not essentially rule out a diagnosis of rabies in all cases, which is a major limitation of antemortem testing (2). Antemortem brain biopsy is not recommended as a primary test, as it is impractical and false-negative results can be obtained due to sampling error especially in the early stages of illness (16).

Serological tests, such as the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody virus neutralization test (FAVN), which detect neutralizing antibodies, and ELISA for detection of specific anti-rabies antibodies, have

Table 5.1. Human rabies antemortem diagnostic tests

Test	Target	Clinical samples	Advantages	Limitations	Additional comments
Nucleic acid amplification (NAA) Conventional and real-time reverse transcriptase polymerase chain reaction (RT-PCR) as well as other methods	Viral RNA	Saliva	Easy, non-invasive sample collection; high sensitivity	Serial/pooled samples need to be tested for increased sensitivity due to intermittent virus shedding (at least 3 serial samples at 3–6 h intervals)	Rapid turnaround time (3–4 h); amplicons from conventional (not real-time) PCR can be used for genetic characterization Risk of amplicon contamination leading to false-positive results higher in conventional PCR
		Nuchal skin biopsy/hair follicles	High sensitivity in all stages of illness	Requires full thickness biopsy and adequate hair follicles for increased sensitivity	Quality of clinical specimens vital; appropriate storage and transport conditions must be maintained
		CSF	Sample usually available in the context of encephalitis	Invasive technique for sample collection, low sensitivity	Requirement for infrastructure and stringent quality control Can also be performed on other clinical samples such as urine and tears, but sensitivity low
Rapid fluorescent focus inhibition test (RFFIT) Fluorescent antibody virus neutralization test (FAVN) Enzyme-linked immunosorbent assay (ELISA)	Virus neutralizing antibodies (RFFIT, FAVN) or antibodies against rabies glycoprotein (ELISA)	CSF Blood (serum)	Diagnostic of rabies irrespective of previous vaccination status Valuable to monitor the immune response within the central nervous system and thus a possible clearance of the rabies virus in patients being treated with experimental protocols Diagnostic of rabies in individuals not vaccinated previously Valuable in estimation of post-vaccinal antibody titres	Low sensitivity, late appearance of specific antibodies (>8 days); short survival and late seroconversion in rabies limit diagnostic value Titre in serum must be interpreted cautiously in previously vaccinated individuals; significant rise in paired sera to be demonstrated for confirmation of diagnosis	Sensitivity of test rises with increased duration of survival For RFFIT and FAVN: long turnaround time (2–3 days); requirement for cell culture facility, fluorescence microscope and biosafety measures; test procedure cumbersome Test (RFFIT, FAVN) is sensitive to cytotoxicity in poor-quality sera; nonspecific inhibitors of virus in sera may produce false-positive results Adequately validated ELISA for antibody detection can be used if RFFIT or FAVN is unavailable; these tests are rapid, inexpensive and safe since live virus is not required Detection of antibodies to other viral antigens, (e.g. nucleoprotein) may also be useful, as they may appear before neutralizing antibodies

Table 5.1. Continued

Test	Target	Clinical samples	Advantages	Limitations	Additional comments
Direct fluorescent antibody test (DFAT)	Viral antigen	Corneal smear	Easy and rapid technique	Low sensitivity and specificity	Requirement for expensive fluorescence microscope
		Nuchal skin (frozen section)	Moderate sensitivity at all stages of illness; can also be used for postmortem rabies diagnosis if brain tissue unavailable	Not recommended because of risk of corneal scarification	Operator expertise required for interpretation Preferable to use more sensitive viral RNA detection methods (such as RT-PCR) on skin samples
Rabies tissue culture infection test (RTCIT)	Live virus	Biological fluids (CSF, saliva)	Enables propagation of virus and valuable for obtaining large quantities of virus for molecular characterization and other studies	Cumbersome, time-consuming technique; requires multiple sections with hair follicles to be examined Cryostat required to prepare frozen sections of skin – not practicable in all settings	Low sensitivity, longer turnaround time (1–3 days), requires fluorescence microscope, cell culture and adequate biocontainment facilities, and operator expertise, requires infectious virus in the sample

CSF, cerebrospinal fluid; RNA, ribonucleic acid

a limited role in diagnosis of rabies, due to the rapid progression of illness and short survival. However, detection of specific anti-rabies virus antibodies in serum (in unvaccinated individuals) and CSF is valuable for diagnosis in cases, especially where survival is prolonged beyond a week (7, 14). Moreover, an inverse correlation has been observed between the presence of anti-rabies virus antibodies in CSF and the detection of viral RNA in clinical samples (14, 17). Therefore, a combination of several tests conducted on multiple clinical samples, with serial sampling whenever feasible (because of intermittent virus shedding in saliva, and a potential rise in antibody titres in serial CSF and serum samples) is recommended to increase the sensitivity of antemortem diagnosis (7, 10). A suggested algorithm for antemortem diagnosis is illustrated in Fig. 5.1.

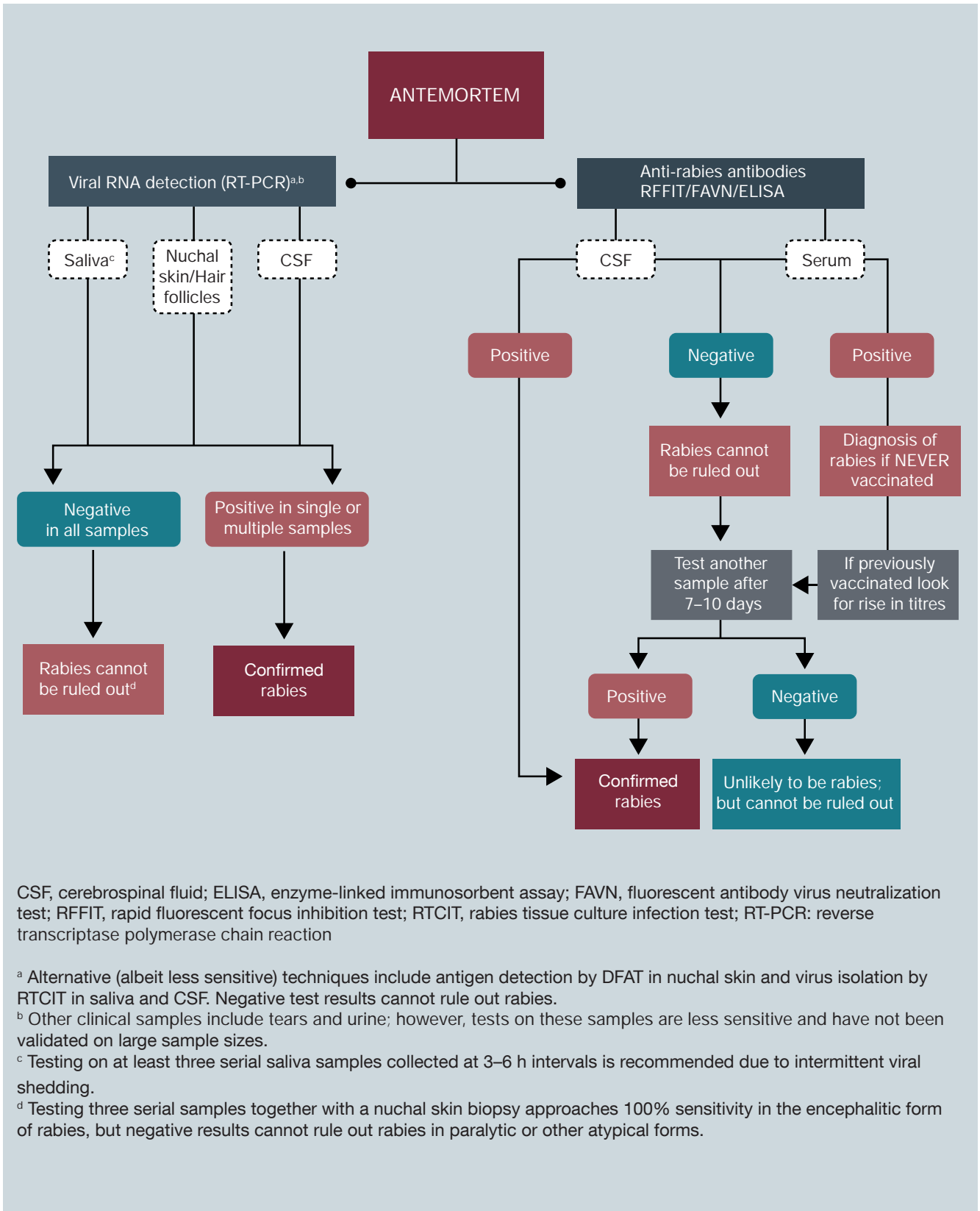


Fig. 5.1. Algorithm for human rabies antemortem diagnosis

Postmortem diagnostic tests

Demonstration of rabies virus nucleoprotein antigens in brain tissue by the direct fluorescent antibody test (DFAT) is the most widely used test for postmortem rabies diagnosis, when brain tissue is available for testing (see [Chapter 11](#)). The direct rapid immunohistochemistry test (DRIT), for detection of viral antigens in brain tissue, has been found to be comparable to the DFAT in the diagnosis of animal rabies and has the additional advantage of applicability under field conditions, as an expensive fluorescence microscope is not required for interpretation of results (see [Chapter 12](#)) (18). While there is a need for adequate validation of this test specifically for diagnosis of human rabies, it is not expected to vary as compared to the tens of thousands of tests conducted upon multiple other mammalian species. Other tests for postmortem diagnosis, and their advantages and limitations, are summarized in Table 5.2.

Postmortem brain tissue can be obtained by craniotomy; however, this procedure continues to be a challenge due to declining rates of autopsies related to religious, cultural and other factors. Brain biopsy is therefore the preferred post-mortem sampling technique which can be obtained via the orbital or transnasal route using biopsy needles or through the occipital route through the foramen magnum using lumbar puncture needles. The transnasal or orbital route will contain fragments of orbitofrontal cortex, whereas the transforamen magnum route will obtain samples from the cerebellum and brain stem (16, 19–22).

Wherever brain tissue is not available for confirmation of a diagnosis post-mortem, which is often the case, testing for the presence of viral RNA using nucleic acid amplification techniques can be done on nuchal skin and other samples obtained postmortem (7, 10, 14, 15). However, tests for detection of viral RNA conducted on samples other than brain tissue are less sensitive and cannot rule out a diagnosis of rabies in all cases. A suggested algorithm for postmortem diagnosis is illustrated in Fig. 5.2.

Conclusions

Laboratory diagnosis should be systematically applied to any suspected or probable case of human rabies. Substantial technical progress has been achieved during the past few decades, and validated techniques using various clinical specimens are now available for human rabies diagnosis. However, access to laboratory testing remains constrained in several resource-limited areas of the world in which canine rabies is endemic and where most cases of human rabies continue to occur. There is a need therefore to develop and implement new tools suitable for use in decentralized laboratories or on-site in remote rabies endemic areas to ensure reliable data on human rabies.

S

Table 5.2. Human rabies postmortem diagnostic tests

Test	Target	Clinical samples	Advantages	Limitations	Additional Comments
Direct fluorescent antibody test (DFAT)	Viral antigen	Brain tissue	Rapid turnaround time (2–3 h); easy to perform; almost 100% sensitive and specific	Fresh brain tissue ideal for testing; results on autolysed or decomposed tissues less reliable Obtaining postmortem brain tissue challenging	“Gold standard” for postmortem confirmation of rabies on brain tissue Validated and commercially available reagents (conjugated antibodies) Need for expensive fluorescence microscope and skilled personnel to interpret test results limits its use in many developing countries DFAT on nuchal skin biopsy obtained postmortem can be done when brain tissue is not available, but sensitivity is low; negative test result does not rule out rabies
Direct rapid immunohistochemistry test (DRIT)	Viral antigen	Brain tissue	Rapid turnaround time (2–3 h); sensitivity and specificity comparable to DFAT; requires only light microscope for interpretation	Reagents not commercially available; complex protocol (multiple wash steps)	Recommended as an OIE diagnostic test for postmortem diagnosis of rabies in animals Not adequately validated for human rabies diagnosis
Histological identification of “Negri bodies” by Seller’s technique on smears from fresh brain tissue or by H&E staining on sections of formalin-fixed, paraffin-embedded tissues	Inclusion bodies (aggregates of viral particles)	Brain tissue	Easy and rapid technique (Seller’s staining); use of formalin fixed tissues for H&E staining reduces biohazard	Less sensitive than immunohistochemistry methods, especially in autolysed specimens H&E staining on formalin-fixed tissues is time-consuming (takes 5–7 days)	No longer recommended for primary diagnosis, both in humans and animals Negative test result does not rule out rabies
Rapid immunochromatographic diagnostic test (RIDT)	Viral antigen	Brain tissue	Rapid turnaround time (10–20 min); low technological requirement; can be used at point of sampling; suitable for diagnosis in field conditions; kits commercially available	Need for much better standardization and quality control of commercially available kits; variable sensitivity and specificity reported	Not adequately validated for human rabies diagnosis. Helpful for rapid post-mortem diagnosis of animal rabies in developing countries where surveillance is lacking

Table 5.2. Continued

Test	Target	Clinical samples	Advantages	Limitations	Additional Comments
Enzyme-linked immunosorbent assay (ELISA)	Viral antigen	Brain tissue	High sensitivity and specificity; rapid; can analyse several samples simultaneously	Reagents not commercially available	Can be used as a complementary technique
Nucleic acid amplification (NAA) Conventional and real-time reverse transcriptase polymerase chain reaction PCR (RT-PCR) as well as other methods	Viral RNA	Brain tissue	Rapid turnaround time (3–4 h); 100% sensitive and specific	Quality of clinical specimen vital; appropriate storage and transport conditions must be maintained	Amplicons from conventional (not real-time) PCR can be used for genetic characterization
		Nuchal skin biopsy/hair follicles, CSF	Rapid turnaround time (3–4 h);	Low sensitivity compared with brain tissue; should be tested only if brain tissue is unavailable; negative test result cannot rule out rabies in all cases	Requirement of expensive infrastructure and trained manpower and stringent quality control Risk of amplicon contamination leading to false positive results higher in conventional PCR
Rabies tissue culture infection test (RTCIT)	Live virus	Brain tissue	enables propagation of virus and is valuable for obtaining large quantities of virus for molecular characterization and other studies	Long turnaround time (1–3 days); requires fluorescence microscope, cell culture and adequate biocontainment facilities as well as operator expertise	Virus isolation techniques are not feasible for use as routine diagnostic tests; they can be done only in specialized laboratories for confirmatory diagnosis, and more importantly for further characterization of viruses
Mouse inoculation test (MIT)	Live virus	Brain tissue	Enables propagation of virus and is valuable for obtaining large quantities of virus for molecular characterization and other studies	Very long turnaround time (up to 28 days); requires animal facility and specialized laboratory with adequate biocontainment; ethically challenging	The MIT should be replaced by the RTCIT whenever possible
Rapid fluorescent focus inhibition test (RFFIT) Fluorescent antibody virus neutralization test (FAVN) ELISA	Virus neutralizing antibodies (RFFIT, FAVN) or antibodies against rabies glycoprotein (ELISA)	Blood (serum), CSF	Diagnostic of rabies in individuals not vaccinated previously	Results difficult to interpret in previously vaccinated individuals	Negative test result does not rule out rabies, since antibodies appear late in the course of illness (> 8 days)

CSF, cerebrospinal fluid; H&E, haematoxylin and eosin; OIE, World Organisation for Animal Health

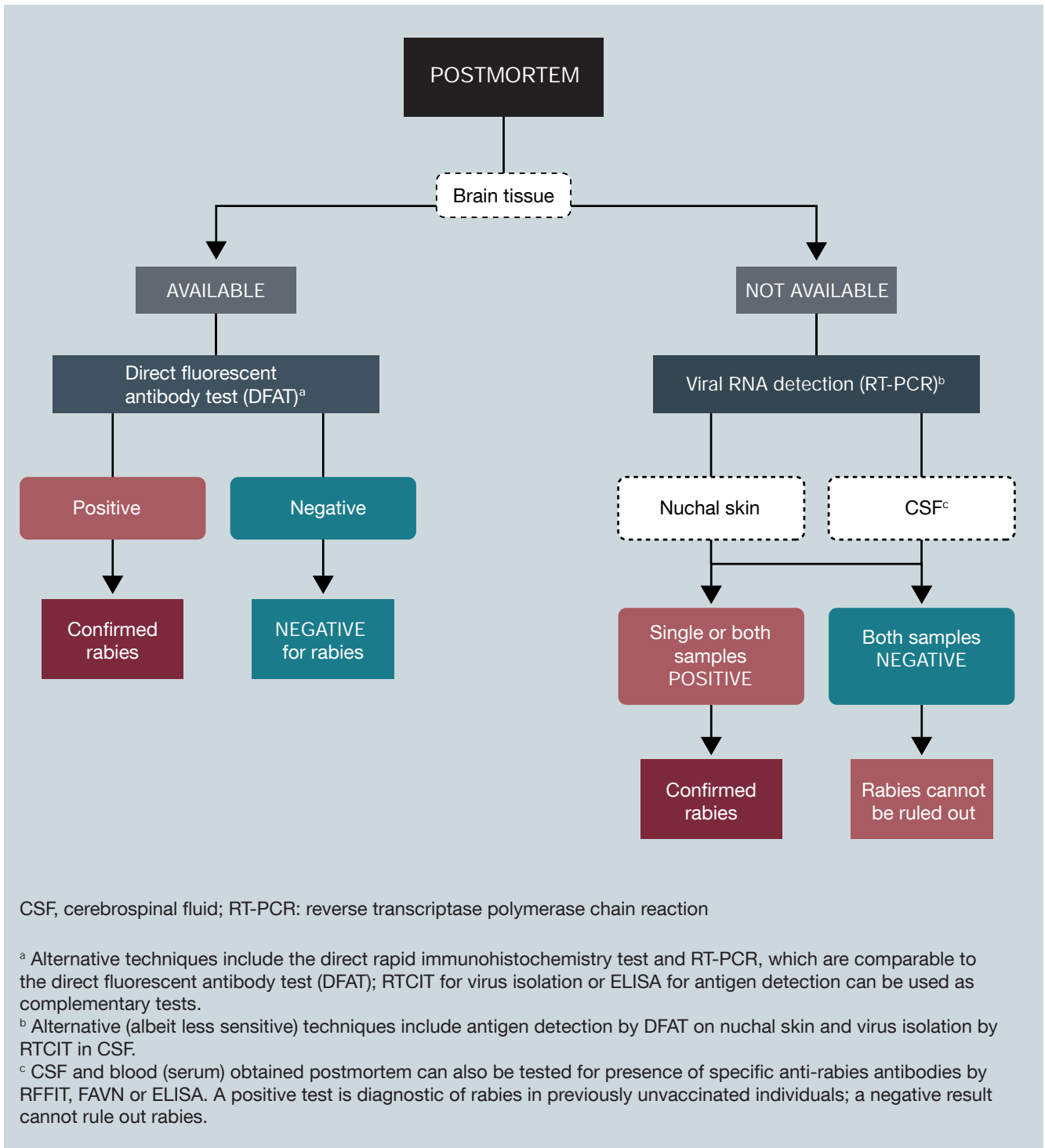


Fig. 5.2. Proposed algorithm for human rabies postmortem diagnosis

References

1. Bourhy H, Dautry-Varsat A, Hotez PJ, Salomon J. Rabies, Still neglected after 125 years of vaccination. *PLoS Negl Trop Dis*. 2010; 4:e839. doi:10.1371/journal.pntd.0000839.
2. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 8 May 2018).
3. Duong V, Tarantola A, Ong S, Mey C, Choeung R, Ly S, et al. Laboratory diagnostics in dog-mediated rabies: an overview of performance and a proposed strategy for various settings. *Int J Infect Dis*. 2016; 46:107–14. doi:10.1016/j.ijid.2016.03.016.
4. Hemachudha T, Ugolini G, Wacharapluesadee S, Sungkarat W, Shuangshoti S, Laothamatas J. Human rabies: neuropathogenesis, diagnosis, and management. *Lancet Neurol*. 2013; 12:498–513. doi:10.1016/S1474-4422(13)70038-3.
5. Willoughby RE Jr. Rabies: rare human infection – common questions. *Infect Dis Clin North Am*. 2015; 29:637–50. doi:10.1016/j.idc.2015.07.006.
6. Karande S, Muranjan M, Mani RS, Anand AM, Amoghimath R, Sankhe S, et al. Atypical rabies encephalitis in a six-year-old boy: clinical, radiological, and laboratory findings. *Int J Infect Dis*. 2015; 36:1–3. doi:10.1016/j.ijid.2015.05.005.
7. Mani RS, Anand AM, Madhusudana SN. Human rabies in India: an audit from a rabies diagnostic laboratory. *Trop Med Int Health*. 2016; 21:556–63. doi:10.1111/tmi.12669.
8. Hellenbrand W, Meyer C, Rasch G, Steffens I, Ammon A. Cases of rabies in Germany following organ transplantation. *Euro Surveill*. 2005;10:E050224.6.
9. Srinivasan A, Burton EC, Kuehnert MJ, Rupprecht C, Sutker WL, Ksiazek TG, et al for the Rabies in Transplant Recipients Investigation Team. Transmission of rabies virus from an organ donor to four transplant recipients. *N Engl J Med*. 2005; 352:1103–11. doi:10.1056/NEJMoa043018.
10. Dacheux L, Wacharapluesadee S, Hemachudha T, Meslin FX, Buchy P, Reynes JM, et al. More accurate insight into the incidence of human rabies in developing countries through validated laboratory techniques. *PLoS Negl Trop Dis*. 2010;4:e765. doi:10.1371/journal.pntd.0000765.
11. Mani RS, Madhusudana SN. Laboratory diagnosis of human rabies: recent advances. *ScientificWorldJournal*. 2013; 2013:569712. doi:10.1155/2013/569712.
12. Fooks AR, Johnson N, Freuling CM, Wakeley PR, Banyard AC, McElhinney, et al. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. *PLoS Negl Trop Dis*. 2009; 3:e530. doi:10.1371/journal.pntd.0000530.
13. Coertse J, Weyer J, Nel LH, Markotter W. Improved PCR methods for detection of African rabies and rabies-related lyssaviruses. *J Clin Microbiol*. 2010; 48:3949–55. doi:10.1128/JCM.01256-10.

14. Mani RS, Madhusudana SN, Mahadevan A, Reddy V, Belludi AY, Shankar SK. Utility of real-time Taqman PCR for antemortem and postmortem diagnosis of human rabies. *J Med Virol*. 2014; 86:1804–12. doi:10.1002/jmv.23814.
15. Dacheux L, Reynes JM, Buchy P, Sivuth O, Diop BM, Rousset D, et al. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin Infect Dis*. 2008; 47:1410–7. doi:10.1086/592969.
16. Mahadevan A, Suja MS, Mani RS, Shankar SK. Perspectives in diagnosis and treatment of rabies viral encephalitis: insights from pathogenesis. *Neurotherapeutics*. 2016; 13:477–92. doi:10.1007/s13311-016-0452-4.
17. Hunter M, Johnson N, Hedderwick S, McCaughey C, Lowry K, McConville J, et al. Immunovirological correlates in human rabies treated with therapeutic coma. *J Med Virol* 2010; 82:255–65. doi:10.1002/jmv.21785.
18. Lembo T, Niezgoda M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht CE. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerg Infect Dis*. 2006; 12:310–3. doi:10.3201/eid1202.050812.
19. Montano Hirose JA, Bourhy H, Sureau P. Retro-orbital route for brain specimen collection for rabies diagnosis. *Vet Rec*. 1991;129:291–2.
20. Tong TR, Leung KM, Lee KC, Lam AW. Trucut needle biopsy through superior orbital fissure for the diagnosis of rabies. *Lancet*. 1999; 354:2137–8.
21. Sudarsanam TD, Chacko G, David RD. Postmortem trucut transnasal brain biopsy in the diagnosis of encephalitis. *Trop Doct*. 2008; 38:163–5. doi:10.1258/td.2007.070091.
22. Sow PS, Diop BM, Ndour CT, Soumare M, Ndoeye B, Faye MA, et al. [Suboccipital aspiration puncture: postmortem brain sampling technique for the virological diagnosis of human rabies encephalitis in Dakar]. *Med Mal Infect*. 1996; 26:534–6 (in French).

Chapter 6

Histopathological techniques in the laboratory diagnosis of human rabies

Introduction

Histopathological examination of the brain is one of the oldest techniques for the diagnosis of rabies and was the gold standard for confirmation of rabies diagnosis before the advent of more advanced techniques such as immunofluorescence, immunohistochemistry and molecular methods. Such evaluation requires close attention to every step from brain removal, fixation, sampling for histopathology, tissue processing, sectioning, and staining. Each of these steps is detailed below.

Brain fixation methods (Step 1)

The brain begins to disintegrate as soon as it is disconnected from its blood supply. The process of arresting this process of deterioration and preserving the morphology of brain tissue is called “fixation”. The two most common ways of fixing the brain are freezing and the use of fixative solutions. Each method has its advantages and disadvantages.

Freezing is a much quicker way of fixing the brain than using fixative solutions (1) and tends to preserve the biochemical and molecular aspects, but it is not appropriate for histology and immunocytochemistry. Two common methods of rapidly freezing the brain include the use of dry ice and snap freezing in liquid nitrogen using pre-cooled isopentane. Freezing of tissues destroys morphology and thereby precludes histopathological examination for diagnosis. It is ideal for employing immunofluorescence on smears and molecular methods, but renders tissues unsuitable for histopathology. The former permits examination of only limited amounts of brain tissues, whereas histopathology allows sampling and detailed examination of all neuroanatomical areas in the brain.

Fixed brain tissues are ideal for histopathological examination and immunohistochemistry. The most commonly used fixatives include 10% neutral buffered formalin, 2.5% glutaraldehyde or 70% alcohol. Fixing the brain with fixative solutions takes longer than freezing, but the brain is much more resilient, easier to handle and provides excellent morphology. A 10% formalin solution is adequate and ideal for performing all histological stains, including immunocytochemistry.

Materials required for brain tissue fixation (1)

Reagents

- formaldehyde, 37–40%
- distilled water
- sodium phosphate, monobasic
- sodium phosphate, dibasic (anhydrous)

Preparation and storage of reagents

Preparation of 10% neutral buffered formalin solution

- | | |
|---|----------|
| • formaldehyde, 37–40% | 100.0 mL |
| • distilled water | 900.0 mL |
| • sodium phosphate, monobasic | 4.0 g |
| • sodium phosphate, dibasic (anhydrous) | 6.5 g |

The salts are dissolved in a mixture of formaldehyde and water, with continuous stirring. The pH should be between 7.0 and 7.5. **The fixative solution should be stored in a container labelled “HAZARDOUS CHEMICAL”.**

Precautions while handling: Avoid contact and inhalation, work in a well-ventilated area and wear protective gloves (2).

Protocol

Whole brain

1. Fix the brain removed at postmortem following craniotomy in 10% neutral buffered formalin.
2. Suspend the brain in a bucket containing 10% formalin, by passing a thread under the basilar artery (Fig. 6.1).
3. Ensure that the brain remains suspended for a minimum period of 2–3 weeks, and that it does not come into contact with the sides or bottom of the bucket. This assures optimum fixation, avoiding distortion of the brain.
4. A slit can be made in the corpus callosum to ensure better formalin penetration.

Precaution: Use at least 2 L of 10% formalin to fix the entire brain for a minimum period of 2–3 weeks.

Brain biopsies

1. Fix the biopsies in 10% neutral buffered formalin, ensuring the volume is at least 10 times that of the tissue size.
2. Immerse the tissue in a wide-neck bottle. For tissue samples of 1–3 mm, 2 h of fixation is sufficient. Larger specimens need to be sliced into thinner sections (0.5–1 cm) during grossing and left overnight for proper fixation, as the penetration rate for formalin is low (1–2 mm/h).

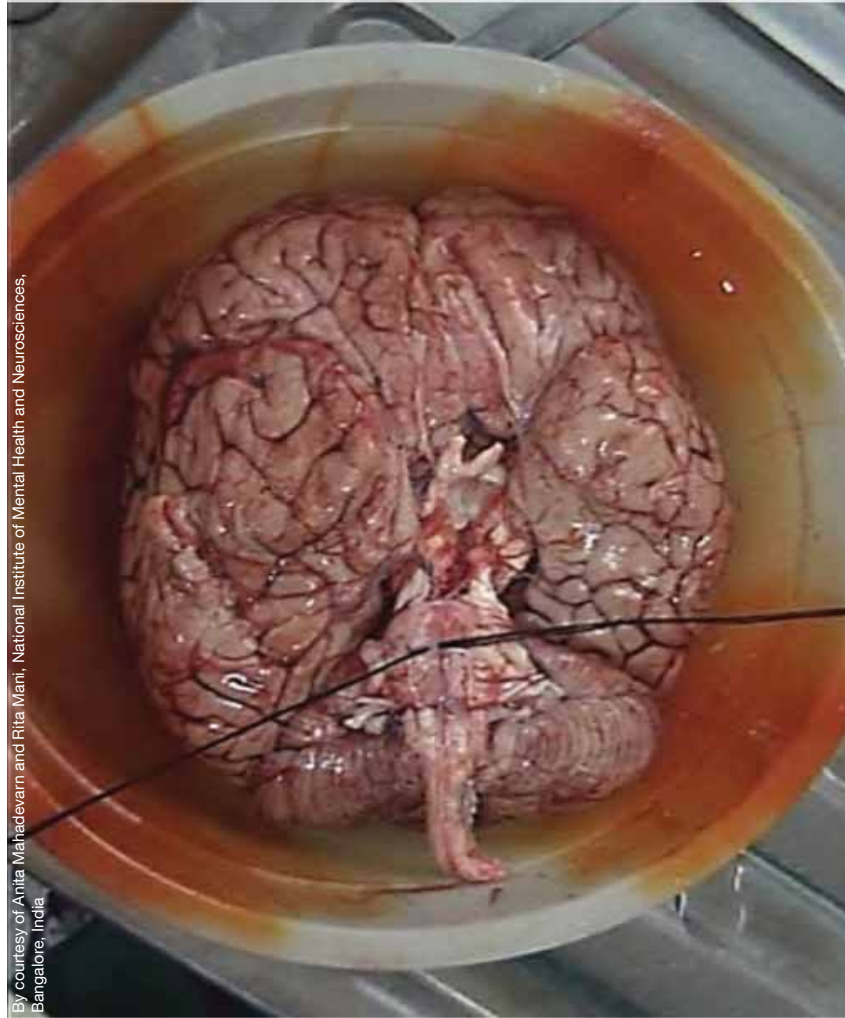


Fig. 6.1. Ideal method for fixing the brain by suspension in 10% neutral buffered formalin using a thread suspended under the basilar artery to prevent tissue distortion

Sampling the brain for processing (Step 2)

Once the brain is fixed optimally, it is sliced serially in the coronal plane into 1 cm thick slabs and the brain stem in the horizontal plane. The gross alterations, if any, are recorded. The different neuroanatomical areas are sampled as per the standard template, as shown (Fig. 6.2).

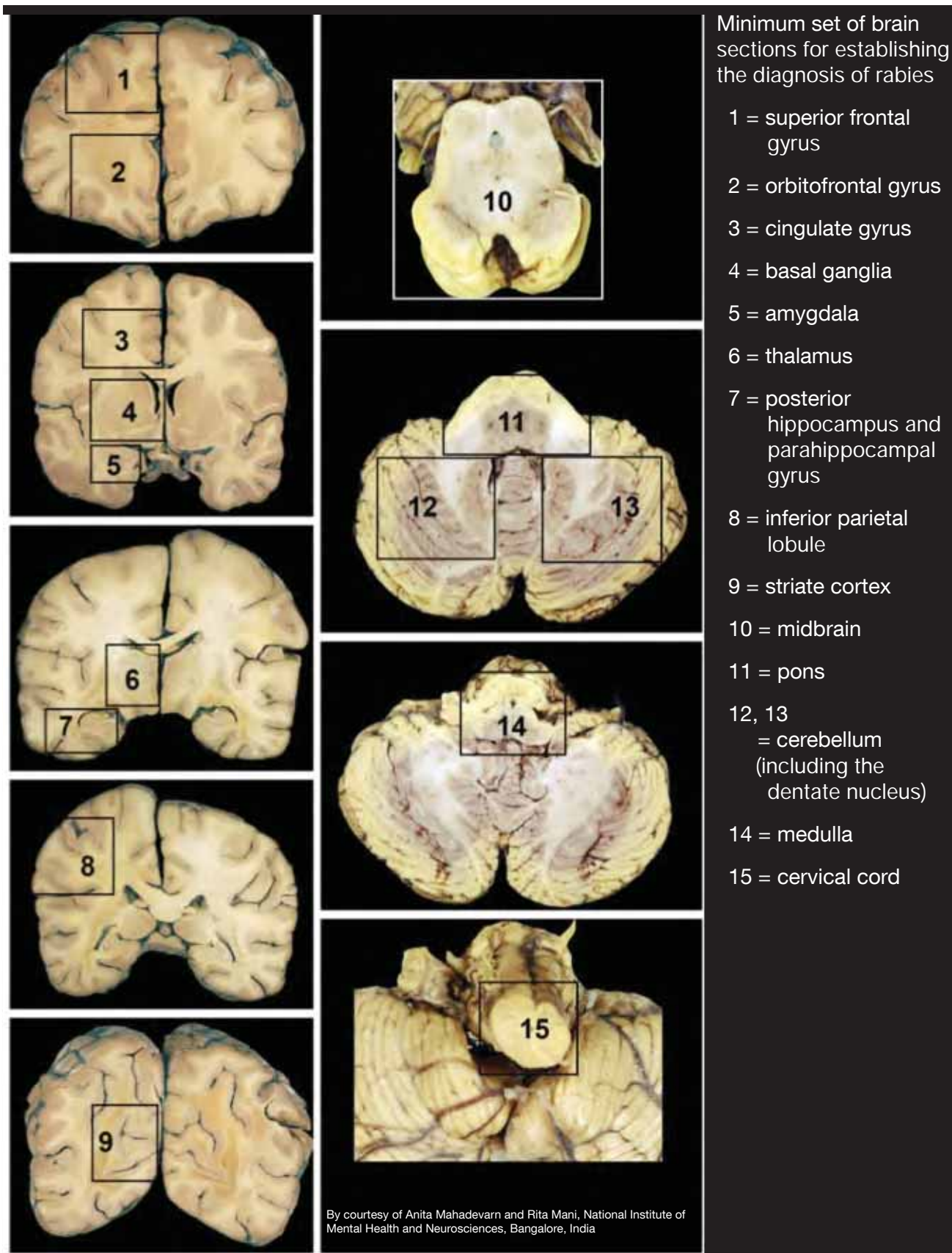


Fig. 6.2. Neuroanatomical sites to be sampled from brain slices

Tissue processing (Step 3)

The sampled tissues, after appropriate fixation in 10% neutral buffered formalin, are then processed for embedding into paraffin wax, preparatory to sectioning and staining.

Reagents (1, 3)

- buffered formalin, 10%
- 70% isopropyl alcohol
- 80% isopropyl alcohol
- 90% isopropyl alcohol
- 100% isopropyl alcohol
- chloroform/xylene (chloroform has slow penetration and is better suited for brain tissues)
- paraffin wax (melting point 60–62 °C)

Equipment

- automatic tissue processor with vacuum
- tissue cassettes

Procedure

Optimum timings must be standardized as per requirement, but the typical schedule given below is followed by most pathology laboratories (1, 3).

Overnight schedule: total processing time (14 h)

- 10% formalin (1 h)
- 10% formalin (1 h)
- 70% isopropyl alcohol (1 h)
- 80% isopropyl alcohol (1 h)
- 90% isopropyl alcohol (1 h)
- 100% isopropyl alcohol (1 h)
- 100% isopropyl alcohol (1 h)
- 100% isopropyl alcohol (1 h)
- chloroform (1 h)
- chloroform (1 h)
- chloroform (1 h)
- paraffin wax (1.5 h)
- paraffin wax (1.5 h)

Notes

1. Vacuum and agitation of the tissue basket is helpful to ensure proper penetration of reagents in the tissues and results in adequately processed tissue.
2. There are no advantages in using shorter processing times. The specimens can be poorly and incompletely dehydrated, cleared and infiltrated, which will cause problems in sectioning, staining and examining the tissues.
3. The reagent solutions are changed once every 10 days and solutions replenished daily to compensate for evaporation.
4. Once processed, the tissues are embedded in paraffin blocks.

Precautions (2)

1. Reagents used in tissue processing, especially formalin, xylene and chloroform, are biohazardous chemicals; gloves and masks should be used when handling these solvents.
2. Alcohols and acetone used are inflammable; adequate fire safety precautions should therefore be taken while handling these reagents.

Sectioning of paraffin blocks (Step 4)

Equipment

- rotary microtome
- slide warming table
- tissue flotation bath
- disposable blades
- egg albumin coated slides/negatively charged slides
- distilled water

Protocol (1, 4)

1. Use a rotary type of microtome to make thin sections (3–4 μm) for examination by light microscopy.
2. Disposable blades provide an excellent cutting edge for paraffin sectioning.
3. For routine tissue sectioning, set the thickness selector at 3–4 μm .
4. Orient the paraffin block in the block holder and secure it. Trim away the excess paraffin using coarse trimming, at 30 or 50 microns, until the entire surface of the tissue is exposed. Automatic Rotary microtomes have “trim” and “fine” buttons available to alternate between trimming and fine sectioning. Place the block in an ice tray to cool (to increase its firmness, as heat during trimming softens the block).
5. Section the block at 3–4 μm to create a ribbon. Place the ribbon gently onto the surface of the flotation bath, as described below.
6. Pre-heat the flotation bath to a few degrees below the melting point of the

paraffin wax being used (usually 45 °C). The use of distilled water in the water bath will help eliminate air bubbles. Tap water may also be used.

- 1% hydrochloric acid 20 mL
- tap water 4000 mL

Slowly add acid to the water. Bring the solution to the boiling point. Cool and add to the flotation bath as needed.

7. Lower the ribbon slowly onto the flotation bath to eliminate wrinkles and entrapped air. Then separate the sections using the edge of a glass slide. Remove any air bubbles with a camel's hair brush. Transfer the sections gently onto a clean pre-labelled slide by lowering it into the water bath and gently lifting up the ribbon.
8. Leave the slides to drain vertically for 15 min, then place them onto a warming table set at 50 °C to remain overnight. Failure to drain the slides will create air bubbles under the tissue and reduce the section's adhesion to the slide. Air bubbles produce section unevenness and staining artifacts, making the final preparation difficult to examine with the microscope.
9. Once the desired number of sections is cut, remove the block from the block holder and seal the exposed surface with molten paraffin. This ensures that the tissues will not dry out and will facilitate re-sectioning of the blocks weeks, months and years later.
10. The most common and routinely used section adhesive is egg albumin. Apply the egg albumin to the surface of the slides, then air dry them for later use. To make Mayer's egg albumin, dissolve 5 g egg albumin flakes in 100 mL distilled water with gentle heat. Filter through coarse filter paper. To 50 mL of filtrate, add 50 mL glycerin and mix. Add a small crushed crystal of thymol to prevent the growth of fungus. Store in the refrigerator. For use, take small quantities in vials.
11. Use of adhesive-coated slides in the water bath promotes the growth of bacteria and fungi. Clean the water bath daily with 1% sodium hypochlorite solution to prevent such contamination.
12. Skim the water surface of the flotation bath after cutting every case to prevent cross-contamination of tissue sections.
13. Pre-mark the slides with the case number using a glass marking pencil or bar code before starting the cutting.

Precaution: Care should be taken to avoid injuries while handling microtome blades.

Staining (Step 5)

Haematoxylin–eosin staining is the standard basic stain used for morphological analysis of sections. The staining method involves application of haemalum, which is a complex formed from aluminium ions and oxidized haematoxylin. This posi-

tively charged haemalum complexes in an acid medium to the negatively charged phosphate remains of the cell nucleus, i.e. DNA. It colours the nuclei of cells blue when washed in weak alkali, such as tap water, followed by saturated lithium carbonate. The nuclear staining is followed by counterstaining with an aqueous or alcoholic solution of eosin Y, which colours cytoplasm and other eosinophilic structures in various shades of red, pink and orange. Adding phloxine to the eosin intensifies the counter stain.

Reagents (1, 5)

Harris haematoxylin

- | | |
|-----------------------|-----------|
| • haematoxylin | 5.0 g |
| • 100% ethyl alcohol | 50.0 mL |
| • potassium alum | 100.0 g |
| • distilled water | 1000.0 mL |
| • mercuric oxide, red | 2.5 g |

Using a 2000 mL flask, completely dissolve the alum in distilled water with the aid of heat. In another glass container, vigorously shake to dissolve the haematoxylin in alcohol, at room temperature. Remove the alum and distilled water and slowly mix the two solutions. Bring the mixture to a boil as rapidly as possible, within approximately 1 min or less. Remove from the heat and slowly add the mercuric oxide; if it is added too rapidly the reaction is exothermic and the solution will spill out of the flask. Reheat the solution until it turns dark purple, then plunge it into a basin or sink of cold water to cool. The solution is now ready for use. Add 20 mL of glacial acetic acid to intensify the nuclear stain. Always filter the solution before each use.

Reagent stability: The solution is stable for about 6 months; a 1000 mL stock solution may be prepared and 500 mL used as a working solution. It is essential to filter the solution every day before use and to remove any surface muck with blotting paper before every use.

Eosin

Eosin stock solution

- | | |
|--------------------------|----------|
| • eosin Y, water soluble | 5 g |
| • distilled water | 100.0 mL |

Eosin–phloxine working solution

Combine in a 1000 mL cylinder:

- | | |
|------------------------|----------|
| • eosin stock solution | 100.0 mL |
| • 100 % ethyl alcohol | 400.0 mL |
| • acetic acid, glacial | 2.5 mL |
| • 1% phloxine | 500 µL |

1% acid alcohol

- 70% alcohol 99 mL
- concentrated hydrochloric acid (HCl) 1 mL

Saturated lithium carbonate

- Add 4 g of lithium carbonate to 500 mL of distilled water.

Protocol (1,5)

1. Deparaffinize the slides by taking them through the xylene followed by decreasing strengths of alcohols, beginning from absolute and moving through 95%, 80%, 70% and 50% and hydrate to water, before staining.
2. Stain in freshly filtered Harris haematoxylin (5 min).
3. Wash in running tap water (2–5 min).
4. Differentiate in 1% acid alcohol (1–2 dips).
5. Wash briefly in tap water.
6. Place in weak ammonia water until sections are bright blue.
7. Wash thoroughly in running tap water (10 min).
8. Place in 80% ethyl alcohol (1–2 min); this preserves the strength of the eosin solution.
9. Counterstain in eosin–phloxine solution (2 min).
10. Dehydrate and clear through 95% alcohol, absolute alcohol, acetone and xylene (2 min each).
11. Mount with DPX (disterene plasticized by tricresyl phosphate or dibutylphthalate) medium.

Results

Nuclei	Blue
Cytoplasm	Pink to red
Most other tissue structures	Pink to red

Mounting (Step 6)

The final step in the preparation of the slide is to cover the portion containing the tissue with a thin glass coverslip; this makes the slide permanent and permits microscopic examination. To affix the coverslip, mounting media of three types can be used: natural resins, synthetic resins, and aqueous media. Synthetic resin DPX of refractive index 1.52 is a good permanent mounting media for routine haematoxylin–eosin preparations and for most special stains.

Histopathological evaluation

Histopathological findings of the brain in rabies viral encephalitis reveal the following findings (Figs. 6.3A–E):

1. Meningeal inflammation (Fig. 6.3A)
2. Microglial nodules (Fig. 6.3B)
3. Perivascular inflammation with lymphocytes and histiocytes (Fig. 6.3C)
4. Negri bodies (Fig. 6.3D)

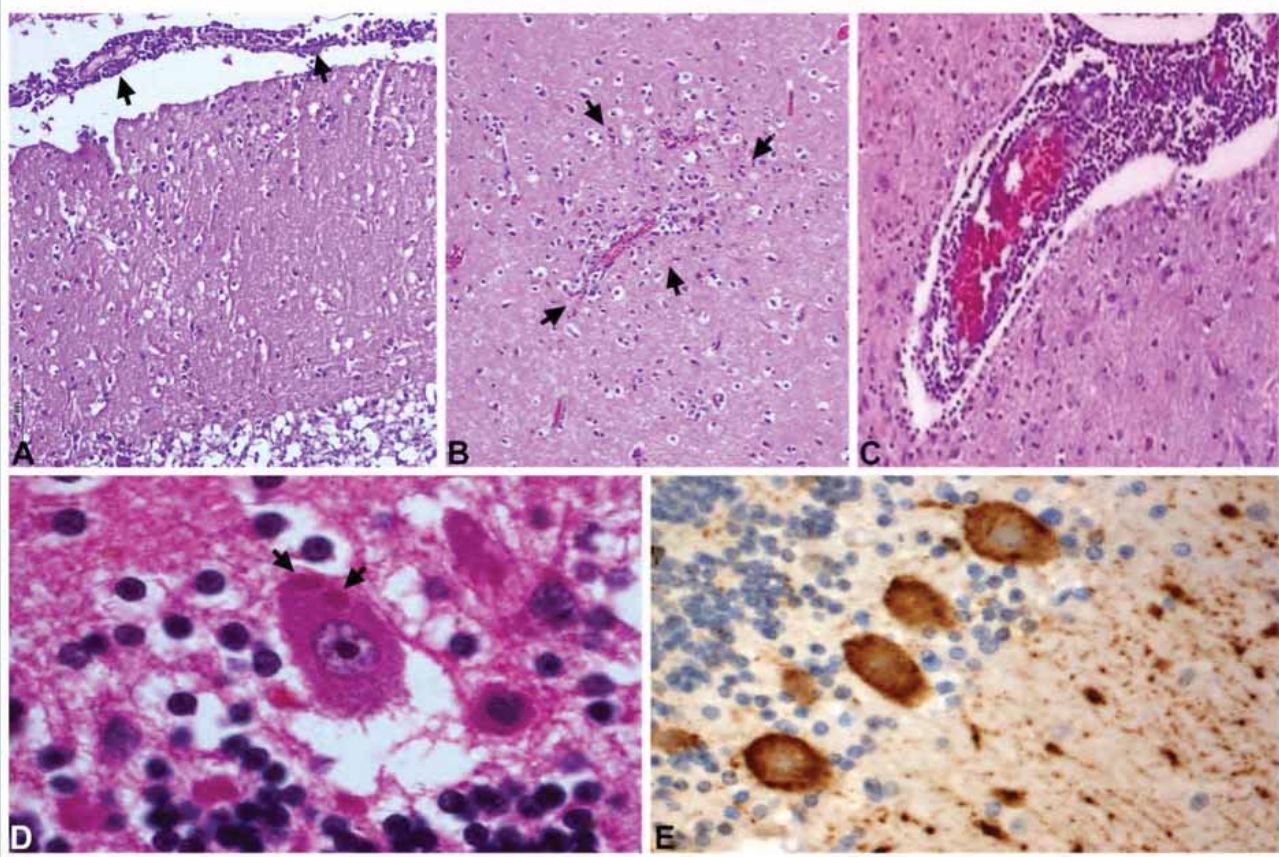
In viral encephalitides, meningeal inflammation with lymphohistiocytic infiltrates are seen in the subarachnoid space, reflecting pleocytosis seen in CSF. The presence of scattered perivascular inflammation and microglial nodules signifies encephalitis. Neuronophagia with virally infected neurons being surrounded by activated microglial cells is a frequent feature. However, intriguingly, perivascular and meningeal inflammation, neuronophagia and microglial nodules are usually not a prominent finding in rabies viral encephalitis.

Historically, Babès (1887) described “rabid nodules” in the motor nuclei of the medulla and the brain stem that represent microglial nodules (“Babès” nodules) (6). Van Gehuchten and Nelis (1900) demonstrated the occurrence of inflammation and proliferation of satellite cells in ganglia (trigeminal and sympathetic ganglia). Yet, it was only in 1903 that Adelchi Negri discovered the “inclusions”, bearing his name, in the hippocampal Ammon’s horn of rabid animals, making it possible to establish an accurate histological diagnosis of rabies; however, his interpretation that inclusions were caused by parasites belonging to protozoa proved to be wrong. Diagnostic utility was shown in a study by Negri’s wife, Lina Negri-Luzzani, in 1913 –. Confirmation of the viral nature of inclusions had to wait for invention of electron microscopy and immunohistochemistry.

Negri bodies are 0.25–0.27 μm round or are oval eosinophilic intracytoplasmic inclusions within the neurons. They are found most frequently in the pyramidal cells of Ammon’s horn, and the Purkinje cells of the cerebellum, as well as in the large neurons of the brain stem. Staining with Giemsa or Seller’s stains can permit differentiation of rabies virus inclusions from other intracellular inclusions. With these stains, Negri bodies appear magenta in colour and have small (0.2–0.5 μm), dark-blue interior basophilic granules; however, these stains are used very infrequently with the advent of immunohistochemistry.

The presence of Negri bodies is highly variable, being detectable in only about 30–50% of cases, occurring more frequently when the incubation period exceeds 3 months (Mahadever and Mani, personal observations). Histological staining for Negri bodies is neither as sensitive nor as specific as other tests. The direct fluorescent antibody test shows rabies virus antigens in nearly 99% of the samples, but this requires the use of fresh unfixed tissues. Hence, the presence of Negri bodies should not be considered essential for the diagnosis of rabies. Immunohistochemistry (Fig. 6.3E) is a highly sensitive and specific method for diagnosis of rabies and should be performed even in cases wherein routine histology detects the presence of Negri bodies.

By courtesy of Anita Mahadevarn and Ritta Mani, National Institute of Mental Health and Neurosciences, Bangalore, India



- A: Mild leptomeningeal inflammation (arrows)
B: Clusters of activated microglia forming "nodules" (arrows)
C: Perivascular lymphohistiocytic infiltrates seen in the grey matter
D: Classical round eosinophilic intracytoplasmic inclusions; "Negri bodies" seen within a cerebellar Purkinje neuron (arrows)
E: Widespread viral antigen within Purkinje neurons and its dendrites seen on immunohistochemistry, compared with fewer numbers of Negri bodies.
[A–D: haematoxylin–eosin stain; E: immunoperoxidase stain with polyclonal antibody to rabies; A–C: objective x10; D: objective x40, E: objective x20]

Fig. 6.3. Spectrum of histopathological changes in the brain in a case of human rabies viral encephalitis

References

1. Bancroft JD, Gamble M editors. Theory and practice of histological techniques, 5th edition. Edinburgh: Churchill Livingstone; 2002.
2. Laboratory safety guidance. United States Department of Labour Occupational Safety and Health Administration; 2011 (OHS 3404-11R; <https://www.osha.gov/Publications/laboratory/OSHA3404laboratory-safety-guidance.pdf>, accessed 27 April 2018).
3. Prophet EB. Tissue processing: dehydration, clearing, and infiltration. In: Prophet EB, Mills B, Arrington JB, Sobin LH, editors. Laboratory methods in histotechnology. Washington (DC): Armed Forces Institute of Pathology; 1992:29–31.
4. Achstetter VA. Microtomy. In: Prophet EB, Mills B, Arrington JB and Sobin LH, editors. Laboratory methods in histotechnology. Washington (DC): Armed Forces Institute of Pathology; 1992:45–52.
5. Allen TC. Hematoxylin and eosin. In: Prophet EB, Mills B, Arrington JB and Sobin LH, editors. Laboratory methods in histotechnology. Washington (DC): Armed Forces Institute of Pathology; 1992:53–58.
6. Kristensson K, Dastur DK, Manghani DK, Tsiang H, Bentivoglio M. Rabies: interactions between neurons and viruses. A review of the history of Negri inclusion bodies. *Neuropathol Appl Neurobiol.* 1996; 22:179–87. doi/pdf/10.1111/j.1365-2990.1996.tb00893.

Chapter 7

Brain removal

Introduction

Techniques for postmortem laboratory diagnosis of rabies in animals are conducted on brain material. The brain tissue can be collected either in a necropsy room after opening the skull and removing the entire brain, or in the field, without opening the skull, by sampling the tissue through the foramen magnum or through the bottom of the eye socket. Both techniques are presented here.

General requirements

Staff in contact with suspect specimens should be vaccinated against rabies with a licensed vaccine and must follow all biosafety recommendations. Ideally, depending upon the relative risk of viral exposure, the staff should be serologically sampled every 6 months to 2 years and receive a booster dose of vaccine if the virus neutralizing antibody titre falls below 0.5 IU/mL of serum (1).

Whichever method is chosen, some common requirements exist.

- Use one set of instruments per animal to avoid any cross- contamination of specimens.
- Clean and decontaminate any contaminated instruments chemically (2, 3) or thermally (e.g. boiling in water).
- Decontaminate all biological waste, ideally by incineration. Where this is not possible, the waste should be buried in quicklime in an isolated place selected to avoid any water pollution and at a depth that cannot be reached by carnivores. Safe disposal of these wastes is an important aspect of laboratory management. Waste should be disposed of in compliance with local legislation.
- Clean any apparatus used in the postmortem room (table tops, vice, eye-socket forceps, etc.) between specimens. All surfaces and instruments should be washed and disinfected after every diagnostic session and the floor, walls and other structures of the postmortem room disinfected and washed down daily.

In the laboratory, wearing protective clothing (e.g. gowns, aprons, gloves, visors, masks, goggles and boots) will protect the operator's skin and mucous membranes against splashing and spillage. All protective clothing must be removed when leaving the rabies laboratory and kept near the exit. In the field, use of PPE, including gloves, apron or gown and goggles, is a minimum requirement.

RABV is easily inactivated by heat or chemical treatment. Iodine, quaternary ammonium compounds and disinfectant (e.g. Virkon) are some of the most

commonly employed disinfectants. However, attention must be drawn to the decreasing activity of some disinfectants as quaternary ammonium compounds in the presence of hard water or large amounts of organic matter. If molecular techniques are to be used, the selected disinfectant must not only inactivate RABV but also disrupt nucleic acids (2).

Brain removal in the laboratory necropsy room

Materials

- scalpels, knives
- dissection forceps
- head fixation system such as:
 - a vice with parallel clamping jaws
 - forceps that allow stabilization of the head through the eye sockets

To open the skull, whatever the system used, it should be possible to follow the principle “one blade/saw, one head” with:

- a strong blade with a hammer
- a medical plaster vibrating saw
- a hand butcher saw
- disposable or sterile spoons
- Petri dishes

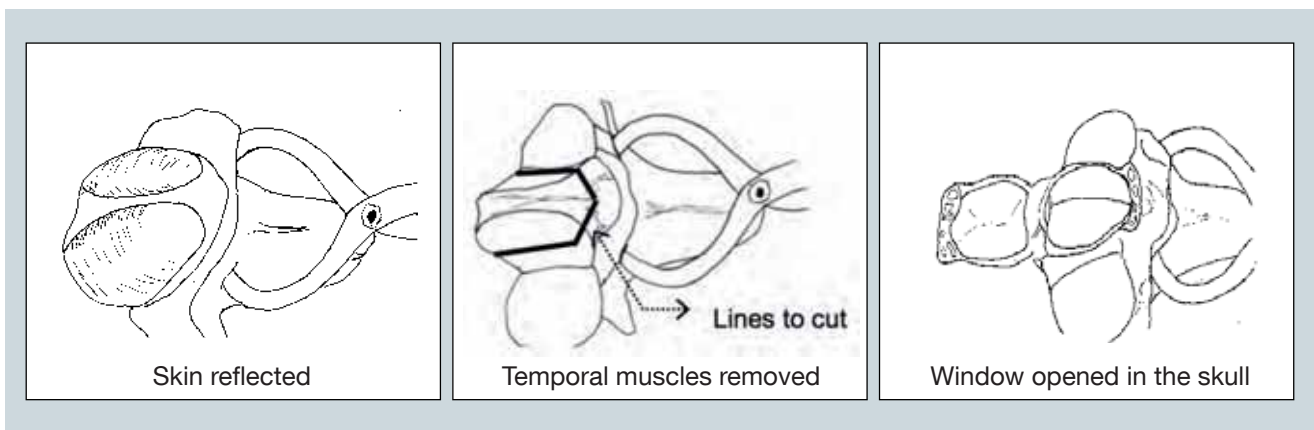


Fig. 7.1. Opening the skull

Protocol

Two main methods of brain removal in the laboratory exist.

Opening a section of the skull (Fig. 7.1)

1. Stabilize the head firmly.
2. Cut the skin along a median line on the top of the head and reflect it.
3. Remove the temporal muscles.
4. Open a window in the skull to access the brain.
5. Remove any dura mater that is still adherent to the brain.
6. Use a spoon to carefully isolate the brain from the skull and scalpel with forceps to collect the cerebellum and medulla oblongata.
7. Transfer the central nervous system to the dissection table for sampling.

Cutting a longitudinal section of the head

1. Maintain firmly the head in a vice.
2. Using a butcher's saw, cut the head longitudinally.
3. Collect the CNS from each half head.
4. Transfer the CNS to the dissection table for sampling.

Brain removal in the field

Brain sampling by opening the skull is hazardous when conducted outside of the laboratory. However, in field conditions, it may be necessary to collect samples of brain tissue for rabies diagnosis; this can be done without opening the skull, either through the foramen magnum or through the posterior wall of the eye socket.

The examples given below are for animals the size of a dog, and the tools used must be adapted to the size of the animal.

Diagnosis with reference methods performed on a full thickness, cylindrical specimen of brain tissue collected via the foramen magnum is as reliable technique as that performed on the hippocampus after opening the skull and dissecting the brain (4). Either this technique or a ventral approach via the throat can be used to access the spinal cord and brainstem, as described in the DRIT protocol (Chapter 12).

Materials

- 2–5 mL pipettes
- drinking-straws (≥ 5 mm in diameter)
- scalpels
- transport tubes with or without a field preservative solution (e.g. glycerol-saline)

Foramen magnum sampling procedure

Brain samples may be taken through the foramen magnum (5) by using a plastic drinking-straw (≥ 5 mm in diameter) or a 2 mL disposable plastic pipette (6). The steps of this procedure are successively:

1. Access to the foramen magnum

- a) of the entire animal:
 - i) Bend the head to expose the occipital region.
 - ii) Cut the skin and neck muscles over the joint between the occipital bone and the atlas vertebra.
 - iii) Open the atlanto-occipital joint by cutting the dorsal membrane and the meninges to access foramen magnum.
- b) of the head alone: check that the foramen magnum is easily accessible.

2. Collection of brain samples (Fig. 7.2)

- a) Enter the straw through the foramen magnum and turn it while pushing it towards one eye. By rotating and screwing the straw this way, samples can be collected successively from the brainstem, base of the cerebellum, hippocampus, thalamus and frontal cortex.

Note: Be careful not to push the straw too close to the top of the skull because it may then be blocked by the section of dura mater that covers the cerebellum.

- b) Pinch the straw between the fingers and gently withdraw it while continuing to turn it.



Fig. 7.2. Straw sampling in a dog

3. Transfer of the sample

- a) If the sample is to be analysed immediately, the cylinder of brain tissue is processed according to the operating instructions of the test. Hence, if the test uses the brainstem, its location is noted in the tube.
- b) If the sample must be sent to a laboratory, the straw, with the sample inside, is cut in the transport tube. If a rapid transport with cold packs is not available, a glycerin-based saline preservative solution may be used; it is then important to let the brain tissue inside the straw.

Variation: sampling through the eye socket

Cerebral tissues sampled through the eye socket are collected in the same way but they are taken in reverse order (7).

1. Push the eyeball to the outer side of the eye socket.
2. Introduce a trocar in the inner angle of the orbit to make an entry through the posterior wall of the eye socket.
3. Introduce through this hole a plastic drinking-straw or a 2 mL disposable plastic pipette, screwing it in the direction of the foramen magnum.
4. Pinch the straw between the fingers and gently withdraw it from the head.

Salivary gland removal

For epidemiological studies, the presence of RABV in salivary glands or its antigens or nucleic acid allows an evaluation of the role of reservoir and “non-reservoir” species in the dissemination of rabies and provides an additional source of material for isolation (Fig. 7.3).

1. With the ventral side of the neck up, make a longitudinal section of the skin over the trachea and reflect it.
2. The mandibular salivary gland is a well-defined gland under the mandibular lymph node, behind the posterior wall of the masseter muscle and under the jugular vein.
3. The parotid salivary gland is not as well defined as the mandibular one, lying on the ear duct.

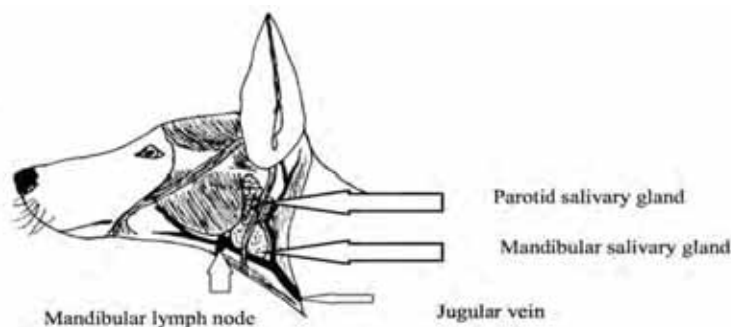


Fig. 7.3. Disposition of canine mandibular and parotid salivary glands

References

1. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 8 May 2018).
2. Aiello R, Zecchin B, Tiozzo Caenazzo S, Cattoli G, de Benedictis P. Disinfection protocols for necropsy equipment in rabies laboratories: safety of personnel and diagnostic outcome. *J Virol Methods*. 2016; 234:75–9. doi:10.1016/j.jviromet.2016.03.017.
3. Wu G, Selden D, Fooks AR, Banyard A. Inactivation of rabies virus *J Virol Methods*. 2017;243:109–12. doi:10.1016/j.jviromet.2017.02.002.
4. Barrat J, Halek H. [Simplified and adapted sampling and conservation techniques for the diagnosis of rabies in Mediterranean countries]. *Maghreb Vét*. 1987; 3:57–60 (in French).
5. Barrat J, Blancou J. Simplified technique for the collection, storage and shipment of brain specimens for rabies diagnosis. Geneva: World Health Organization; 1988 (WHO/Rab. Res./88.27; http://www.who.int/rabies/en/simplified_technique_collection_brain_specim_rabies_diagnosis.pdf, accessed 27 April 2018).
6. Bourhy H, Sureau P. [Laboratory methods for the diagnosis of rabies]. Paris: Institut Pasteur; 1990 (in French).
7. Montano Hirose MA, Bourhy H, Sureau P. Retro-orbital route for brain specimen collection of rabies diagnosis. *Vet Rec*. 1991; 129:291–292.

Part 2. Detection of virus

Chapter 8

Virus isolation in animals: the mouse inoculation test

Introduction

Isolation of pathogens in animal models, including the mouse inoculation test (MIT) for rabies diagnosis, has been used in global laboratory practice for more than a century (1). The advent of cell tissue cultures has facilitated a substantial reduction of animal use. Use of animals is discouraged when other methods are available. However, there is still no alternative to animal use in many areas of infectious disease research: for example, pathogenicity studies, evaluation of safety and efficacy of biologicals and other pharmaceutical preparations, or the absence of cell culture capacity in a given diagnostic laboratory, which is still common in developing countries (2, 3). Although weaning and adult mice demonstrate less susceptibility to lyssaviruses than some other mammals (such as Syrian hamsters, particularly via peripheral inoculation routes), they offer operational convenience because of their small body size, which allows housing of several animals in a cage, relatively inexpensive husbandry, and convenient handling offered by their long tails. Inoculated intracranially, mice (particularly those aged 1–3 days) demonstrate high susceptibility to lyssaviruses similar to that of nerve cell tissue cultures.

Isolation of lyssaviruses in mice has several advantages and disadvantages from isolation in cell culture, such as the rapid tissue culture infection test (RTCIT). For example, mice are usually more resistant than other small mammals to bacterial contaminants and toxic substances, which may be present in the diagnostic material obtained from dead animals in the field. Such samples frequently cannot be inoculated into cell culture, or inoculation of cell culture requires high dilutions of the samples, which reduces the sensitivity of the test. The use of mice allows accumulation of high virus titres at first inoculation, as the infection causes disease only after replication of lyssaviruses in the mouse brain. Conversely, most conventional cell cultures used to isolate lyssaviruses must be passaged every 3–5 days, thus not providing sufficient time for virus amplification until several passages (sometimes 10 or more) have been made (4). Such multiple passages *in vitro* may alter virus properties due to the accumulation of mutations in viral genomes, particularly in the absence of host immune response that occurs *in vivo*. In fact, the opposite and disadvantageous consequence of long virus replication in the mouse brain before the animal becomes ill is that MIT takes longer to complete (3–5 weeks) than RTCIT (2–3 passages, 4–5 days each) to ensure negative results.

In pathogen discovery studies, inoculation of animals facilitates isolation of different infectious agents causing diseases with their further identification and characterization (5). Such pathogens can be missed in cell culture, particularly if they replicate slowly and do not produce strong cytopathic effect. The MIT does not require a sterile environment and CO₂ incubator, and it can be performed in

relatively basic field laboratories with a vivarium. Therefore, operational choice between MIT and RTCIT depends on the aims of the study, the quality of samples and the capacity of the laboratory.

Choice of animals and their husbandry

All conventional strains of albino laboratory mice demonstrate nearly identical susceptibility to lyssaviruses. Suckling mice (aged 1–3 days) are preferable because of their high susceptibility. As was demonstrated for several attenuated rabies virus (RABV) strains, mice aged 1–7 days were susceptible to both intracranial and intramuscular challenge, whereas mice aged 3 weeks were not susceptible to these viruses inoculated by any of the routes. Nevertheless, weanling mice (aged 3–4 weeks) can be used for routine isolation of lyssaviruses from samples collected in the field. The use of older mice is not recommended because of their decreased susceptibility and because their skulls are too thick for conventional challenge via narrow gauge needles. Forced use of needles on thick skulls will immediately increase traumatic effects. Mice of both sexes are equally susceptible, but an attention should be paid to not put young adult mice of different sexes in one cage to avoid unwanted breeding. The use of females is more practical, as males may fight more frequently.

The animals must be in good general health. This will make them more resistant to adverse effects from intracranial inoculation and will eliminate nonspecific morbidity and mortality during the test. Generally, at least 3 or more days of quarantine observation is required for mice after their delivery from a breeding facility to the diagnostic laboratory for them to adapt to the new environment, decrease any stress caused by transportation and identify any potential health problems. Mice that become ill and demonstrate unexpected clinical signs during the experiment (e.g. diarrhoea) should be removed immediately from the experiment and submitted to the vendor or to a veterinary laboratory for diagnostic purposes (if mice have been inoculated with lyssaviruses, the laboratory where such a diagnosis is performed must be licensed for work with these pathogens, and diagnostic staff must be vaccinated appropriately).

Housing of mice depends on the size of the cages used. It is unlikely that mice can transmit lyssaviruses among each other during the observation period; therefore, properly marked mice inoculated with different specimens can be co-housed. However, to avoid mislabelling, it is always preferable to keep in each cage the mice inoculated with only one specimen. A group of 5–10 weanling mice is usually used per specimen. Suckling mice are housed with a dam, one litter per cage, and are inoculated with one specimen (marking of suckling mice is difficult and unreliable, except tattooing). If a litter is large and some of the suckling mice are not used for inoculation, they should be euthanized to avoid misinterpretation of test results.

Preparation of the inoculum

Suspensions of tissues, cell cultures or saliva are commonly used for the MIT. The tissues must be rigorously homogenized using suitable means of preparation. All non-disposable equipment used for homogenization must be sterilized by autoclaving. Ideally, all steps of preparation of the inoculum should be performed in a biosafety cabinet to contain dispersion of droplets.

Brain tissue is soft and can be homogenized easily. Other organs, particularly those with a large amount of connective tissues (e.g. salivary glands), may represent difficulties. A pestle and mortar are the “classical” homogenization tools and can be efficiently used if the amount of tissue is relatively large (0.3 g or more). Abrasives such as sterile sand or glass shards can be added to improve homogenization of tough tissues. For small amounts of brain tissue, sterile wooden or plastic sticks can be used to homogenize the sample in a microtube with a flat or cone bottom (1.5–2.0 mL Eppendorf or similar tube; a screw cap is preferable to control leakages). For small amounts of tough tissues, it may be necessary to use metal or polymer beads, placed in a microtube together with the tissue sample, and subject the tube to high-speed vibration in an electric homogenizer for 5–10 min. Homogenization supplies, instruments and diluents should be pre-cooled at 4 °C to reduce enzymatic destruction of virions during preparation of the inoculum.

One of the most commonly used diluents for preparation of the suspension is a sterile isotonic NaCl (sodium chloride) solution, although mice are resistant even for inoculation with distilled water. An addition of 2–20% of animal serum will increase virus stability during the preparation and inoculation period. However, the donor animal for the serum supplement should never be vaccinated against rabies. If such information is unavailable, dog, cat and cattle sera should not be used for preparation of the inoculum. Commercial equine, sheep or rabbit sera are usually safe for use. The serum must be heat-inactivated at 56 °C for 30–45 min. If sterility of the diluent is not warranted by the manufacturer, it must be filtered through a bacteria-retaining filter. Many laboratories routinely use cell culture medium (for example, the minimum essential medium) for preparation of the animal inoculum. It usually contains 2–5% fetal bovine serum certified for the absence of RABV antibodies, pathogens and toxic substances. Although antimicrobial and antifungal medicines are not needed to inoculate mice with fresh sterile tissue material, their use is advised for inoculation with salivary gland suspensions or other samples that can be contaminated with bacteria or fungi. A mixture of penicillin (500 IU/mL) and streptomycin (1500 IU/mL) has been used for such purposes historically, and more antibiotics are now available commercially. Usually, antibiotics may be already present in the minimum essential medium and other cell culture media.

A suspension (by weight) of 10% is typically used to inoculate animals, although this is an operational choice and the concentration can be increased 2-fold or decreased without significant changes in the sensitivity of the test or adverse effects for inoculated animals. The required amount of diluent is usually added to the tissue specimen gradually during homogenization. When homogenization is performed with beads in a closed tube, the full amount of diluent can be added to the tube at the beginning. After homogenization, the suspension should be centrifuged to remove gross particles. While such particles may not adversely affect inoculated animals, they may clog the syringe needles during inoculation.

Centrifugation is usually performed at 150–200 *g* for 10–15 min, ideally in a cooling centrifuge (4 °C). If bacterial contamination is expected in the specimen, the suspension supernatant can also be subjected to filtration via a 0.45 µm filter attached to a syringe. The use of 0.22 µm filters is less practical: it does not add much benefit for removal of bacterial contamination but absorbs micro-particles of tissue that may contain the pathogen of interest in a greater quantity than the clean supernatant. The prepared inoculum should be kept in a container with pre-cooled ice packs or on wet ice until use. Freezing should be avoided, because each freeze–thaw cycle may reduce virus titre.

Inoculation procedure

Preparation

The inoculation can be performed in a biosafety cabinet or on the laboratory bench, depending on institutional requirements. As lyssaviruses are not transmitted by the aerosol route except under highly specific circumstances (e.g. a high concentration of aerosol particles, or an artificially concentrated virus preparation), animal inoculation on the bench is usually considered safe. The bench, all surfaces and instruments around must be washable; appropriate personal protective equipment (PPE) should be used to avoid self-exposure and to protect the animal from human infections that may be introduced by the operator performing the test. The minimum PPE includes a laboratory coat with long secured sleeves, double latex gloves, a face shield and a hair net to protect against droplets of inoculum. There is absolutely no efficient way to protect an operator from self-stick injuries by syringe needles except by exercising cautious behaviour and good inoculation skills. Hands should not be crossed during the procedure: mice and mouse containers should be placed at one side, whereas syringes and sharps containers should be placed on the other side from the operator. Syringes should not be re-capped, to reduce the risk of needle stick injuries. If this is not possible, staff must be trained in the safe one-hand capping technique, or special capping devices (available in the market) should be used.

Anaesthesia

Ideally, mice should be anaesthetized prior to intracranial injection. This is the only option approved by the American Veterinary Medical Association (AVMA) and the American Association for Laboratory Animal Science (AALAS). Similar requirements have been introduced in many countries worldwide, and their implementation is enforced by the local Institutional Animal Care and Use Program (IACUC).

In general, there are two major options for anaesthesia of mice, with inhalational or injectable medicines. The most convenient inhalational anaesthetic used for this purpose is isoflurane (6). Isoflurane has many advantages over injectable agents: minimal animal handling, large margin of safety, ease of anesthetic control, low cost of anaesthetic agent, no controlled medicines and quick recovery times. The primary disadvantages are the initial cost of equipment and the need to control human exposure to gas (which is avoidable if the room satisfies the ventilation requirements standard for BSL2 and BSL3 facilities).

Minimum components of equipment include:

- isoflurane vaporizer
- supply gas (oxygen)
- supply gas regulator
- flowmeter (0–1000 mL/min)
- induction chamber
- connection tubing and valves

The flowmeter should be set between 500 mL/min and 1000 mL/min, and the vaporizer to 5%. The incubation chamber can be pre-filled with the gas, and group of animals placed in the chamber. Anaesthesia typically occurs after 1–2 min of incubation, after which time the animals (individually) can be removed; the operator then has 1–2 min to inoculate the mouse before it awakens, which is usually sufficient time. Using this technique, a group of five mice can be inoculated during 4–6 min before another group of mice is placed in the chamber for the next sample.

Other inhalational agents (halothane, sevoflurane, etc.) are used less frequently. Ether, used for animal and human anaesthesia historically, is not approved by AVMA and AALAS due to its potential side-effects and poor safety characteristics.

Among the injectable anaesthetics, ketamine in combination with other agents (e.g. xylazine) is used most frequently. Table 8.1 shows examples of such combinations, which are administered in a volume of 0.1–0.2 mL, usually intraperitoneally (7). The anaesthesia usually takes effect after 0.5–1 min, and lasts 5–15 min. If needed, the anaesthetic can be reversed by atipamezole (1 mg/kg) but this is usually not necessary, because if several samples are inoculated in several groups of mice, the first groups will wake up before the last one is completed. Combinations of anaesthetics can be prepared in advance in designated sterile containers, which must be properly labelled and stored until the expiration date (the earliest among the medicines used in the combination).

Other injectable anaesthetics (barbiturates, benzodiazepines, opioids) are used in mice less frequently. Tribromoethanol may cause unpredicted effects in mice aged older than 16 days.

If anaesthetics or equipment for anaesthesia are not available in the diagnostic laboratory, which frequently occurs in developing countries, or if national and institutional regulations do not require anaesthesia, mice may be inoculated without sedation (and this method was used for decades worldwide). One must consider that needle-stick injuries caused during inoculation are momentary, whereas longer lasting adverse effects, if they occur, are caused not by the needle stick itself but by trauma. Therefore, a short-term anaesthesia will not protect mice from such effects. Instead, precautions must be taken to avoid excessive trauma, and the technique described below may help in this regard.

Medicines	Dosage	Comments
Ketamine + xylazine	100/10 mg/kg	α 2 agonist reversible with atipamezole 1 mg/kg
Ketamine + medetomidine	Female 75/1 mg/kg Male 50/1 mg/kg	α 2 agonist reversible with atipamezole 1 mg/kg; may cause moderate respiratory depression
S-Ketamine + medetomidine racemic ketamine + medetomidine	Female 75 mg/kg + 0.25 mg/kg Male 50 mg/kg + 0.25 mg/kg	α 2 agonist reversible with atipamezole 1 mg/kg; action shorter and awakening faster than racemic ketamine
Ketamine + dexmedetomidine	50–75/0.5–1 mg/kg	α 2 agonist reversible with atipamezole 1 mg/kg; may cause moderate respiratory depression

Table 8.1. Use of ketamine in combination with other medicines for injectable anaesthesia of mice aged 3–4 weeks

Inoculation technique

The volume of inoculum used for intracranial inoculation of weanling mice is 30 μ L; smaller volumes (10–15 μ L) should be used for suckling mice. Intramuscular or subcutaneous inoculation of mice is usually performed in 50 μ L volumes. These doses can be measured accurately in a 0.5 mL insulin syringe. The needles should be 0.40–0.45 mm in diameter (26 or 27 gauge) and 1–2.5 cm long. Larger needles traumatize the brain substantially, whereas larger syringes do not allow accurate measurement of inoculation doses. Ideally, the syringe should be disposable, with an attached needle which does not have dead space. The use of such a syringe will help to minimize air bubbles. If bubbles do occur, they must be removed into a sterile soft substance (such as sterile cotton) to avoid aerosolization. Sterilized glass syringes with detachable needles can also be used, although they are less convenient. Ideally, an individual syringe should be used to inoculate each mouse. This is especially important for disposable syringes as their needles are made of soft metal and become blunt after each intracranial injection. Even if one syringe is used to inoculate a group of several mice, under no conditions should the same syringe be used to inoculate other samples without sterilization, to avoid cross-contamination.

Generally, right-handed individuals handle a mouse in the left hand and a syringe in the right hand. For sedated mice, no special handling technique is needed except that the animal must be fixed by grasping the hair scruff and pressing the head gently but firmly to the surface of an inoculation board, which will allow a precise needle stick to penetrate the skull, without moving or rotating the skull under the pressure of the needle. Sedated suckling mice, which have very soft skulls, can be easily handled by forceps during all inoculation procedures. This eliminates the risk of needle stick injury for an operator. If not sedated, it is easy to take a mouse at the tip of the tail by the thumb and the second finger, put the animal on the bench, clutch the base of the tail between the fourth and fifth fingers, release the thumb and second finger, and grasp by the mouse's scruff. The mouse handled tightly at the base of the tail and at the scruff is sufficiently

immobilized to allow manipulations on the head, even without sedation. Non-sedated suckling mice are typically handled on their neck scruff by finger and thumb, which warrants sufficient immobilization.

The syringe is taken by the right hand (the index finger on the plunger, and the barrel supported between the thumb and the third and fourth fingers) and pushed through the skull with a quick thrust. Although inoculation can be done at different areas of the calvarium, it is usually easy to make the injection slightly aside of the central line (to avoid puncture of the venous sinus), in the middle of the imaginary line between the mouse's right eye and right ear (Fig. 8.1). The needle should easily penetrate the skull; it should be inserted 1–3 mm into the brain tissue. Too deep a penetration and injection of inoculum into the basal area of the brain can be very traumatic. After injecting the desired volume of inoculum, the needle should be removed gently and slowly (during several seconds) to prevent reflux of the inoculum. If such reflux occurs, it will be seen on the mouse's fur as a drop of transparent bloody liquid; it will mean that the amount of the inoculum in the mouse brain is reduced.

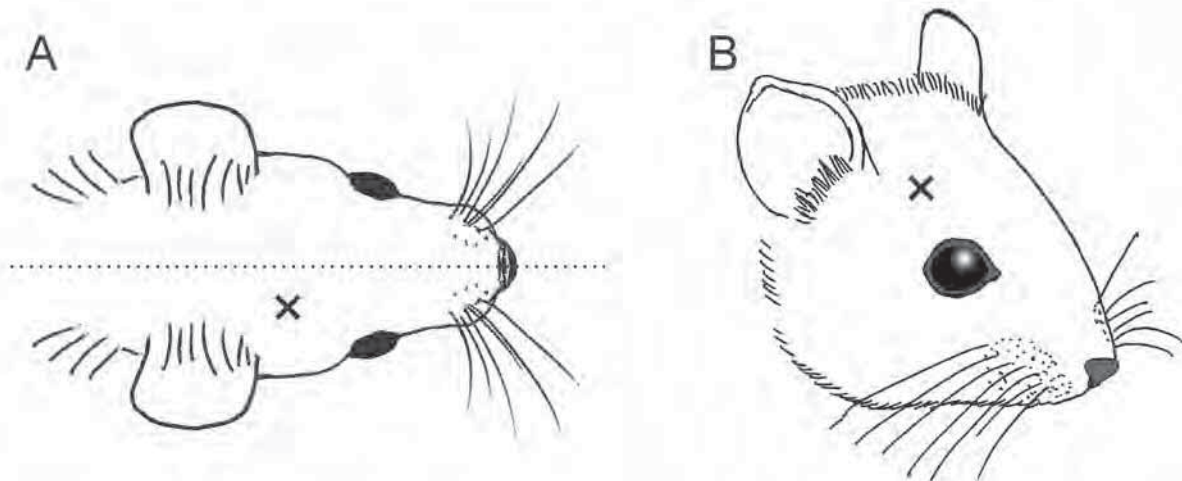


Fig. 8.1. Place of intracranial inoculation on the head of a mouse aged 3–4 weeks: A, dorsal view; B, facial view

Peripheral inoculation is rarely performed for diagnostic purposes as the susceptibility of mice to lyssaviruses injected via peripheral routes is limited. The example when peripheral inoculation may be used for diagnostic purposes is a significant bacterial contamination or toxicity of the inoculum that kills mice if injected intracranially. Intramuscular challenge is usually performed into the deltoid or gastrocnemius muscles. An assistant is needed to stretch the leg of the mouse for immobilization. Subcutaneous inoculation can be performed under the scruff of the neck, when handled as described above for intracranial challenge.

Contamination of work surfaces, protective wear and instruments during the procedure may occur via droplets of inoculum from the syringes or from the animal's fur. To avoid potential exposure and further spread of the virus, all materials should be rigorously decontaminated with disinfectants immediately after completion of the inoculation. Outer gloves (which also can be contaminated) should be replaced before the decontamination procedure. Disposable syringes are discarded in sharps containers and autoclaved together with other disposable supplies used for inoculation. Non-disposable syringes and needles should be disinfected chemically, rinsed in water and autoclaved before next use.

Cages must be labelled promptly and carefully, indicating the age of the animals, the inoculation date, the inoculum used, the number of animals and any other useful information. Incomplete labels may cause missing specimens or data.

Observation and euthanasia

The inoculated mice should be observed closely for ~ 2 h after the procedure. This period of time is usually sufficient for recovery from anaesthesia or from inoculation trauma. If mice continue to behave abnormally (e.g. slow movements, ataxia, continuous one-side rotation), the observation should be continued for another hour, and a decision on euthanasia is made.

The incubation period of RABV in mice inoculated intracranially is usually 4–20 days, during which time the animals should be checked twice a day to register early clinical signs. During other times (starting from the first post-inoculation day) they should be checked at least daily. The observation period should extend for at least 40 days, unless fixed strains of RABV with short incubation periods, such as challenge virus standard strain (CVS), are used. The number of healthy, sick and dead animals should be recorded daily on designated cards or record sheets. Clinical signs should be recorded for sick animals. The following clinical signs are suggestive of rabies: ruffed fur; tremours when handled by forceps; increased agitation with chaotic fast movements or jumps; lack of coordination in legs, best noted when a mouse is placed on a bench and starts to move; ataxia; paresis; paralysis; prostration.

Clinical signs and death occurring during the first 2 days after inoculation are usually attributed to trauma or contamination of the inoculum with bacterial or other viral pathogens. Whenever clinical signs occur, a decision on euthanasia should be made promptly based on the pain scores approved by the IACUC. Usually, mild clinical signs (pain scores of 1–2) require increased observation whereas severe signs (pain scores of 3–4) require immediate euthanasia.

The euthanasia is performed via intoxication with carbon dioxide or other means (8). Placing the animals in a chamber pre-filled with carbon dioxide causes severe distress. Therefore, the animals must be placed in a chamber filled with ambient air into which carbon dioxide is supplied with gradually increasing concentration, at a rate of 10–30% of volume per min. Mice must be observed closely to ensure euthanasia via the absence of breathing and heart beats and cyanotic skin. As alternatives to carbon dioxide, an overdose of anaesthetics used during inoculation or barbiturates can be used. In every case, euthanasia on an unconscious animal should be confirmed by a secondary method, such as cervical dislocation or thoracic opening. After completion of each experiment, cages must be carefully cleaned with disinfectants and autoclaved.

Postmortem examination

Even if mice develop neurological signs or die within the expected timeframe after inoculation, diagnosis of rabies should not be assumed without laboratory confirmation. Similar clinical signs may be caused by a number of other viral or bacterial pathogens. Most commonly, the confirmation is made by detection of lyssavirus antigens in the mouse brain via the DFAT, although other methods for detection of antigens or RNA can be used as well.

Removal of the brain should be performed in a biosafety cabinet. The mouse carcass is placed on clean absorbing material (such as a gauze pad or a paper towel), ventral surface downwards. For a right-handed operator, the mouse head should be directed to the left. The skin of the neck and head is pulled by forceps and cut away. The skull is grasped by forceps in the orbits, and the calvarium is cut away by curved scissors or a scalpel exposing the brain. The brain is lifted by the same scissors or scalpel, removed from the skull and placed on a clean gauze pad or paper towel. A section of the brain just anterior to the cerebellum is used to make impressions on a microscope slide.

Troubleshooting

Usually the MIT produces acceptable results, but in some instances it fails. The most common reasons for such failures are addressed below and appropriate corrective actions are suggested.

If mice do not recover completely after inoculation and die shortly thereafter, this may result from trauma or from toxicity of the inoculum. Trauma usually produces distinct morbidity or mortality patterns in different mice, whereas toxicity of the inoculum produces a uniform result in all inoculated animals. To reduce trauma, the needle should not be inserted too deeply during inoculation, as described above, and long, inappropriately gauged needles should not be used. Also, younger mice usually recover better after inoculation than older mice. If the toxicity of the inoculum is the reason for failure, it should be determined whether the diluent or the tissue specimen is toxic. If the diluent is toxic, all samples prepared with it will produce uniform mortality patterns. A mock mouse inoculation with the same diluent can be performed to confirm such toxicity. If this is the case, the diluent must be replaced. If the toxicity originates from a specific tissue sample (and only mice inoculated with this sample demonstrate early morbidity or mortality), the sample can be diluted, and several 10-fold dilutions can be used for inoculation. Even though such dilutions will reduce the sensitivity of the MIT, this may be the only way to isolate a particular virus.

Sometimes, mice recover after inoculation but develop clinical signs and succumb within 48 h or later but in the absence of lyssavirus antigens in their brains. This usually results from bacterial or other viral contamination of the inoculum. To remove bacterial contamination, the inoculum can be filtered through a 0.45 µm filter attached to a syringe. Should viral contamination occur, there is no realistic way to separate an unwanted virus from a lyssavirus present in the sample. The exceptions include only some mouse viruses which are not pathogenic for other animals. In such a case the inoculum can be injected intracranially or intramuscularly into other mammalian species (for example, Syrian hamsters) where the murine virus should not replicate but the lyssavirus will.

If a lyssavirus cannot be isolated from a sample that demonstrates abundant presence of viral antigens or RNA, this can result from significant degradation of infectious virus (for example, due to inappropriate storage) or from high titres of virus-neutralizing antibodies in the specimen. If a sample is degraded, a higher concentration of the inoculum can be used (for example, 20% tissue suspension), and newborn (aged 1–3 days) mice inoculated to increase the sensitivity of the MIT. If virus-neutralizing antibodies are present in the sample, several serial 10-fold dilutions of the inoculum can improve the chances of virus isolation. The dilutions should be made promptly after preparation of the original inoculum, and cold diluent should be used at each step to decrease binding of antibodies to the virus released from the tissue cells during the homogenization process.

Virus titration

Quantitation of virus via determination of an infectious or lethal dose can be performed using titration in cell culture or in animal models, including mice. The titres obtained in animal models and in cell culture usually correlate, although the titres of infectious virus may not necessarily correlate with antigenic load determined by ELISA or with the number of genome copies determined by quantitative RT-PCR. Cell culture offers several advantages for virus titration compared with animal models. The test in cell culture is more rapid, and eventually is less costly; however, it requires cell culture capacity. Mouse inoculation can be performed in a more basic BSL2 laboratory with a vivarium. Other examples where determination of lethal doses in animals is preferable include the evaluation of susceptibility *in vivo* and pathogenesis studies, potency tests for biologicals, and comparison of virulence via intracranial and peripheral routes.

Several serial dilutions of viral inoculum are used for a titration. Most commonly, 10-fold dilutions are used for determination of virus titre in mice, although other dilution factors (for example, 2-fold, 3-fold, 5-fold) can be used as well. The less the dilution factor, the more precise is the titre determined. However, a decrease of the dilution factor will increase the number of animals used. For example, seven 10-fold dilutions (seven groups of mice) are usually sufficient to determine lyssavirus titres in most field samples. To cover the same titre range via 5-fold dilutions, 10 groups of mice are needed. Even 21 groups of mice would be needed to cover this range via 2-fold dilutions. The size of the group inoculated with one dilution can also vary. Usually 4–5 mice per group are used for generic titrations, but groups as large as 10 or even 20 mice can be used for such important procedures as determination of vaccine potency test for statistical comparisons.

All dilutions are inoculated in mice following the standard MIT procedure described above. It is preferable that the inoculation is made by one operator to improve comparability of the results between groups. The observation period for mice inoculated intracranially can usually be reduced to 30 days. Longer incubation periods are rare, and even if 1–2 mice are missed because of the observation shortening, they will not affect the obtained titre significantly. However, for mice inoculated peripherally the observation should be at least 45 days (preferable 60 days) because longer incubation periods in such animals occur more frequently.

After completion of the experiment the mice from the end-point dilution that caused mortality and from at least one dilution above are examined for the presence of viral antigens in their brains. The titres are calculated via the Reed & Muench or Spearman–Kärber methods, as described elsewhere.

References

1. Koprowski H. The mouse inoculation test. In: Meslin F-X, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*, 4th edition. Geneva: World Health Organization; 1996:80–7.
2. Markotter W, Kuzmin IV, Rupprecht CE, Nel LH. Lagos bat virus virulence in mice inoculated by the peripheral route. *Epidemiol Infect.* 2009; 137:1155–62. doi:10.1017/S0950268808001945.
3. Faber M, Li J, Kean RB, Hooper DC, Alugupalli KR, Dietzschold B. Effective preexposure and postexposure prophylaxis of rabies with a highly attenuated recombinant rabies virus. *Proc Natl Acad Sci U S A.* 2009; 106:11300–5. doi:10.1073/pnas.0905640106.
4. Bourhy H, Rollin PE, Vincent J, Sureau P. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immuno-diagnosis for rapid laboratory diagnosis of rabies. *J Clin Microbiol.* 1989; 27:519–23 (<https://pdfs.semanticscholar.org/f162/ed980cfb1abab567aeeb-d8a2feef088ca73c.pdf>, accessed 1 May 2018).
5. Shope RE, Murphy FA, Harrison AK, Causey OR, Kemp GE, Simpson DI, et al. Two African viruses serologically and morphologically related to rabies virus. *J Virol.* 1970; 6:690–2. PMID:5530013.
6. Gargiulo S, Greco A, Gramanzini M, Esposito S, Affuso A, Brunetti A, et al. Mice anesthesia, analgesia, and care, Part I: anesthetic considerations in preclinical research. *ILAR J.* 2012; 53:E55–69. doi:10.1093/ilar.53.1.55.
7. Taylor BJ, Orr SA, Chapman JL, Fisher DE. Beyond-use dating of extemporaneously compounded ketamine, acepromazine, and xylazine: safety, stability, and efficacy over time. *J Am Assoc Lab Anim Sci.* 2009; 48:718–26. PMID:19930819.
8. AVMA guidelines for the euthanasia of animals: 2013 edition. Schaumburg (IL): American Veterinary Medical Association; 2013.

Chapter 9

Virus isolation in cell culture: the rabies tissue culture infection test

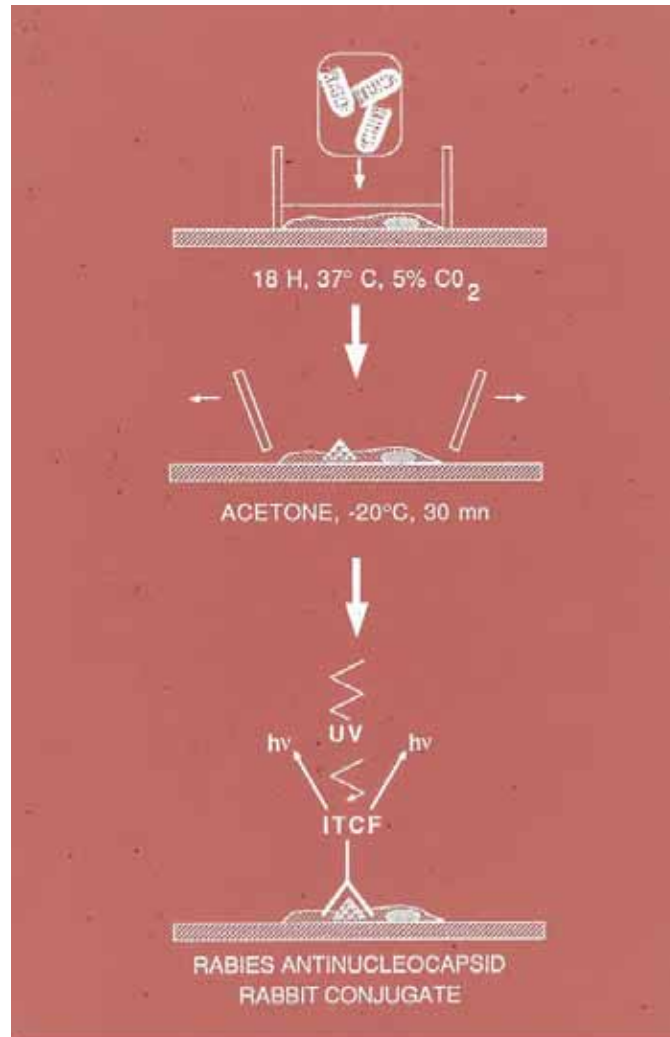
Introduction

The aim of the rabies tissue culture infection test (RTCIT) is to detect *in vitro*, using cell culture techniques, the presence of infectious rabies virus (RABV) or other lyssaviruses in tissue suspensions or in liquid biological samples. The RTCIT, now well-developed and standardized, is widely implemented and is considered using brain biopsy specimens as a conventional routine test for the postmortem diagnosis of rabies in animals and humans and acting as a confirmatory method to the direct fluorescent antibody test (DFAT), which is the gold standard technique. Application of this test can also be extended to the analysis of other types of sample, including body fluids such as saliva and cerebrospinal fluid (CSF), in the diagnosis of human rabies, both postmortem or antemortem.

Initially, the first attempts to isolate RABV in cell culture from suspected samples were performed in the 1970s using various cell lines, including baby hamster kidney (BHK-21), chick embryo-related or neuroblastoma cells (1–6). The latter were demonstrated to be more susceptible to field isolates of RABV than other cell lines tested, probably due to their neuronal origin (7,8). Virus isolation in neuroblastoma cell culture was further demonstrated to be at least as efficient as animal inoculation, especially for small quantities of virus, and was rapidly used routinely to replace RABV isolation in newborn mice in different laboratories in the early 1980s (9, 10).

Viral infection in cells produces specific inclusions detectable generally 18–24 hours after infection (ranging as early as 4–5 h up to 5 days) by direct immunofluorescence, after cell fixation and staining with a specific anti-rabies fluorescent conjugated antibody (1–4, 11) (Fig. 9.1). Therefore, virus isolation in cell culture provides more rapid results than inoculation in animals (10–21 days or longer) and remains less expensive. This technique is also more ethical because it replaces the use of animals (12). Thus, RTCIT represents a reference technique for isolation of lyssaviruses, and should replace the mouse inoculation test (MIT) whenever possible (13, 14).

Virus isolation is generally performed at the postmortem diagnosis of rabies on brain specimens to confirm the results obtained by the detection of lyssavirus antigens using the DFAT, especially when the results are negative or uncertain (13, 14). Compared with the DFAT, this technique has demonstrated an overall concordance of 99.75%, with a specificity of 100% and a sensitivity of 94.37–8.74% (11). However, a major limitation of this technique is the preservation of the infectivity in the submitted samples, as suboptimal conditions of samples (such as decomposed specimens) could induce false-negative results (15).



Reproduced with permission from: Dacheux L, Bourhy H. Virus isolation in cell culture: the rabies tissue culture infection test. In: Rupprecht C, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention. Vol 2. New York (NY): Elsevier; 2015:25–31.

Fig. 9.1. Schematic representation of the RTCIT (rabies tissue-culture infection test) protocol, based on the use of a rabies anti-nucleocapsid FITC (fluorescein isothiocyanate)-conjugated antibody (of rabbit origin)

The RTCIT can also be applied to the antemortem (and postmortem) diagnosis using biological fluids such as CSF, lacrimal fluid or saliva. However, the sensitivity of the test will vary according to the intermittence of viral excretion and the level of viral load, the number of consecutive cell passages performed before fixation of the cells and the immunological status of the patient, as the presence of specific RABV antibodies, especially those with neutralizing activity, may negatively affect the rate of positivity (14). The quality of those samples, similarly to other diagnostic tests, is of utmost importance and the samples should therefore be frozen immediately after collection, preferentially in dry tubes (medium containing preservatives should not be used).

In addition to diagnosis, virus isolation represents a necessary step to amplify any field isolate for its further characterization.

Protocol

The RCTIT technique can be performed using either multiple chamber slides (e.g. 8-chamber slides [such as Lab-Tek]) or microplates (e.g. 96-well plates).

Reagents and equipment

Reagents

- cell culture medium DMEM10 (see [Annex 1](#) for composition)
- suspension medium (grinding medium, see [Annex 3](#) for composition)
- neutral PBS
- 80% acetone (diluted in PBS)
- trypsin (sterility of each new batch should be tested after inoculation of brain heart infusion broth)
- DEAE-dextran (given at a final concentration of 25 µg/mL)
- Reagents for DFAT including: specific anti-rabies fluorescent conjugated antibody (either polyclonal or monoclonal antibodies, generally labelled with the fluorescein isothiocyanate (FITC) fluorophore (commercially available or validated homemade antibodies), used at the recommended dilution with appropriate diluent, counter staining (Evans Blue, generally at 1/2000 dilution) and mounting medium (80% glycerol diluted in PBS).
- Supernatant of homogenized, controlled infected and naive brain tissues, used as positive and negative controls, respectively (e.g. mouse brain tissues).

Equipment

- 10 Broek-type glass homogenizers or glass rods adapted to the plastic centrifuge tubes (15 mL tubes)
- Class 2 biosafety cabinet for cell culture and manipulation of RABV, using standard biosafety measures
- eight chamber slides (e.g. Lab-Tek, glass or Permanox slides) or microplates (e.g. 96-well plates) with flat bottom, clear colour, adapted to adherent cell culture
- dry incubator, used at 37 °C ± 2 °C
- humid, CO₂ controlled incubator used for routine cell maintenance and for the RTCIT procedure, used at 5 % CO₂ ± 2% and 37 °C ± 2 °C
- refrigerated centrifuge
- fluorescence microscope with appropriated filters for the wavelength of the fluorescent dye (for FITC: maximum excitation wavelength at 490 nm and emission wavelength at 520 nm). In particular, the microscope should be equipped with a long focal lens if using microplates (e.g. 96-well plates) to allow the plate to be read in an inverted position. This step could be simplified using directly an inverted fluorescence microscope.
- appropriate virucidal solution.

Choice and maintenance of neuroblastoma cell cultures

- Murine neuroblastoma cells, such as Neuro-2a (ATCC CCL-131, https://www.lgcstandards-atcc.org/products/all/CCL-131.aspx?geo_country=fr), should be used, due to their high sensitivity to RABV and other lyssavirus infection.
- Maintain the neuroblastoma cells in 75 cm² plastic flasks in cell culture medium (Annex 1) at 37 °C ± 2 °C in a 5% CO₂ ± 2% humidified incubator. Trypsinize the cells twice a week. Details regarding maintenance and conservation of murine neuroblastoma cells are described in Annex 2.

Preparation of the cell suspension

1. Trypsinize one flask of subconfluent cells for the cell suspension preparation and resuspend the cells at a concentration of 4 × 10⁵ cells/mL (for 8-chamber slides) or at a concentration of 2 × 10⁵ cells/mL (for 96-well microplates) in DMEM10 in a 15 mL tube.
2. This preparation can be kept until 3 days at 4 °C.

Preparation of the inoculum

For brain samples

1. Prepare a 30% suspension (mass/vol) of the brain specimen in the suspension medium (grinding medium, Annex 3) using 10 Broek-type glass homogenizers or glass rods adapted to plastic centrifuge tubes (e.g. 15 mL tube). Different brain tissues should be selected after autopsy or necropsy, including the brainstem, cortex, cerebellum and hippocampus (when available). The suspension must be performed in a class 2 biosafety cabinet.
2. Clarify the suspension by centrifugation at 1500 × *g* for 5 min at 4 °C.

For liquid samples

1. Saliva samples may be diluted 1/2 (vol/vol) in suspension medium, as well as lacrimal samples. Sterile CSF specimens may be inoculated undiluted.

Inoculation

Inoculation using 8-chamber slides (Fig. 9.2)

1. Distribute 400 µL of the neuroblastoma cell suspension (4 × 10⁵ cells/mL) per chamber (160 000 cells/chamber) to reach an 80% confluent cell monolayer.
2. Add 50 µL of each clarified brain homogenate in one chamber per sample and mix gently (up and down pipetting), being careful not to generate aerosols.
3. Include at least one positive control (fixed or field isolate) and one negative control (confirmed negative brain) for each series of slides, preferably on a separate slide or on the same slide as the samples, but using a well as distant as possible from the wells used for the tested samples.

4. Incubate for 18–24 h at $37\text{ °C} \pm 2\text{ °C}$ in a $5\% \text{CO}_2 \pm 2\%$ humidified incubator. A longer incubation period may be required (e.g. 60 h during weekends), in which case, it is suggested to reduce the number of cells (80 000 cells/chamber).

Inoculation using microplates (e.g. 96-well plates) (*adapted from 7,13*)

1. Distribute 200 μL of the neuroblastoma cell suspension (4×10^5 cells/mL) per well (40 000 cells/well) in four wells of a 96-well plate to reach an 80% confluent cell monolayer.
2. Add 100 μL of each clarified brain homogenate in each of the four wells and mix gently (up and down pipetting). Include at least one positive (fixed or field isolate) and one negative (confirmed negative brain) control for each series of plates.
3. Incubate for 2–4 days at $37\text{ °C} \pm 2\text{ °C}$ in a $5\% \text{CO}_2 \pm 2\%$ humidified incubator. Changing of cell medium can be eventually performed during this incubation period.

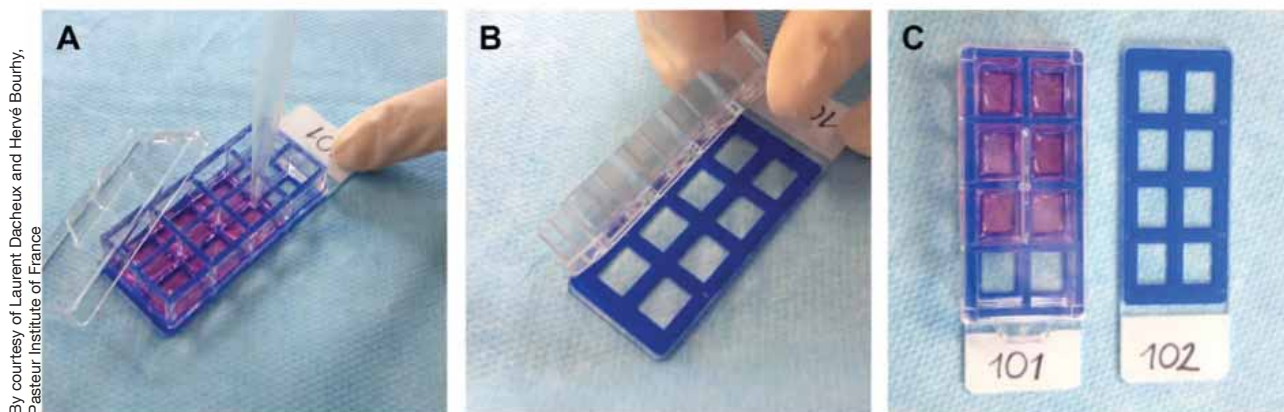


Fig. 9.2. Illustrations of 8-well chamber slides; A: Depositing the cell medium into the 8-well chamber slides; B: Removing the 8-well incubating chambers; C: Slides with and without 8-well incubating chambers

Fixation of cells and virus detection

Fixation using 8-chamber slides (e.g. Lab-Tek) (Fig. 9.2)

1. Discard carefully the medium from each chamber in a validated virucidal solution.
2. Fix the cells with cold 80% acetone for 30 min at 4 °C .
3. Dry the slides at room temperature for 5–15 min.
4. Stain with the specific anti-rabies FITC-conjugated antibody (50 μL per chamber, using an appropriate dilution with addition of counter-staining) during 30 min at $37\text{ °C} \pm 2\text{ °C}$ in a humidified incubator.
5. Remove the plastic chambers' frame.
6. Rinse the slides in two successive baths of phosphate-buffered saline (PBS). This washing must be very gentle to prevent removal of the cells.
7. Cover with mounting medium (80% glycerol in PBS) and coverslip.
8. Read using the fluorescence microscope.

Inoculation using microplates (e.g. 96-well plate) (*adapted from 7,13*)

1. Discard carefully the medium from each well in an appropriate virucidal solution and perform one wash with PBS.
2. Fix the cells with cold 80% acetone for 30 min at 4 °C.
3. Dry the slides at room temperature for 5–15 min.
4. Stain with the specific anti-rabies FITC-conjugated antibody (50 µL per well, using an appropriate dilution with addition of counter-staining) for 30 min at 37 °C ± 2 °C in a humidified incubator.
5. Wash twice the wells with PBS. This washing must be very gentle to prevent removal of the cells.
6. Add a drop of mounting medium in each well (80% glycerol in PBS).
7. Read using the fluorescence microscope.

Interpretation of results

The presence of bright green fluorescent inclusions in the cytoplasm of the cells can be observed using the fluorescence microscope (Fig. 9.3). Optimal final magnification with 8-chambers slides is 400x, and with microplates (e.g. 96-well plate) a final magnification of 160–200x is adapted using a microscope equipped by a longer focal lens to read the plates in an inverted position (this step can be simplified using an inverted fluorescence microscope).

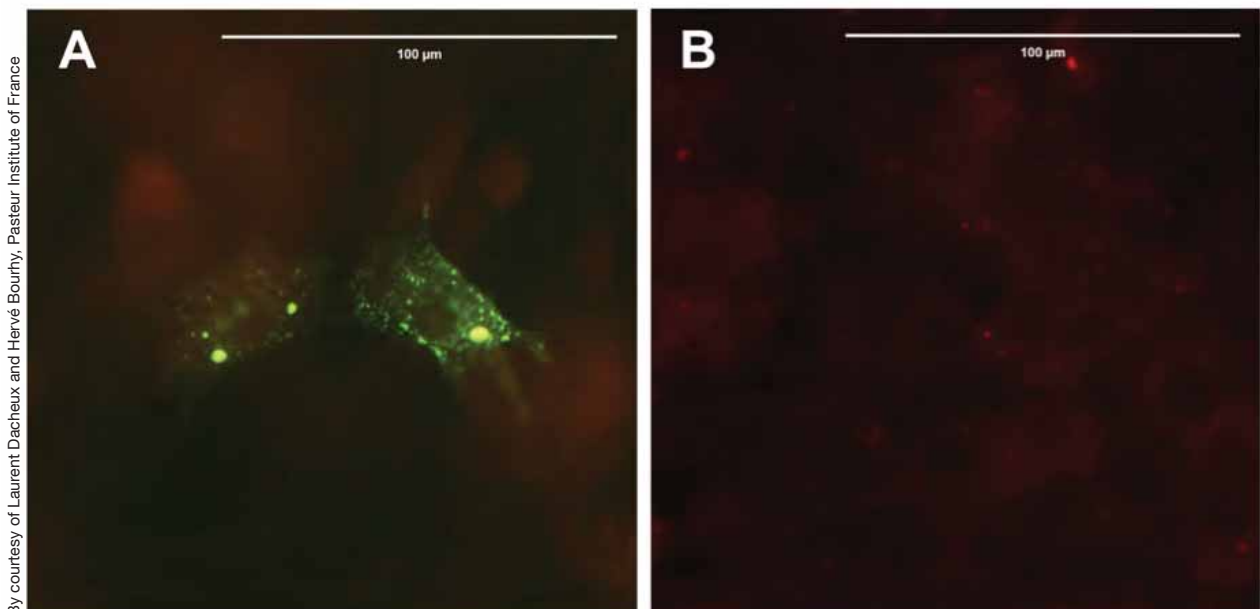


Fig. 9.3. Examples of microscopic observations (magnification 400x) of RTCIT slides after labelling with a specific anti-rabies FITC-conjugated antibody

A: Positive (presence of infection) slide, with the observation of bright green fluorescent inclusions in the cytoplasm indicating RABV infection

B: Negative (absence of infection) slide, with cells observed in red after counter staining with Evans Blue

Discussion

The RTCIT represents a technique of reference for lyssavirus isolation (13, 14). This chapter describes a validated protocol of RTCIT using 8-chambers slides (e.g. Lab-Tek) which has been demonstrated to provide reliable and rapid results, as fast as 18–24 h after inoculation (11), as well as the initial method performed in microplates (7, 13).

Whatever the technique used, suboptimal sample conditions, such as decomposed brain, are a major limitation that could induce a loss of infectivity and yield false-negative results (15). Cytotoxicity can also result from such samples due to the presence of infection-inhibiting factors in brain suspensions, limiting the robustness of the test (10, 11). However, the addition of antibiotics, reduction of the time before changing media or dilution of samples can reduce this cytotoxicity.

Variations in this technique exist and mainly include the type of material used for cell culture (with other multi-well plastic plates or glass cover-slips) as well as the process of inoculation with incubation time, further passages and use of cell permeability agents. Longer incubation periods (2–4 days) can be routinely conducted, especially for negative samples, to increase sensitivity. Replacement of cell medium during this longer incubation period is not necessary but can be done. In addition, successive passages can also be performed (up to three passages may be considered to increase sensitivity), and the supernatants kept for further passages if necessary. In this case, the time required to finalize the assay should be compatible with the limited delay available in some cases so as to inform the physician in charge of the decision regarding prophylaxis for the exposed patients. Permeability agents, such as diethylaminoethyl (DEAE)-dextran, can be added to the cell suspension immediately before use, to give a final concentration of 25 µg/mL. The use of cell lines other than neuroblastoma cells has been suggested but is not recommended (13, 14). Cell culture tests and their variations should be fully validated before use.

Although the RTCIT is relatively easy to perform, some critical parameters must be emphasized. Implementation of the test requires that strict standard operating procedures for cell culture be followed, with appropriate equipment and proficiency of staff. In parallel, because detection is based on the direct immunofluorescence of viral antigens, similar technical recommendations related to the DFAT must also be followed, such as the choice and validation of the specific anti-rabies fluorescent conjugated antibody, dilution and incubation conditions, washing steps and mounting medium (13, 14, 16). In particular, the final reading step is of utmost importance and must be performed by trained operators (up to two different readers per slide or microplate), using adapted magnification as well as a regularly controlled and well-maintained fluorescence microscope. Participation of the laboratory to a proficiency test is strongly suggested for this technique (17).

References

1. Larghi OP, Nebel AE, Lazaro L, Savy VL. Sensitivity of BHK-21 cells supplemented with diethylaminoethyl-dextran for detection of street rabies virus in saliva samples. *J Clin Microbiol.* 1975; 1:243–5.
2. Rudd RJ, Trimarchi CV, Abelseth MK. Tissue culture technique for routine isolation of street strain rabies virus. *J Clin Microbiol.* 1980; 12:590–3.
3. Smith AL, Tignor GH, Emmons RW, Woodie JD. Isolation of field rabies virus strains in CER and murine neuroblastoma cell cultures. *Intervirology.* 1978; 9:359–61.
4. Smith AL, Tignor GH, Mifune K, Motohashi T. Isolation and assay of rabies serogroup viruses in CER cells. *Intervirology.* 1977; 8:92–9.
5. Portnoi D, Favre S, Sureau P. Use of neuroblastoma cells (MNB) for the isolation of street rabies virus from field specimens. Atlanta (GA): United States Centers for Disease Control and Prevention Rabies Information Exchange; 1982:6:35–6.
6. Umoh JU, Blenden DC. Comparison of primary skunk brain and kidney and raccoon kidney cells with established cell lines for isolation and propagation of street rabies virus. *Infect Immun.* 1983; 41:1370–2.
7. Webster WA, Casey GA. Virus isolation in neuroblastoma cell culture. In: Meslin FX, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*, 4th edition. Geneva: World Health Organization; 1996:96–104.
8. Rudd RJ, Trimarchi CV. Comparison of sensitivity of BHK-21 and murine neuroblastoma cells in the isolation of a street strain rabies virus. *J Clin Microbiol.* 1987; 25:1456–8.
9. Sureau P. [Rapid technics for the laboratory diagnosis of rabies]. *Arch Inst Pasteur Tunis.* 1986; 63:183–97 (in French).
10. Rudd RJ, Trimarchi CV. Development and evaluation of an in vitro virus isolation procedure as a replacement for the mouse inoculation test in rabies diagnosis. *J Clin Microbiol.* 1989; 27:2522–8.
11. Bourhy H, Rollin PE, Vincent J, Sureau P. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immunoassay for rapid laboratory diagnosis of rabies. *J Clin Microbiol.* 1989; 27:519–23.
12. Russell WMS, Burch RL. *The principles of humane experimental technique.* London: Methuen; 1959.
13. *Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees).* Paris: World Organisation for Animal Health; 2013.
14. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 8 May 2018).

15. McElhinney LM, Marston DA, Brookes SM, Fooks AR. Effects of carcase decomposition on rabies virus infectivity and detection. *J Virol Methods*. 2014; 207:110–3.
16. Dean DJ, Abelseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin FX, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*, 4th edition. Geneva: World Health Organization; 1996:88–95.
17. Robardet E, Picard-Meyer E, Andrieu S, Servat A, Cliquet F. International interlaboratory trials on rabies diagnosis: an overview of results and variation in reference diagnosis techniques (fluorescent antibody test, rabies tissue culture infection test, mouse inoculation test) and molecular biology techniques. *J Virol Methods*. 2011; 177:15–25.
18. Pouliott C, Dupuis M, Appler K, Brunt S, Rudd R, Davis A. Susceptibility of neuroblastoma cells to rabies virus may be affected by passage number. *J Virol Methods*. 2017; 247:28–31.

Annex 1. Composition of cell culture medium DMEM10

- Dulbecco's modified Eagle's medium (DMEM) with high glucose (4500 mg/L), supplemented with L-alanyl-L-glutamine (GlutaMAX) (862 mg/L) and pyruvate (110 mg/L) (supplemented medium commercially available)
- 10% of heat inactivated fetal calf serum (FCS)
- Optionally: appropriate antibiotics such as gentamicin at final concentration 50 µg/mL
- Each new reconstitution of cell culture medium should be tested for sterility (for example, after inoculation and incubation of a small volume in brain heart infusion broth). The quality of new batches of FCS should also be tested, in comparison with previous validated batches (including the evaluation of cell multiplication and the presence of RABV neutralizing activity).

Annex 2. Maintenance and conservation of Neuro-2a murine neuroblastoma cells

- Cells are subcultured twice a week in 75 cm² plastic flasks in culture medium at 37 °C ± 2 °C and in a 5% CO₂ ± 2% humidified incubator.
- For each passage, culture medium is discarded and cells are rinsed twice with PBS or trypsin, then incubated by adding 2 mL of trypsin at room temperature. After the cell layer detaches from the flask surface, trypsin is inactivated by adding 9 mL of culture medium. The suspension is carefully mixed by pipetting to disperse cell clumping. Cell counting is performed using, for example, Malassez or Kovak slides. Optionally, trypan blue can be added (dilution of 1:2) to perform a viable cell counting. A total of 3–4 × 10⁶ cells is then seeded into a new T75 flask into 15 mL of DMEM10 as the final volume.
- The number of cell passages should be registered and a limit number of cell passages has to be defined (e.g. 30 passages after thawing the initial aliquot), based on the literature as well as on laboratory experience, to avoid cell degeneration and loss of sensitivity (18). Sufficient stocks of cells (stored in liquid nitrogen) have to be prepared periodically.
- Ideally, maintenance of neuroblastoma cells should use DMEM10 without addition of antibiotics, to avoid low and/or unnoticed bacteria contamination. In this case, an aliquot of cells obtained from the last trypsinization should be maintained in parallel, in a small T25 cm² plastic flask with cell culture medium supplemented with antibiotics. This aliquot will be used as temporary back-up in case of contamination, until fresh cells from a new frozen aliquot are amplified.

Annex 3. Composition of suspension (grinding) medium

- 300 mL of Dulbecco's modified Eagle medium (DMEM) with high glucose (4500 mg/L), supplemented with L-alanyl-L-glutamine (GlutaMAX) (862 mg/L) and pyruvate (110 mg/L)
- 200 mL of heat inactivated FCS
- 200 mg of vancomycin
- 15 mg of gentamicin
- 350 µg of amphotericin B
- The final concentration of each compound of the suspension medium is: 40% of heat inactivated FCS, 0.4 mg/mL of vancomycin, 30 µg/mL of gentamicin and 0.7 µg/mL of amphotericin B for a final volume of nearly 500 mL. After homogenization, the suspension medium is aliquoted (for example under a volume of 80 mL) and stored frozen (≤ -20 °C). Each new batch should be confirmed as negative for RABV by the RTCIT before use.

Chapter 10

Transmission electron microscopy in rabies diagnosis, ultrastructural studies and research

Introduction

Electron microscopy (EM) deals with the elucidation of fine structural features underlying biological organization. The electron microscope has made outstanding contributions by illuminating the morphological details which are not clearly observed by the venerable light microscope. Thus, EM has enhanced our understanding of the basic organization of the cell. Furthermore, EM has been of great use in unravelling the organization of complex synaptic relations between neurons, in analysing the ultrastructure of pathogens and in examining the morphology of various cellular organelles such as mitochondria, rough endoplasmic reticulum and Golgi.

Historically, EM has long been used in the characterization of rabies virus (RABV) in the central nervous system (CNS). Negri bodies and RABV particles have been found in neurons and neuronal processes in CNS tissues, including Ammon's horn of the hippocampus and the cerebellum, brainstem and cerebral tissues. Using thin section EM, in 1962 Matsumoto (1) confirmed the viral nature of the Negri bodies, describing a matrix containing elongated particles. The morphology of the viral nucleocapsid was found by negative stain EM to be a single-stranded helix (2). Immunofluorescence and EM were instrumental in understanding the pathogenesis of RABV in experimental animals (3). Also, EM has assisted in the description of cases in which RABV was transmitted by corneal transplantation and of solid organs from infected donors to recipients (4, 5). Negri bodies were shown to contain N and P proteins by immuno-gold EM, and viral mRNA and genomic and anti-genomic RNA were detected by fluorescent in situ hybridization (6).

EM has also been used to confirm confocal microscopy studies on the presence of Toll-like receptor-3 (TLR3) in Negri bodies in RABV-infected cells (7). Tomographic reconstruction analysis by using cryo-EM found that the interface between the M, M/N protein layers and the viral envelope allows for the straightening of the lipid bilayer membrane in both the characteristic bullet-shaped virions and in spherical/pleomorphic particles (8). Moreover, in vesicular stomatitis virus studies, cryo-EM elegantly explained the packaging of viral RNA and N and M proteins to form the bullet-like morphology of the rhabdovirus nucleocapsid (9). These are a few of the examples demonstrating the utility of EM in diagnostics and applied research. Relevant examples of transmission electron microscopy (TEM) are shown in Figs. 10.1–10.5. This chapter describes a protocol for the use of TEM in related studies.

By courtesy of B. K. Chandrasekhar Sagar, National Institute of Mental Health and Neurosciences, Bangalore, India and Cynthia S. Goldsmith, United States Centers for Disease Control and Prevention

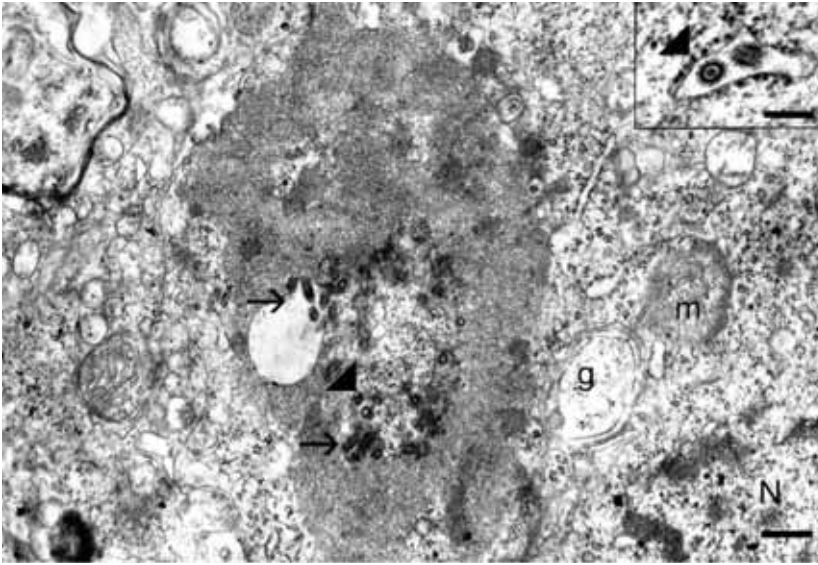


Fig. 10.1. Electron microscopy image of a RABV-infected murine motor neuron showing a granular Negri body having many longitudinally cut bullet-shaped (arrow) and cross-sectional (arrow head) RABV particles; inset shows a highly magnified cross-sectional virus particle (arrowhead) in dilated rough endoplasmic reticulum, with a dark nuclear core in the centre and the outer glycoprotein membrane with spikes

g, Golgi complex; m, mitochondria; N, nucleus

Scale bar: 500 nm
Inset: 100 nm

By courtesy of B. K. Chandrasekhar Sagar, National Institute of Mental Health and Neurosciences, Bangalore, India and Cynthia S. Goldsmith, United States Centers for Disease Control and Prevention

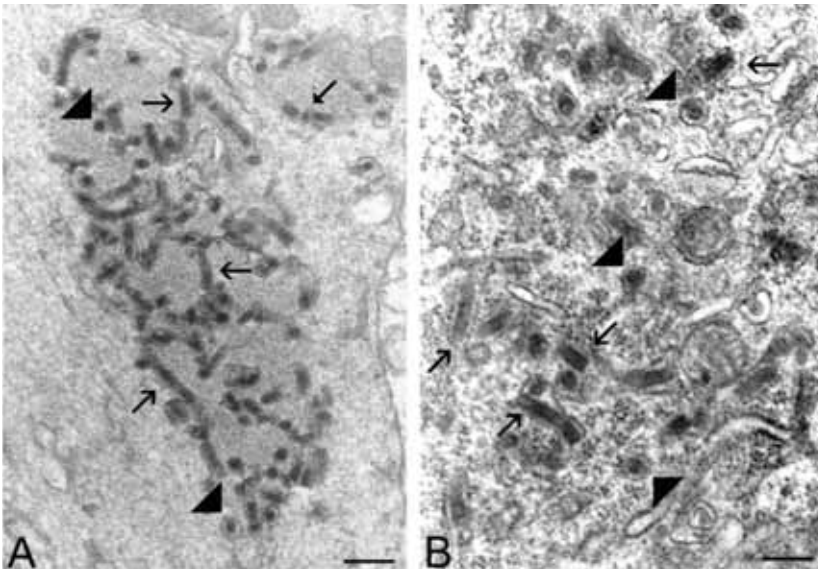


Fig. 10.2 (A, B). Electron microscopy images of RABV CVS strain-infected murine neuronal cells showing many longitudinal (arrows) and cross-sectional (arrowheads) RABV particles in the cytoplasm; cytoplasmic granulation of the Negri body is conspicuous in A

Scale bars: A, 200 nm; B, 100 nm

By courtesy of B. K. Chandrasekhar Sagar, National Institute of Mental Health and Neurosciences, Bangalore, India and Cynthia S. Goldsmith, United States Centers for Disease Control and Prevention

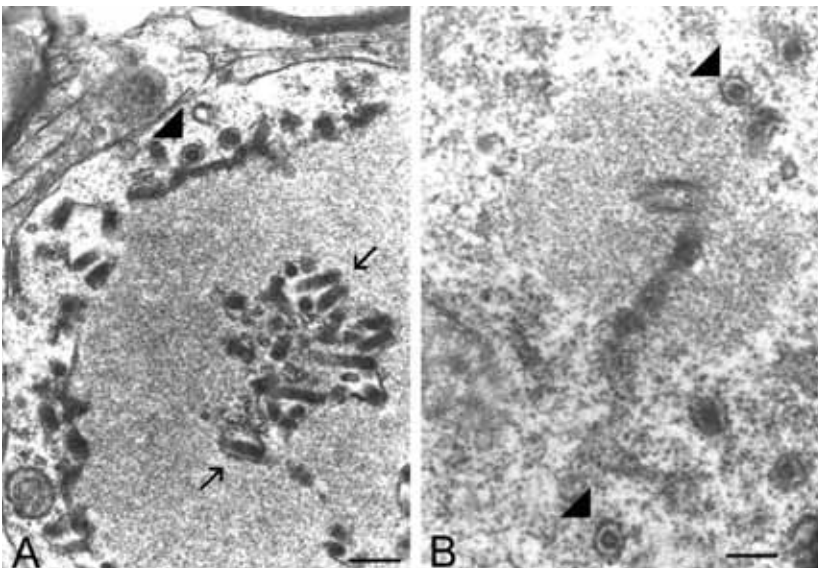


Fig. 10.3 (A, B). Electron microscopy images of RABV-infected murine brainstem motor neurons showing granular Negri bodies having longitudinal (arrow) and cross-sectional (arrowhead) RABV particles

Scale bars: A, 200 nm; B, 80 nm

By courtesy of B. K. Chandrasekhar Sagar, National Institute of Mental Health and Neurosciences, Bangalore, India and Cynthia S. Goldsmith, United States Centers for Disease Control and Prevention

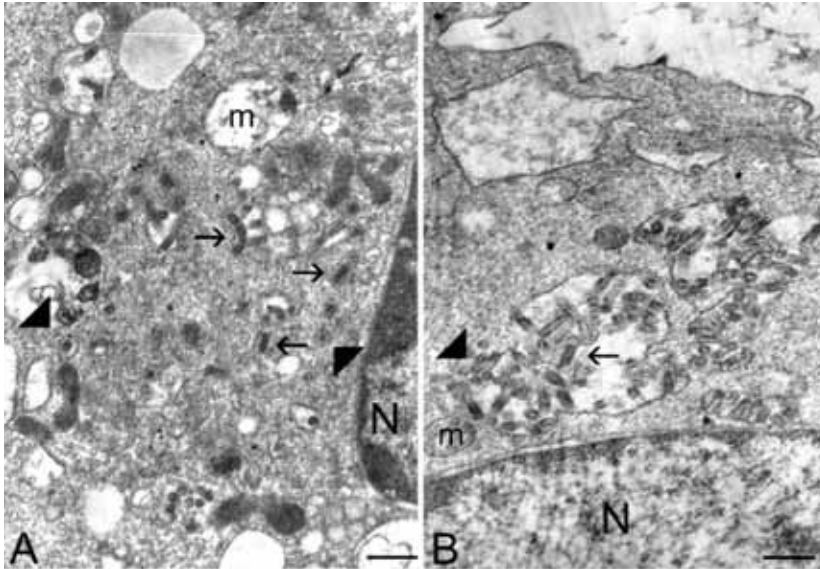


Fig. 10.4. Electron microscopy images depicting cultured baby hamster kidney cells containing many longitudinal (arrows) and cross-sectional (arrowheads) rabies virus particles in the cytoplasm (A) and in the dilated rough endoplasmic reticulum (B)

g, Golgi complex; m, mitochondria; N, nucleus

Scale bars: A, 300 nm; B, 320 nm

Images A–C by courtesy of B. K. Chandrasekhar Sagar, National Institute of Mental Health and Neurosciences, Bangalore, India and Cynthia S. Goldsmith, United States Centers for Disease Control and Prevention; image D by courtesy of Maureen Metcalfe and Sherif Zaki, United States Centers for Disease Control and Prevention

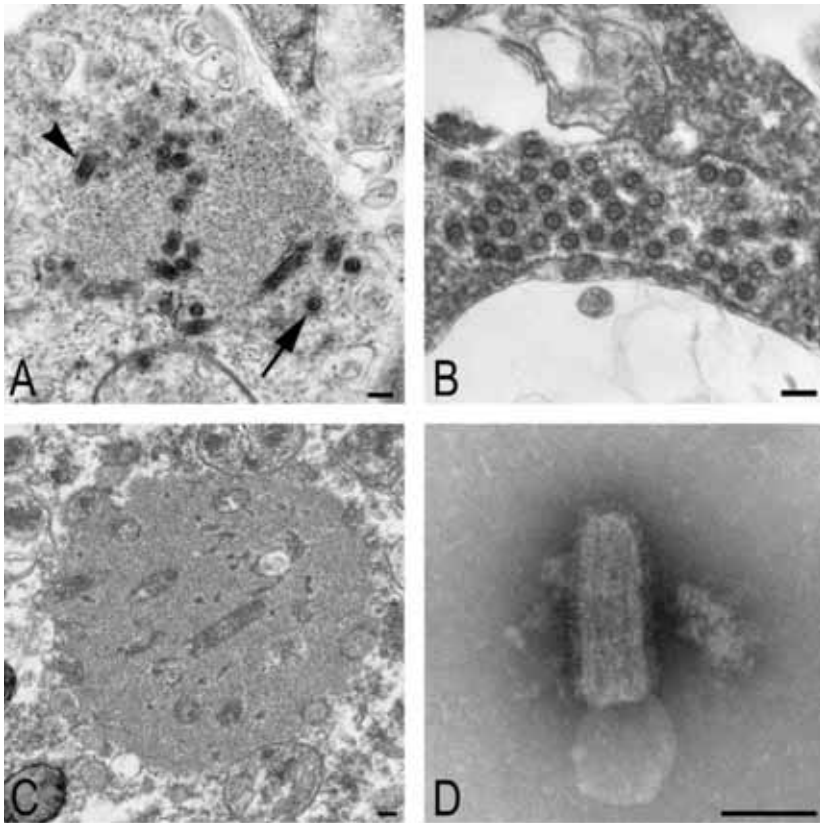


Fig. 10.5. Electron microscopy images of the central nervous system from fatal human rabies cases: (A) typical Negri body composed of granular material and virions cut longitudinally (arrowhead) and in cross-section (arrow); (B) RABV particles cut in cross-section, within the cisternae of intracytoplasmic membranes; (C) another Negri body with unusually large tubular particles (approximately 130 nm in diameter) with dense material within the particle; (D) from homogenized brain tissue, a negative stain electron microscopy image of a bullet-shaped RABV virion with short surface projections and an internal, coiled nucleocapsid

Scale bars: 100 nm

Specimen preparation for transmission electron microscopy

All reagents must be used under a chemical fume hood or in a well-ventilated area.

Fixation

Chemical fixation is the most widely used method of preserving biological specimens for TEM. The goal of fixation is to preserve the structure of the cell with minimum alteration from the living state. The fixative provides stable bonds that will hold the molecules together, accomplished by the formation of inter- and intracellular cross-links of proteins. The cross-links are formed not only between their own reactive groups but also between reactive groups in the tissues (7).

Glutaraldehyde is used as a primary fixative because of its capacity to stabilize most proteins without coagulation. The other fixatives used are acrolein, para-formaldehyde and osmium tetroxide. Osmium tetroxide is used as a post-fixative because it preserves lipids and imparts electron density to cell components. Double fixation – pre-fixation with glutaraldehyde and post-fixation with osmium tetroxide – is the standard procedure for preserving tissues (8, 9).

The factors that affect the quality of fixation are the size of the specimen, osmolarity, pH and the duration of fixation.

Size. Homogeneity in fixation depends primarily on the size of the specimen; thus, small specimen size is of utmost importance in achieving uniform fixation. The ideal size of the tissue should not exceed 3 mm³.

Osmolarity. The osmolarity of the fixative affects the size and shape of the cell. The addition of nonelectrolytes, e.g. sucrose, act as osmotic stabilizers and minimize extraction of cellular constituents. For most mammalian tissues, the recommended osmolarity ranges from 500 to 700 mOsm.

pH. The pH of the fixative must remain close to the pH of the tissue because any change will alter the structure of tissue proteins, including changes in consistency of protoplasm, the selectivity of cell membranes and the activity of enzymes. Since the average pH value of most tissue is 7.4, the best preservation of fine structure is obtained by keeping the pH of the fixative within narrow limits of 7.2 to 7.4.

Duration of fixation. For RABV-infected specimens, a duration of 24 h at 4 °C is currently recommended.

Washing

Following fixation in the primary fixative, the tissues are washed in 0.1 mol sodium phosphate or sodium cacodylate buffer.

Post-fixation (secondary fixation)

The tissues are post-fixed in 1% osmium tetroxide for 1–2 h at 4 °C.

Dehydration

To facilitate infiltration with liquid resin, which is a prerequisite for embedding, dehydration should be carried out gradually, using absolute ethanol (glass distilled).

Clearing

The tissues are cleared with a clearing agent to facilitate infiltration, using either propylene oxide or acetone.

Infiltration and embedding

A complete and uniform penetration of tissue specimens by a suitable embedding medium is a requirement for satisfactory sectioning. Infiltration involves a gradual replacement of the clearing agent with an embedding medium that facilitates a complete impregnation of the interstices of a tissue specimen with the medium. This is accomplished by gradually decreasing the concentration of the solvent and increasing the concentration of the embedding medium, as described by Sagar and colleagues (10). This makes the tissue blocks sufficiently strong to enable the cutting of ultrathin sections.

Embedding is carried out by placing the tissues in BEEM (better equipment for electron microscopy) capsules, or, where orientation of the specimens is required, polyethylene flat embedding moulds, using the same embedding medium to fill the container.

Polymerization

The liquid embedding medium containing the tissues is polymerized in an oven at 60 °C for 24–48 h. After complete polymerization, the blocks are ready for sectioning.

Precautions and other considerations

Refer to [Chapter 3](#) (Biosafety) in this manual. If working with unfixed virus, laboratory workers should receive pre-exposure rabies vaccination.

Protocol A: Human or animal brain tissue

Within a Biosafety Level 2 cabinet and using proper protection (e.g. gloves, laboratory coat, safety glasses and closed-toe shoes), the infected human or animal brain tissues are cut into small pieces (1 x 1 mm³) and fixed in 3% buffered glutaraldehyde for 24 h.

1. Wash the specimens thoroughly in 0.1 mol sodium phosphate buffer pH 7.2 for 1–2 h (2–4 changes).
2. Post-fix in 1% osmium tetroxide for 1 h at 4 °C.
3. Wash thoroughly in 0.1 mol sodium phosphate buffer pH 7.2 for 1 h (2–4 changes).
4. Dehydrate in 70% ethanol for 1 h, 80% ethanol for 1 h and en-bloc stained with 2% uranyl acetate in 95% ethanol at 4 °C for 1–2 h (en-bloc staining gives better, fine structural preservation of membrane structures and cell joints, protein, myofibrils and mitochondria).
5. Follow with absolute ethanol (2 changes of 30 min each).
6. Clear in propylene oxide (2 changes of 15 min each at room temperature).
7. Follow with propylene oxide and Araldite 1:1 (overnight on rotator at room temperature).
8. Follow with propylene oxide and Araldite 1:3 (3 h on rotator at room temperature).
9. Follow with pure Araldite (3 h on rotator at room temperature).
10. Embed in polyethylene flat moulds with a label at the proper orientation, and heat in an oven at 60 °C for 24–48 h for polymerization.

Protocol B: Cell cultures

Within a Biosafety Level 2 cabinet and using proper protection (e.g. gloves, laboratory coat, safety glasses and closed-toe shoes), infected culture cells are scraped from the flask, transferred to a conical centrifuge tube and pelleted for 10 min at 1200 × r/min in a table top centrifuge. The medium is pipetted off and disposed as infectious waste.

1. Gently layer 3% buffered glutaraldehyde onto the pellet, without disturbing the pellet, and allow it to fix for 24 h.
2. Remove the glutaraldehyde and dispose of it as hazardous chemical waste.
3. Gently layer 0.1 mol sodium phosphate buffer onto the pellet for 30 min.
4. Fashion a small spatula by using a razor blade to whittle one end of a round wooden applicator stick.
5. Coax the pellet from the sides of the centrifuge tube by using the spatula, remove it from the tube with a plastic transfer pipet (with the end cut off) and place it in a container with 0.1 mol sodium phosphate buffer.
6. Continue with Protocol A, beginning at the step of post-fixation with 1% osmium tetroxide.

Protocol C: Alternate processing (by using an electron microscopic laboratory-grade microwave with a water-filled ColdSpot®, available with the PELCO BioWave®, Ted Pella, Inc.)

Begin after the glutaraldehyde fixation step of Protocol A.

At 150 watts:

1. Rinse twice with 0.1 mol sodium phosphate buffer (40 s each).
2. 1% osmium tetroxide (40 s). Place vials on ice, during the time that the embedding resin is prepared.
3. Rinse twice with water (40 s each).

At 550 watts:

4. Dehydrate in 70% ethanol (40 s), 80% ethanol (40 s) and en-bloc stain with 2% uranyl acetate in 95% ethanol (40 s).
5. Rinse twice with 100% ethanol (40 s each).
6. Rinse twice with acetone (40 s each).
7. To control for heat, place 900 mL of tap water in a plastic beaker at the front of the microwave. Change water after each step.
8. Rinse with acetone and resin 2:1 (5 min).
9. Rinse with acetone and resin 1:2 (5 min).
10. Rinse with pure resin, four changes (15 min each).
11. Transfer specimens to BEEM capsules or flat moulds with a label and fill with resin.
12. Polymerize in an oven at 60 °C for 24–48 h. Note that if speed is necessary, specimens can be polymerized at 95 °C for 75 min, or until resin is cured.

Processing for negative stain electron microscopy

Within a Biosafety Level 2 cabinet and using proper protection (e.g. gloves, laboratory coat, safety glasses and closed-toe shoes), supernatant from homogenized brain tissue or a cell culture is mixed 1:1 with 5% buffered paraformaldehyde and allowed to fix for 24 h.

1. Place 5 µL of this solution on a sheet of Parafilm.
2. Place a 300-mesh formvar or carbon-coated EM grid (that has been treated to improve hydrophilicity) on top of the drop of the solution.
3. Allow the specimen to adsorb for 10 min.
4. Blot the grid to remove the solution and rinse on a drop of water (3 min).
5. Stain (30 sec) with 2% phosphotungstic acid (pH 7.3) and blot.

Ultramicrotomy of sections

To obtain a high resolution, the thickness of the section should be about 50 nm. The plastic blocks are cut by using an ultramicrotome. Initially, 1 μm thick sections are collected on a glass slide and stained by using 1% toluidine blue, then viewed under a light microscope to find areas of interest and to study light microscopic features. Next, 40–50 nm thick ultrathin sections are collected on copper grids.

Staining

Stain grids by using uranyl acetate and lead citrate. A small clean piece of dental wax is placed in a Petri dish and drops of uranyl acetate (centrifuged or filtered) are placed on the dental wax. The grids are floated sections side down on the surface of the drops and stained for 1–2 h, or for 40 s in an EM-grade microwave at 550 watts, then washed thoroughly in distilled water, and dried. A pellet of NaOH (sodium hydroxide) or KOH (potassium hydroxide) is placed on the dental wax, drops of lead citrate (cooled) are added, and the grids are floated sections side down of the surface of the drops. The lid of the Petri dish is immediately returned to avoid atmospheric CO_2 coming into contact with the staining reagent. Sections are stained for 5–7 min, washed and dried. Washing is done by holding the grid in EM-grade tweezers under the jet of double distilled water from a wash bottle. The grids are dried and preserved in a grid box.

After proper staining, the ultrathin sections are scanned by using TEM, and representative areas are photographed or saved as a digital file.

References

1. Matsumoto S. Electron microscopy of nerve cells infected with street rabies. *Virology*. 1962; 17:198–202. PMID:14471291.
2. Hummeler K, Tomassini N, Sokol F, Koprowski H. Morphology of the nucleoprotein component of rabies virus. *J Virol*. 1968; 2:1191–9.
3. Murphy FA, Harrison AK, Winn WC, Bauer SP. Comparative pathogenesis of rabies and rabies-like viruses: infection of the central nervous system and centrifugal spread of virus to peripheral tissues. *Lab Invest*. 1973; 29:1–16. PMID:4580585.
4. Baer GM, Shaddock JH, Houff SA, Harrison AK, Gardner JJ. Human rabies transmitted by corneal transplant. *Arch Neurol*. 1982; 39:103–7. PMID:7036961.
5. Srinivasan A, Burton EC, Kuehnert MJ, Rupprecht C, Sutker WL, Ksiazek TG, et al. Transmission of rabies virus from an organ donor to four transplant recipients. *N Engl J Med*. 2001; 352:1103–11. doi:10.1056/NEJMoa043018.
6. Lahaye X, Vidy A, Pomier C, Obiang L, Harper F, Gaudin Y, et al. Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication. *J Virol*. 2009; 83:7948–58. doi:10.1128/JVI.00554-09.
7. Menager P, Roux P, Megret F, Bourgeois JP, Le Sourd AM, Danckaert A, et al. Toll-like receptor 3 (TLR3) plays a major role in the formation of rabies virus Negri bodies. *PLoS Pathog*. 2009; 5:e1000315. doi:10.1371/journal.ppat.1000315.
8. Guichard P, Krell T, Chevalier M, Vaysse C, Adam O, Ronzon F, et al. Three dimensional morphology of rabies virus studied by cryo-electron tomography. *J Struct Biol*. 2011; 176:32–40. doi:10.1016/j.jsb.2011.07.003.
9. Ge P, Tsao, J, Schein S, Green TJ, Luo M, Zhou ZH. Cryo-EM model of the bullet-shaped vesicular stomatitis virus. *Science*. 2010; 327:689–93. doi:10.1126/science.1181766.
10. Johannessen JV, editor. *Electron microscopy in medicine. Vol 1: Instrumentation and techniques*. New York (NY): McGraw-Hill International Book Company; 1978.
11. Dykstra MJ, Reuss LE. *Biological electron microscopy: theory, techniques and troubleshooting*, 2nd edition. New York (NY): Plenum Publishers; 2003.
12. Mascorro JA, Bozzola JJ. Processing biological tissues for ultrastructural study. In: Kuo J, editor. *Electron microscopy methods and protocols*, 2nd edition. Totowa (NJ): Humana Press; 2007:19–34.
13. Chandrasekhar Sagar BK, Ravindrakumar MT, Gayathri N. *An introduction to electron microscopy as a diagnostic tool*. Bangalore: National Institute of Mental Health & Neurosciences; 2014.

Annex. Buffers, fixatives, stains and embedding media

Buffers

0.1 mol sodium phosphate buffer

Recipe 1

Solution A: Dissolve 1.56 g NaH_2PO_4 in 100 mL of quartz double distilled water.

Solution B: Dissolve 7.05 g NaH_2PO_4 in 500 mL of quartz double distilled water.

Prepare 0.1 mol phosphate buffer by adding 1:5 ratio of solution A and B respectively. Adjust the pH to 7.2 by adding solution A or B.

Recipe 2

Dissolve 2.92 g sodium phosphate (monobasic) and 7.62 g sodium phosphate (dibasic) in 500 mL dH_2O . Adjust the pH to 7.3 with liquid sodium hydroxide.

0.1 mol sodium cacodylate buffer

Dissolve 10.7 g sodium cacodylate in 500 mL of quartz double distilled water. Add 17.1 g of sucrose and mix until it dissolves. Adjust pH 7.2 with 0.1 N HCL. This solution is stable for 15 days at 4 °C.

Fixatives (these solutions are stable for 1 month at 4 °C)

3% glutaraldehyde: glutaraldehyde is often supplied as 25%

- glutaraldehyde (25%) 12 mL
- 0.1 mol sodium phosphate
or 0.1 mol sodium cacodylate buffer 88 mL

2.5% glutaraldehyde

Other distributors supply glutaraldehyde as 10% in a 10 mL ampoule, sealed under dry nitrogen.

- glutaraldehyde (10%) 10 mL
- 0.1 mol sodium phosphate
or 0.1 mol sodium cacodylate 30 mL

5% paraformaldehyde

- EM-grade paraformaldehyde (20%) 10 mL
- 0.1 mol sodium phosphate
or 0.1 mol sodium cacodylate 30 mL

Stains

1% toluidine blue

Dissolve 2 g sodium tetraborate (Borax) or sodium carbonate in 100 mL hot distilled water (50 °C), bring down to room temperature then add 1 g toluidine blue. Stir well until it dissolves, and filter the solution before use by using a syringe filter.

Uranyl acetate

Several different uranyl acetate solutions have been employed for staining ultra-thin sections. Alcoholic solutions give better penetration of stain into sections than aqueous solutions. Prepare saturated uranyl acetate solution in 50% ethanol and keep in a brown bottle or away from light. Take 10 mL of solution and centrifuge before use, or use a syringe and syringe filter.

Lead citrate

Dissolve 200 mg of lead citrate in 90 mL distilled water. Add 0.1–0.2 g sodium hydroxide to dissolve lead citrate, then add distilled water to make up 100 mL (pH 12.0).

Embedding media

Ingredients are added and mixed thoroughly. Remove air bubbles by heating at 60 °C for 5 min or by placing under a vacuum.

Preparation of Araldite CY 212 embedding medium

- araldite CY 212 – epoxy resin 10 mL
- DDSA (dodecenylsuccinic anhydride) – hardener 10 mL
- dibutyl phthalate – plasticizer 1 mL
- DMP 30 (tris dimethylaminomethyl phenol) – accelerator 0.4 mL

Preparation of Epon-substitute/Araldite 502 embedding media

- Epon-substitute 812 10 mL
- araldite 502 5 mL
- DDSA (dodecenylsuccinic anhydride) 26 mL
- dibutyl phthalate 0.5 mL
- DMP 30 (tris dimethylaminomethyl phenol) 0.65 mL
(or 1.5% of DMP 30)

Part 3. Demonstration of antigens

Chapter 11

The direct fluorescent antibody test

Introduction

Historically, before the advent of modern laboratory techniques, diagnosis of rabies was based upon clinical signs (1). During the late 1800s, nonspecific histological evidence of central nervous system (CNS) inflammation and, later, demonstration of intra-cytoplasmic inclusions within neurons such as Negri bodies formed a basis for early laboratory diagnosis, coupled with accompanying behavioural alterations or a history of a bite from a suspect rabid animal. Based upon the predilection of lyssaviruses for infection of the CNS, the brain became the organ of choice for routine diagnosis of rabies. Gradually, the combination of clinical observations, improved laboratory techniques and epidemiological surveillance resulted in the accumulated evidence that, without primary replication in the CNS, dissemination to other anatomical sites did not occur, including the salivary glands. As the major portal of viral exit, the salivary glands might seem to have been a more preferable tissue than the CNS for laboratory diagnosis, except that multiple glands contribute to the composition of saliva and salivary excretion was known to be intermittent.

Hence, salivary glands were not selected as appropriate samples for primary diagnostic focus. By the late 1950s, Goldwasser and Kissling had demonstrated the utility of the fluorescent antibody techniques in detecting rabies virus (RABV) antigens in the CNS of rabid animals (2). The fundamental principle of the test depends upon the microscopic detection of viral antigens using fluorescein isothiocyanate (FITC)-conjugated antibodies to RABV. In the ensuing decades, further improvements to the direct fluorescent antibody test (DFAT) were made and greater experience was obtained in its application as a standard diagnostic technique (3, 4, 5). By the 21st century, the DFAT was employed for diagnosis of rabies in millions of cases of suspect animals and was documented to detect all lyssaviruses. As both the sensitivity and the specificity of the DFAT approach 100%, it remains the gold standard for routine veterinary and human laboratory diagnosis of rabies today (6, 7).

This chapter describes one standard operating procedure that details pre-analytical, analytical and post-analytical facets of the DFAT, based on the United States *Protocol for postmortem diagnosis of rabies in animals by direct fluorescent antibody testing* (8).

The brainstem, for example pons, medulla and thalamus, is the most critical tissue for diagnostic testing of rabies. The RABV antigens are widespread throughout the brain in most rabid subjects. However, those with only a sparse amount of viral antigens tend to have more present in the brainstem than in the other CNS regions. Furthermore, examination of a complete cross-section of the brainstem tests the maximum ascending and descending nerve tracts, which is

important because of the possibility of unilateral spread of RABV, particularly in large animals (Fig. 11.1). Along with the brain stem, either cerebellar or hippocampus tissue may be examined. A more confident diagnosis may be achieved by testing additional tissue from one or both of these regions, but a definitive diagnosis cannot be made without a full cross-section of the brainstem.

By courtesy of Lilian Orciari, United States Centers for Disease Control and Prevention, and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

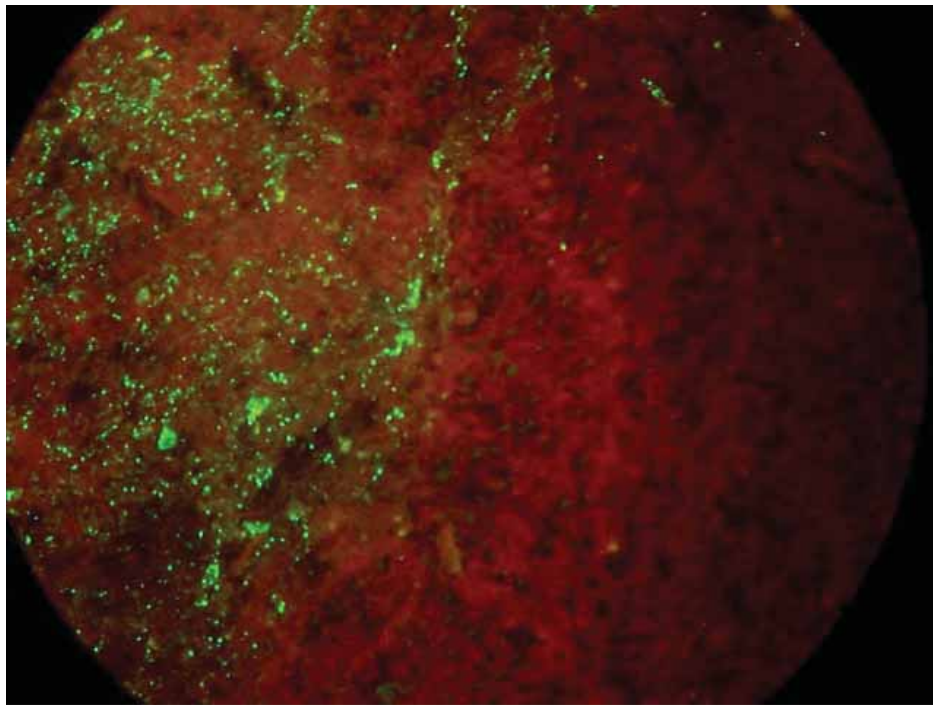


Fig. 11.1. Unilateral antigen distribution

A DFAT was performed on a transverse cross-section of brainstem from a cow infected with rabies. Unilateral distribution of RABV antigens is demonstrated, and a full cross-section of the brainstem must be tested to rule out rabies. Sagittal section of brain stem or an aliquot from the only the right side might miss the presence of RABV antigens in the focal areas.

Specimen shipment

Proper collection, shipping and handling of rabies specimens is essential for a rapid and reliable diagnosis of rabies in animals. In each locality, submitters should be provided with guidelines for proper packaging of suspect specimens and shipping requirements. Intact heads or entire carcasses (if a small mammal, such as a bat) may be submitted to the laboratory. Fresh, unfixed, dissected brain tissue is acceptable but must include a complete transverse cross section of the brainstem and aliquots of tissue from the cerebellum (vermis, right and left) lobes if available. If the cerebellum is unavailable for testing, then the right and left hippocampi may be substituted. Care must be taken to avoid trauma to the brain during euthanasia or sample processing. A submission form should accompany the specimen and should include basic information such as the submitter's name and contact information, animal type and identifier, date of collection, history of expo-

sure in the human or pet, for example bite or scratch, animal owner, geographical location and vaccination history. Sample identifiers are essential when submitting more than one sample at a time, and specimens should be labelled clearly and packaged separately in leak-proof containers. Rapid delivery or shipment of specimens to the laboratory is vital, especially if human rabies post-exposure prophylaxis (PEP) is delayed, pending a laboratory result. Specimens should be kept chilled, not frozen, prior to shipment to the primary testing laboratory. Frozen heads must thaw before they can be safely necropsied, and freezing may delay testing. Testing can occur on frozen tissues, but repeated freeze–thaw cycles may compromise test results and storage in frost-free freezers should therefore be avoided.

Materials

Reagents

- acetone (of American Chemical Society grade)
- conjugate diluent (sterile 0.01 mol phosphate buffered saline, pH 7.4–7.6 with optional Evans Blue counterstain 1–4 $\mu\text{L}/\text{mL}$)
- FITC-conjugated anti-RABV antibody reagents (in the USA, two different conjugates are required in each test)
- 20% glycerol–TRIS buffer coverslip mounting fluid (see Annex)
- phosphate buffered saline (PBS) 0.01 mol, pH 7.4–7.6, for washing slides
- specificity control FITC-conjugated non-RABV antibodies (species and isotype match for one anti-RABV conjugate)

Supplies and equipment

For necropsy/brain removal and dissection

- autoclave
- absorbent blotters (bibulous paper, natural paper towels, disposable wipes, etc.)
- bone chisel (Councilman)
- closed-front gown or coveralls
- containers for acetone fixation and PBS soaks (e.g. Coplin jars, polypropylene 5-place slide holders, staining dishes)
- disinfectant (refer to [Chapter 3](#) on Biosafety)
- disposable laboratory mat, towels or newspapers to cover work surface
- face shield
- forceps (small and large)
- hammer
- incinerator
- large plastic bags for disposal of animal heads and waste
- microscope slides (ideally, polytetrafluoroethylene (PTFE) [Teflon]-coated or pre-ringed with two 15–16 mm diameter wells for routine tests and three 11.5 mm wells for confirmatory tests, with frosted ends)
- microscope slide boxes

- necropsy apron (optional)
- Petri dishes, paper plates or suitable containers for dissection of brain tissues
- powder-free gloves (nitrile or latex)
- restraining device (or method of securing animal heads)
- rubber gloves (heavy duty)
- sample containers (e.g. plastic screw cap tubes or jars, ointment tins)
- scalpels
- tongue depressors for lifting and retracting the brain

For DFA testing

- coverslips (22 mm x 50 mm)
- fluorescence microscope, ideally equipped with HBO 100W or metal halide illuminator (current LED – less suitable), Plan-Apochromat lens 20x (> 0.75 NA) and Plan-Fluorite 40x (> 0.75 NA or oil). Inverted microscopes, although useful for cell culture techniques, are not recommended for routine DFA testing.
- freezers (non frost-free –20 °C flammable-storage, and –70 °C)
- humidified slide chamber (tray with lids or Petri dish may be substituted)
- incubator (37 °C)
- refrigerator (4 °C)
- slide trays (stainless steel or other nonporous autoclavable)
- syringe filters, low protein binding 13 mm or 25 mm diameter 0.45 µm (for amounts > 10 mL, a 25 mm size filter is required)
- syringes 5 mL or 10–20 mL respectively

Biological materials

Unfixed brain impression (positive and negative) control slides should be prepared in advance and may be stored for up to 1 month at –20 °C or at –70 °C or below for up to 6 months.

- RABV indigenous positive (+ 3–4 antigen distribution from two rabies virus variants seen in the region) needed for conjugate titration and routine testing.
- negative brain tissues for control slides.

Methods

Once specimens are received at the testing laboratory, they are given an accession number, and all pertinent information is recorded. All samples and associated materials, such as submission forms, animal heads, carcasses, slides, Petri dishes or paper plates and containers with saved reference material, are labelled carefully with accession numbers. Use of printed labels may be best. Extreme care must be taken to prevent cross-contamination between samples throughout the testing procedure. Each sample must be processed on a clean work surface with a clean,

separate set of instruments. The laboratory personnel performing the necropsy should wear appropriate personal protective equipment (PPE). At a minimum, this includes a closed-front laboratory coat, gown or coveralls, heavy rubber gloves or double gloves, shoe covers or rubber boots, safety glasses and a face shield. A N95 mask should be worn if a biological safety cabinet is not used for necropsy (see [Chapter 3](#) on Biosafety for additional information). Necropsy one sample at a time to avoid cross-contamination.

Most animal brains can be removed using a hammer and chisel method. Brain tissues (brainstem and cerebellum) from large (livestock) animals may be retrieved via the foreman magnum using a long-handled scalpel and spatula or spoon. Smaller mammals, such as bats, can be processed easily with a scalpel or scissors and forceps.

Protocol

1. For average medium-sized animals, place the specimen in a restraining device to anchor the head.
2. Make a midline incision through the skin and muscle using a scalpel or boning knife, then remove the skin and muscle from each side to fully expose the skullcap to the level of the ears.
3. Position a chisel blade behind the eye sockets and cut into the bone by striking the chisel with a hammer.
4. Make two lateral cuts above the ears and a final cut at the back of the skull where the spinal cord enters. Use bone cutters if needed to cut through any adhering bone. Bone cutters are essential for cat specimens since the cerebellum is encased in bone.
5. Cut away dura mater, the membrane covering the brain, then sever the underlying nerves and cut the brain stem region near the spinal cord.
6. Lift the brain out using forceps and a tongue depressor and place it in a sterile Petri dish or in another suitable container.

Notes: After necropsy of specimens, instruments must be handled appropriately; disposable scalpel blades and disposable plastic forceps must be discarded in a sharps container, and reusable stainless steel instruments decontaminated by placing in disinfectant and autoclaved between uses.

Heads or carcasses should be saved until diagnostic results are finalized and reported before discarding.

If test results are unusual, it may be necessary to verify animal type or species. All brain tissue samples should be saved for at least 6 months, and stored frozen at $-20\text{ }^{\circ}\text{C}$ to $-70\text{ }^{\circ}\text{C}$. Some specimens, such as those with unusual or unique lyssavirus variants, should be saved indefinitely.

Occasionally, samples demonstrating signs of decomposition are received for rabies testing. A sample should be considered unsatisfactory for testing if: (i) the cross-section of the brain stem is incomplete; (ii) the brain material is damaged beyond recognition; or (iii) the brain material is decomposed, for example liquefied, discoloured (brown or green) or desiccated (Fig. 11.2).

The brain material from unsatisfactory samples may be tested; however, if negative, the test should be reported as inconclusive due to insufficient sample or sample condition.

By courtesy of Lillian Orciari, United States Centers for Disease Control and Prevention, and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA



Fig. 11.2. Decomposed cow brain

Severe autolysis of tissues is noted in this brain sample. The brain tissues are discoloured, demonstrating both brown (cerebellum) and greenish coloration (cerebral hemispheres). The structural characteristics of the cerebellum are not recognizable; however, the structure of the cerebrum is still recognizable. The brain stem (not shown) was liquid, unrecognizable and was not retrievable.

7. Following necropsy, dissect the appropriate tissues needed for slide preparation.
8. Extract a complete cross-section of brainstem and cerebellum (right, left and vermis) using a sterile scalpel and forceps.
9. Place these pieces on an absorbent paper (bibulous paper, paper towel, e.g. Kimwipe).
10. Prepare touch impressions by pressing a slide gently against the tissues on the paper and blotting several times to remove excess tissue.
11. Make duplicate impressions from the same piece of tissue on the same microscope slide.

Note: Always be consistent when making impressions, and try not to lose any tissue. If the brain is from a large mammal, such as livestock, it may require multiple slides to test a full cross-section of brainstem.

Cut the cross sections in half, then make duplicate impressions from each half.

12. Place unused portions of the brain stem and cerebellum in a non-breakable container, such as an ointment tin or plastic screw cap jar.
13. Place the slides from each specimen in a separate container for fixation and allow to air dry for approximately 15–30 min prior to fixing.
14. When slides are completely dry, add chilled ($-20\text{ }^{\circ}\text{C}$) acetone to each container for fixation.
15. Fix a negative control slide and a positive control slide at the same time as the test slides also in separate containers. Positive control slides should be made from tissue from a strongly positive specimen infected with the most common RABV variant present in the area. Negative control slides should be made from a specimen that tested negative and had minimum background and nonspecific fluorescence (positive and negative control slides can be made from the brain stem or cerebellum tissue, air dried and stored frozen at $-70\text{ }^{\circ}\text{C}$ for up to 6 months).
16. Fix test and control slides in acetone for at least 1 h to overnight at $-20\text{ }^{\circ}\text{C}$ (nomore than 24 h).
17. Following fixation, discard acetone appropriately into a waste bottle, and allow slides to dry at room temperature.
18. Place the slides in a moisture chamber. Two anti-RABV reagents, preferably two different monoclonal conjugates, are used for DFA testing. Use of two different conjugate products reduces the chance of non-recognition of any one viral variant. Polyclonal anti-rabies reagents are also an option, but nonspecific binding can be problematic because of the presence of extraneous antibodies.
19. To prepare fresh working dilution of conjugate, remove the stock conjugate aliquots and mix with the appropriate amount of diluent (PBS plus optional Evans blue) to obtain the working dilution previously determined by titration. The working dilutions of conjugate should be stored at $4\text{ }^{\circ}\text{C}$ and used within 7 days or discarded.
20. Fill two syringes with the working dilutions of the two anti-rabies conjugates, then equip each syringe with a $0.45\text{ }\mu\text{m}$ low protein-binding filter.
21. Discard three drops of conjugate through the filter of the syringe containing the first anti-RABV reagent, then administer by filtering onto the impression in the first well (closest to the frosted end) of all the slides.
22. Start adding conjugate to the positive control slide, follow with the test slides and last, add to the negative control slide. Make sure the reagent completely covers the touch impressions; it may take 2–3 drops depending on the size of each well.
23. Add the second anti-RABV reagent to the duplicate impression in the other well (furthest from the frosted end of slides) in the same manner.

24. Incubate the slides in a humidified chamber at 37 °C for 30 min (Fig. 11.3).

Notes: It is essential that sufficient conjugate is covering the slides and that consistent humidity is maintained while slides are incubating so that the conjugate does not dry on the slides. Dried conjugate may be mistaken for specific staining or for obscure specific staining of lyssavirus antigens.

By courtesy of Lillian Orciari, United States Centers for Disease Control and Prevention, and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

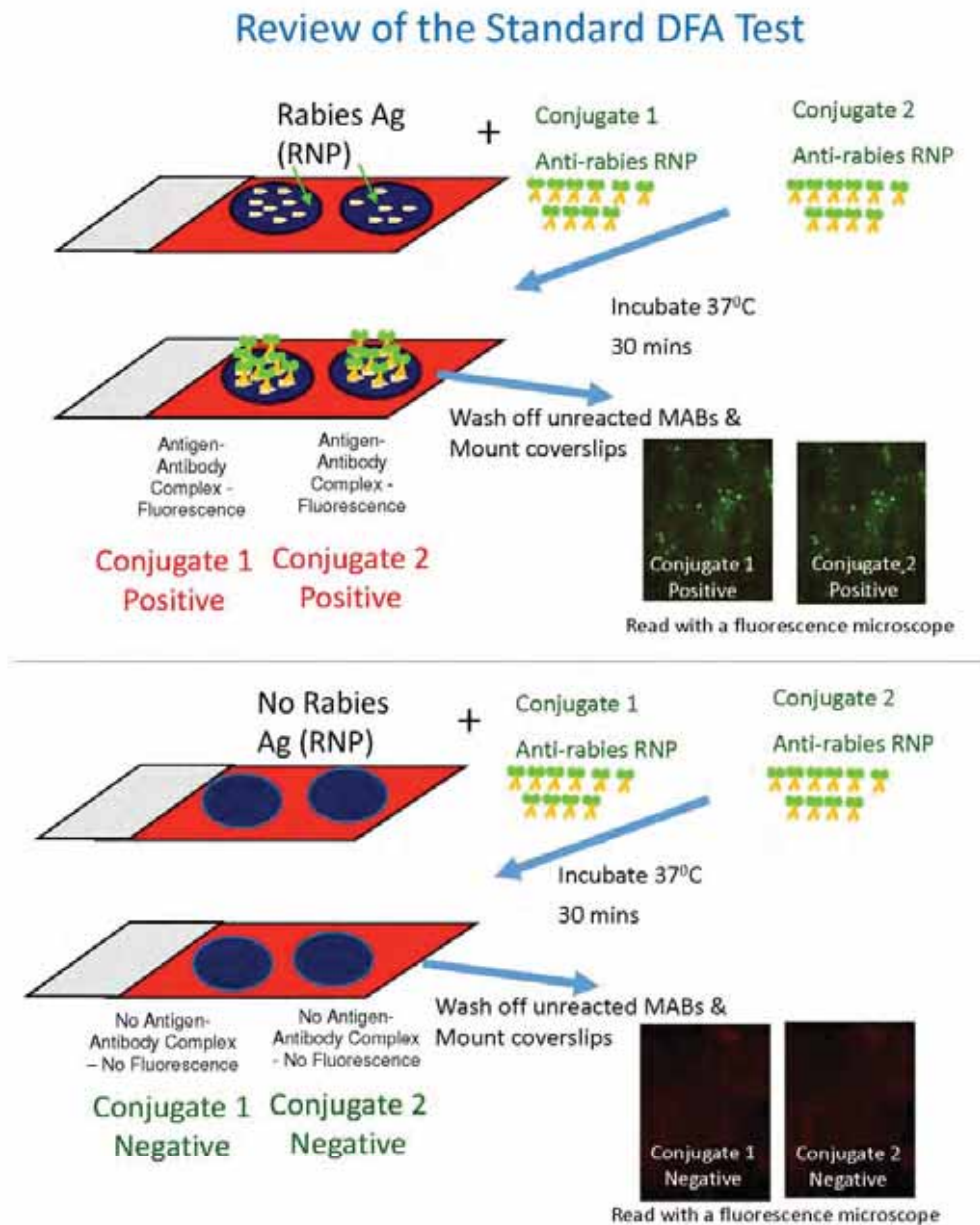


Fig. 11.3. Overview of the standard direct fluorescent antibody test (DFAT)

The basic steps in the DFAT are reviewed in this figure. If present, RABV antigens (e.g. RNP) in acetone-fixed brain impressions will react with the two FITC-labelled anti-rabies conjugates and form specific antigen-antibody complexes. After unreacted anti-rabies antibodies are removed by washing with PBS, specific fluorescence RABV inclusions are observed. If no RABV antigens are present, then no antigen-antibody complexes are formed and no specific fluorescence (i.e. no inclusions) are observed.

25. Remove slides from the incubator, and gently rinse each well of the slide carefully with a stream of PBS from a wash bottle. Do not allow conjugate from one well to wash into another.
26. Transfer each set of slides from a specimen to a rinse container; use one rinse container per specimen. Use separate containers for the positive control and the negative control.
27. Soak the slides in PBS for 5 min.
28. Discard and replace with fresh PBS and soak for another 5 min.
29. Remove slides from the PBS, one by one, and invert them on to clean absorbent paper, turn them over to blot the back of the slides and place them in a slide tray.
30. Add 20% (low) glycerol TRIS-buffered mounting fluid to each of the coverslips first, not directly to the slide, using 1 mL syringe fitted with a 0.45 μm 2 mm (Nalgene) syringe filter.
31. Invert the coverslip with mounting medium onto the slide. The mounting medium used in the DFAT may affect the avidity of the antigen-antibody complex; mounting solutions with high glycerol concentrations are not recommended (9).

Note: If the above-mentioned mounting medium is not used to attach coverslips to slides, small inclusions and sparse amounts of antigen may be missed (Fig. 11.4).

32. Take the slides into the dark room equipped with a fluorescence microscope.
33. Keep the slides in the dark, and observe the test slides within 2 h using a fluorescence microscope. If slides will not be read within 2 h, they can be stored at $-20\text{ }^{\circ}\text{C}$ or below and retain fluorescence.
34. Examine impressions on slides thoroughly at 200x (Plan-Apochromat ≥ 0.75 NA) magnification for 4+ sparkling apple-green fluorescing inclusions.

If further resolution is required to examine the inclusion morphology, observe with 40x neofluorite lens with ≥ 0.75 NA. Ideally, two readers should examine the slides made from each test animal to ensure correct interpretation. Results are recorded for the control slides and for each impression of the test slides on a worksheet. The reactions are rated on staining intensity and antigen distribution. For example, RABV antigens that stain sparkling apple-green fluorescence are rated as 4+ intensity. Substandard samples, such as those with some degree of decomposition, might have a slightly diminished brightness, bright but are not sparkling, 3+. Dull green inclusions with 2+ or less are not diagnostic of rabies, unless additional testing confirms the inclusions are lyssavirus specific.

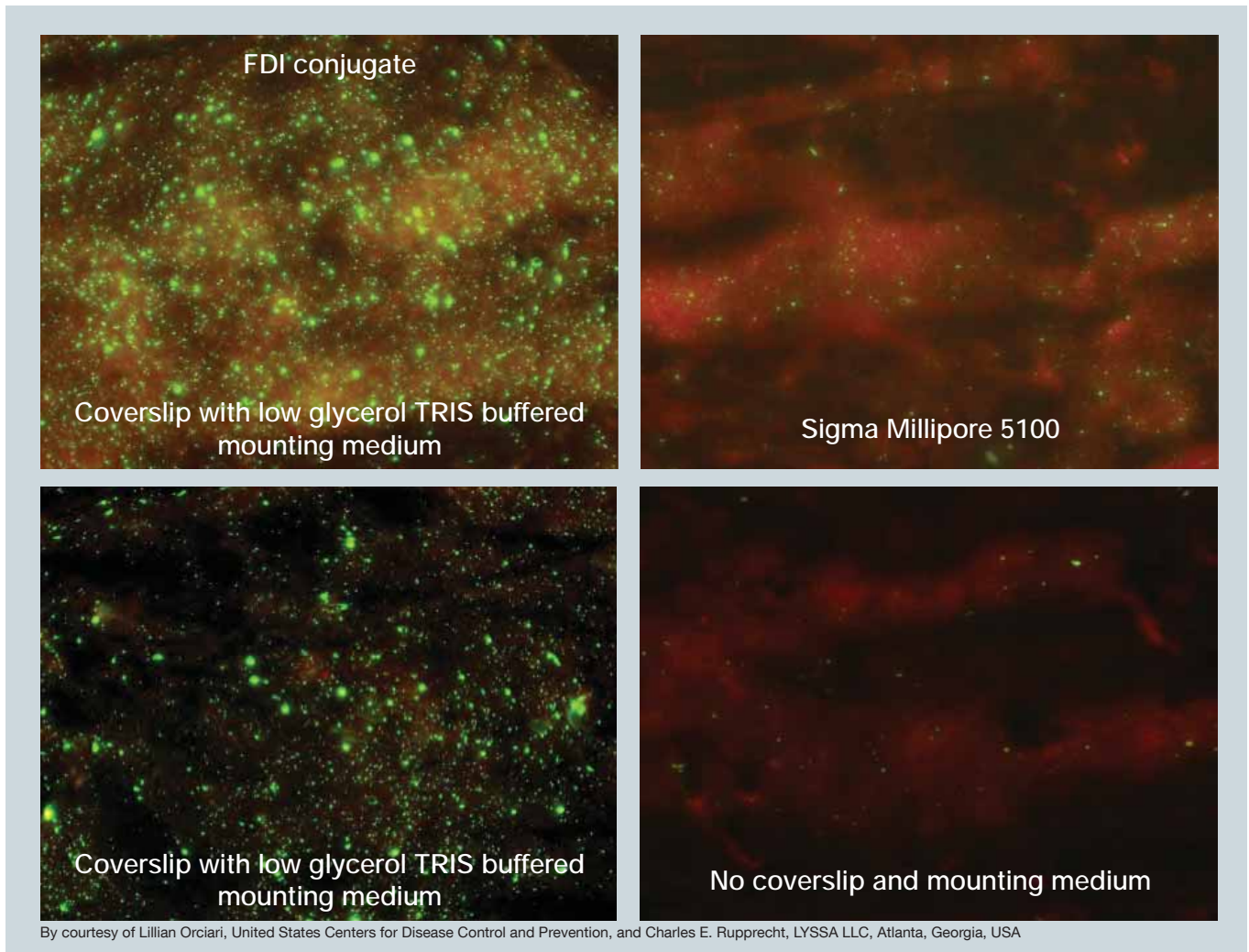


Fig. 11.4. DFAT-positive impressions with and without low glycerol mounting medium

Duplicate brain impressions from the same positive (South Central Skunk RABV) sample were tested with working dilutions of anti-rabies conjugates (Fujirebio Diagnostics Inc. and Sigma Millipore DFA 5100). Photographs were taken of brain impression slides with and without coverslips and mounting medium at 200x magnification.

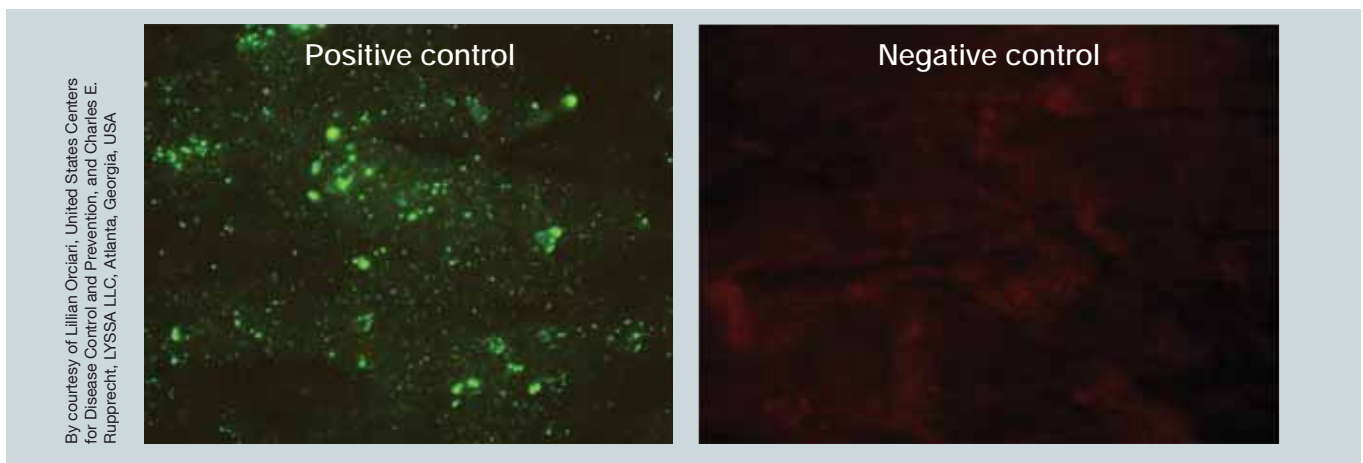


Fig. 11.5. DFAT controls

The positive control impression (left) demonstrates 4+ sparkling apple-green fluorescence and 3–4+ antigen distribution. The working dilution of conjugate should be diluted so that intense apple-green fluorescence is observed in the positive control for each test, including observation of 100% of the small and large inclusions. The negative control slide demonstrates no specific fluorescence.

The distribution of antigens is graded on a scale of 4+ down to 1+. If rated as 4+, inclusions of varying size and shape are observed in nearly all microscope fields, and 3+ inclusions of varying size and shape are numerous in most fields. However, if rated as 2+, 10–50% of the fields have inclusions of varying size and shape, and 1+ have inclusions of varying size and shape seen in < 10% of the microscope fields.

Readers should record the antigen intensity/distribution for each impression for examples 4+/4+, 4+/2+ or 3+/4+ and make a note if the staining intensity is below +4.

The positive control slides must demonstrate the expected 4+ staining intensity 3–4+ antigen distribution (Fig. 11.5). There should be no staining on the negative control slides. The test slides must be remade and retested if the positive and negative control slides do not demonstrate acceptable results.

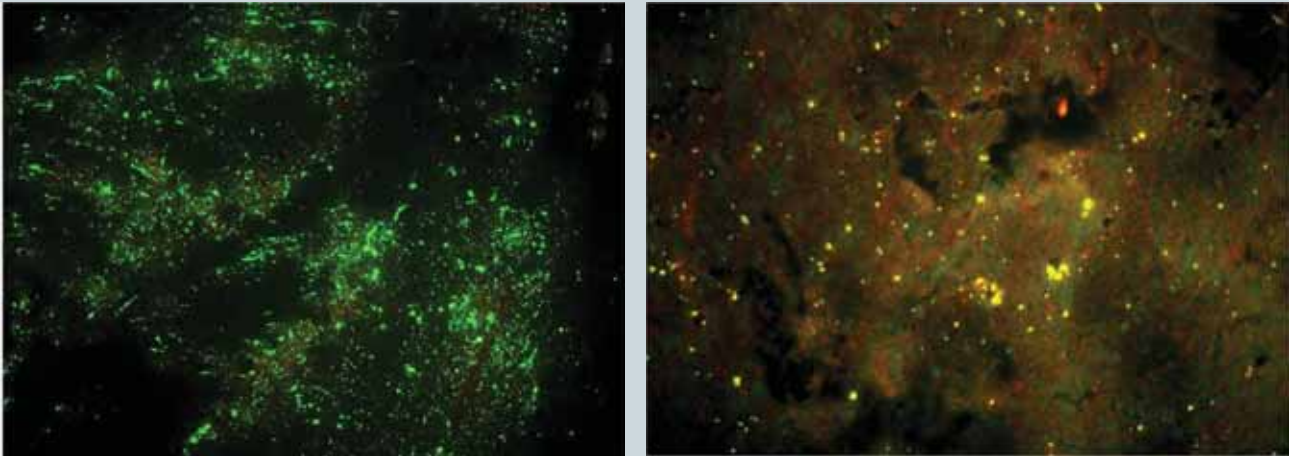
Interpretation of test results

Test results may be reported as negative for rabies, positive for rabies, unsatisfactory, or inconclusive. If both slides are clearly negative for the presence of lyssavirus antigens, the sample is reported out as negative for rabies, provided the proper tissues were tested and in satisfactory condition. If both slides are clearly positive, having at least a 3+ to 4+ intensity and 2+ to 4+ antigen distribution, the sample is reported out as positive for rabies. Sometimes, inexperienced individuals may be concerned when observing yellow or golden autofluorescence in negative samples caused by accumulations of lipofuscin (Fig. 11.6).

Specimens that are not clearly negative or positive require repeat testing. For example, samples with very sparse inclusions less than 10% of fields demonstrating antigen, inclusions with atypical morphology or inclusions with staining intensity of less than +3 brightness, warrant repeat testing. If, after repeat testing with specificity control conjugates, the results are still not clear, the specimen test result is reported out as inconclusive. Alternative, confirmatory testing may be necessary to rule out rabies.

Because PEP of human rabies is often delayed until a test result is attained, repeat testing should be accomplished as quickly as possible. Repeat slides are made from the original brain tissue that is saved after dissecting the brain and making the initial set of slides. Impressions are made from a cross-section of brainstem and cerebellum. However, for the repeat (confirmatory) DFAT, three identical brain impressions are made from each piece of tissue per 3-well slide. Multiple impression slides may be required to test each of the required tissues, full cross-section of brain stem and three lobes of cerebellum.

In the repeat confirmatory DFAT, the brain impression slides are tested with two anti-rabies conjugates and the specificity control reagent, negative control conjugate. The specificity control reagent contains non-rabies antibodies of the same isotypes prepared in the same animal as the anti-rabies conjugate which it is paired.



By courtesy of Lillian Orciari, United States Centers for Disease Control and Prevention, and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 11.6. RABV-positive antigens compared with atypical autofluorescence

The positive control (left) with 4+ sparkling apple-green fluorescence and 4+ distribution is compared with atypical autofluorescence observed in negative sample (right) with accumulations of lipofuscin in the CNS tissue impression. Note the gold–yellow colouration of objects without distinct outlines seen occasionally in older animals and in the cerebellum of cats. This gold autofluorescence is not immunofluorescence or nonspecific binding. The same observation is possible without staining with FITC-labelled conjugates by exposing tissue impressions with lipofuscin to blue light.

Note: For example, currently in the USA, Sigma Millipore Light Diagnostics Rabies DFA Cat# 5100 contains a mixture of two mouse IgG1 monoclonal antibodies and one mouse IgG2 monoclonal antibody, FITC-labelled. The specificity control reagent paired with this anti-rabies conjugate is Sigma Millipore Light Diagnostics Rabies Negative Control (Monoclonal antibody FITC-conjugate) Catalogue #5102.) It is a mixture of the same isotype IgG1 and IgG2 mouse monoclonal antibodies at the same concentration in mg/mL as Sigma Millipore Cat#5100.

Positive and negative control (3-well slides) must be tested in conjunction with the repeat test slides using two anti-rabies conjugates and the specificity control reagent, meaning the negative control conjugate (Figs. 11.7.1–5). The specimen is considered negative for rabies if all brain impressions are negative upon repeat testing with two anti-rabies conjugates and the specificity control conjugate (Fig 11.7.2). Also, if atypical inclusions are present on test slides stained with one anti-rabies conjugate and the specificity control paired with that conjugate, and no inclusion is observed with the second anti-rabies conjugate indicating the staining is nonspecific (Fig 11.7.3), the test is considered negative.

The specimen is positive for lyssavirus antigens if, upon repeat testing, inclusions typical of rabies are observed on impressions tested with one or both of the anti-RABV conjugates, but not the specificity control reagent paired to one of the anti-rabies conjugates (Figs. 11.7.1, 11.7.4).

The specimen results are inconclusive if inclusions are observed on all impressions tested with the two anti-rabies conjugates and specificity controls and are indistinguishable from RABV inclusions or may mask specific staining (Fig. 11.7.5).

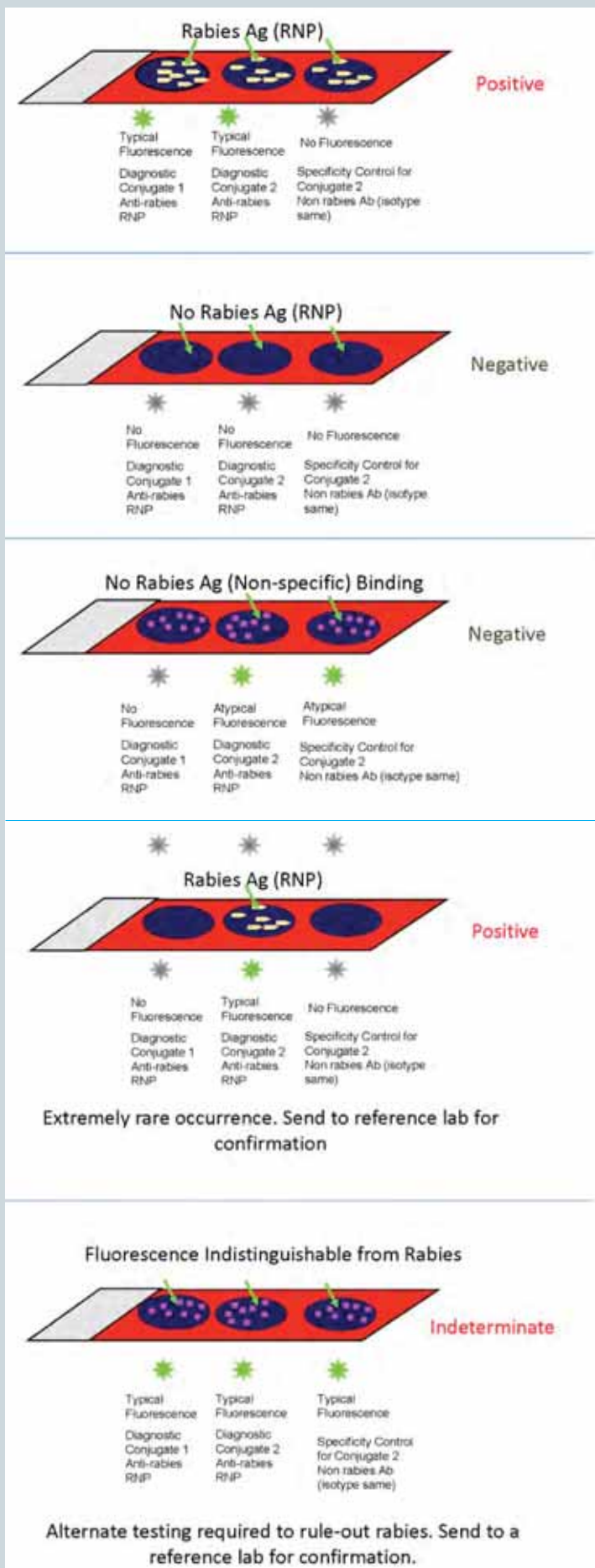


Fig. 11.7.1. If typical RABV antigens fluoresce with both anti-rabies conjugates and the specificity control is negative, then the test is positive

Fig. 11.7.2. If no fluorescence is demonstrated in DFA with both anti-rabies conjugates and the specificity control, then the DFAT is negative

Fig. 11.7.3. If conjugate 1 demonstrates no fluorescence, and atypical fluorescence is demonstrated with conjugate 2 and the same fluorescence is demonstrated in the specificity control paired with conjugate 2, then the test can be reported as negative

Fig. 11.7.4. If anti-rabies conjugate 1 demonstrates no fluorescence, but typical RABV antigens fluoresce with conjugate 2, and the specificity control is negative, then the samples should be considered as positive, but sent to a reference laboratory for confirmation

Fig. 11.7.5. If all three conjugates demonstrate fluorescence indistinguishable from RABV antigens, then the test should be considered as indeterminate and alternative testing is required to rule-out rabies; it may be necessary to send to a reference laboratory for confirmation.

By courtesy of Lillian Orclari, United States Centers for Disease Control and Prevention, and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 11.7. Confirmatory DFAT with specificity control reagent

Alternative tests, such as real-time RT-PCR, DRIT, hemi-nested RT-PCR or isolation in cell culture, may be required to rule out rabies. Of the alternative tests, real-time RT-PCR and DRIT are the most useful for expediency (10–12).

Discussion

The DFAT has proven its utility as an ideal diagnostic method during the past 50 years, in both developed and developing countries (13). The test is highly sensitive and specific, as demonstrated in multiple external inter-laboratory trials (14). Owing to its accuracy and advantages in an anatomical–pathological context, in some countries such as the USA, nearly 100 000 suspect animals may be tested annually (15). Properly controlled, the test can provide rapid, unequivocal results within a few hours, irrespective of lyssavirus species or mammalian taxa (including humans in either antemortem or postmortem situations) (7). The procedure may be used for a variety of purposes, including routine laboratory-based surveillance, risk assessment in bite exposure cases, determination of RABV in experimental animal clinical trials, in vitro assays (such as the rapid fluorescent focus inhibition test [RFFIT; see Chapter 19] and the fluorescent antibody virus neutralization test [FAVN; see Chapter 20] and evaluation of vaccination programmes). Unlike the historical use of conventional Negri body identification using Sellers' stain and the traditional process of holding suspect animals until late in the clinical course, rabid animals may be detected upon the initial onset of illness. Fresh, frozen and fixed tissues can be used (16). Concentration upon the brainstem and cerebellum as the CNS tissues of choice are based in part upon basic pathobiology, relative ease of anatomical access and economy of effort (17). Both monoclonal and polyclonal commercial FITC-conjugates can be utilized. Currently, in the USA, only monoclonal antibody conjugates are available (Table 11.1). In cases of indeterminate results, confirmatory laboratory testing may involve viral isolation, alternative antigen capture assays or relevant molecular tests (18).

Potential drawbacks in consideration of the DFAT include the need for investment in a fluorescence microscope, the necessity for appropriate high-quality, high-intensity arc lamps, regular microscope maintenance and routine participation in proficiency testing as well as the need for use of infectious agents.

Table 11.1. Monoclonal antibody FITC-labelled conjugates for the rabies DFAT internationally available

Manufacturer	Product name	Product number	Antibodies
Fujirebio Diagnostics (FDI)	FITC-Anti-Rabies Monoclonal Globulin	800-900	Contains a mixture of two IgG2a monoclonal antibodies
Sigma Millipore	Light Diagnostics TM Rabies DFA Reagent II (Monoclonal antibody FITC-conjugate)	5500	Contains the same mixture of two IgG2a monoclonal antibodies as FDI 800-090
Sigma Millipore	Light Diagnostics TM Rabies DFA Reagent III (Monoclonal antibody FITC-conjugate)	6500	Contains the same mixture of two IgG2a monoclonal antibodies as FDI 800-090, one antibody Fab fragment
Sigma Millipore	Light Diagnostics TM Rabies DFA Reagent (Monoclonal antibody FITC-conjugate)	5100	Contains the same mixture of two IgG1 monoclonal antibodies and one IgG2 monoclonal antibody
Sigma Millipore	Light Diagnostics TM Rabies Negative Control (Monoclonal antibody FITC-conjugate)	5102	Contains a mixture of IgG1 IgG2 non-rabies monoclonal antibodies

References

1. Rupprecht CE. A tale of two worlds: public health management decisions in human rabies prevention. *Clin Infect Dis*. 2004; 39:281–3.
2. Goldwasser RA, Kissling RE. Fluorescent antibody staining of street and fixed rabies virus antigens. *Proc Soc Exp Biol Med*. 1958; 98:219–23.
3. Beauregard M, Boulanger P, Webster WA. The use of fluorescent antibody staining in the diagnosis of rabies. *Can J Comp Med Vet Sci*. 1965; 29:141–7.
4. Dean DJ, Abelseth MK. Laboratory techniques in rabies: the fluorescent antibody test. *Monogr Ser World Health Organ*. 1973; 23:73–84.
5. Bourhy H, Rollin PE, Vincent J, Sureau P. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immunoassay for rapid laboratory diagnosis of rabies. *J Clin Microbiol*. 1989; 27:519–23.
6. Rabies. In: *Manual of diagnostic tests and vaccines for terrestrial animals*. Paris: World Organization for Animal Health; 2013 [Volume 1. Chapter 2.1.13].
7. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 14 May 2018).
8. Protocol for postmortem diagnosis of rabies in animals by direct fluorescent antibody testing: a minimum standard for rabies diagnosis in the United States. Atlanta (GA): United States Centers for Disease Control and Prevention (<http://www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf>, accessed 14 May 2018).
9. Rudd RJ, Smith JS, Yager PA, Orciari LA, Trimarchi CV. A need for standardized rabies-virus diagnostic procedures: effect of cover-glass mountant on the reliability of antigen detection by the fluorescent antibody test. *Virus Res*. 2005; 111:83–88. doi:10.1016/j.virusres.2005.03.014.
10. Hoffmann B, Freuling CM, Wakely PR, Rasmussen TB, Leech S, Fooks AR, et al. Improved safety for molecular diagnosis of classical rabies viruses by use of a TaqMan real-time reverse transcription-PCR “double check” strategy. *J Clin Microbiol*. 2010; 48:3970–8.
11. Wadhwa A, Wilkins K, Gao J, Condori Condori RE, Gigante CM, Zhao H, et al. A pan-lyssavirus Taqman real-time RT-PCR assay for the detection of highly variable rabies virus and other lyssaviruses. *PLoS Negl Trop Dis*. 2017; 11:e0005258. doi:10.1371/journal.pntd.0005258.
12. Lembo T, Niezgodá M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht CE. Evaluation of a direct, rapid immunohistochemistry test for rabies diagnosis. *Emerg Infect Dis*. 2006; 12:310–3.
13. Tepsumethanon V, Lumlertdacha B, Mitmoonpitak C, Fagen R, Wilde H. Fluorescent antibody test for rabies: prospective study of 8,987 brains. *Clin Infect Dis*. 1997; 25:1459–61.

14. Robardet E, Picard-Meyer E, Andrieu S, Servat A, Cliquet F. International interlaboratory trials on rabies diagnosis: an overview of results and variation in reference diagnosis techniques (fluorescent antibody test, rabies tissue culture infection test, mouse inoculation test) and molecular biology techniques. *J Virol Methods* 2011; 177:15–25.
15. Monroe BP, Yager P, Blanton J, Birhane MG, Wadhwa A, Orciari L, et al. Rabies surveillance in the United States during 2014. *J Am Vet Med Assoc.* 2016; 248:777–88. doi:10.2460/javma.248.7.777.
16. Whitfield SG, Fekadu M, Shaddock JH, Niezgoda M, Warner CK, Messenger SL, Rabies Working Group. A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin-fixed brain tissue specimens. *J Virol Methods* 2001; 95:145–51. doi:10.1016/S0166-0934(01)00304-4.
17. Bingham J, van der Merwe M. Distribution of rabies antigen in infected brain material: determining the reliability of different regions of the brain for the rabies fluorescent antibody test. *J Virol Methods.* 2002; 101:85–94.
18. Fooks AR, Johnson N, Freuling CM, Wakeley PR, Banyard AC, McElhinney LM, et al. Emerging technologies for the detection of rabies virus: challenges and hopes in the 21st century. *PLoS Negl Trop Dis.* 2009; 3:e530.

Annex. Use of reagents and equipment in the DFAT

FITC-conjugated anti-rabies antibody reagents

Manufacturer's instructions should be followed with regard to storage and reconstitution of lyophilized conjugates.

Sigma Millipore Light Diagnostics™ Cat# 5100 (<https://www.emdmillipore.com/>)

Sigma Millipore Light Diagnostics™ Rabies Monoclonal Negative Control Reagent Cat# 5102 (<https://www.emdmillipore.com/>)

Sigma Millipore Light Diagnostics™ Rabies DFA II, FITC conjugate Cat# 5500 (<https://www.emdmillipore.com/>)

Sigma Millipore Light Diagnostics™ Rabies DFA II, FITC conjugate Cat# 6500 (<http://www.emdmillipore.com/>)

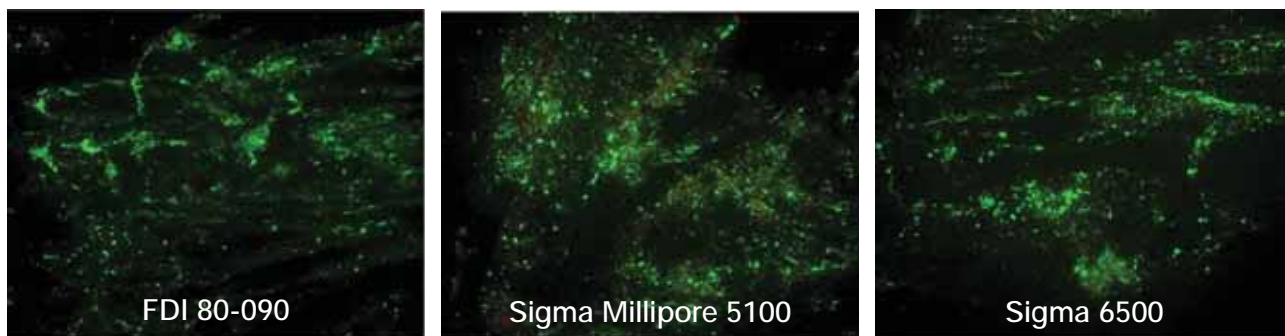
Other polyclonal and monoclonal anti-rabies conjugates are available internationally.

Conjugate titration

Prior to use, the optimal working dilution of a conjugate must be determined by titration. Conjugate titration is the serial dilution of the stock “neat” new lot of anti-rabies conjugate and testing the reactivity or ability of these dilutions of conjugate to detect RABV antigens in brain impressions from a minimum of two predominant viral variants seen in a geographical location (Fig. 11.8). It is best to use indigenous street virus from dogs, wild carnivores or bats rather than laboratory-adapted RABV. Titration should be performed the same as the diagnostic test using the standard protocol and the same supplies, equipment and diluent used in routine testing, with a minimum of two laboratorians observing the slides.

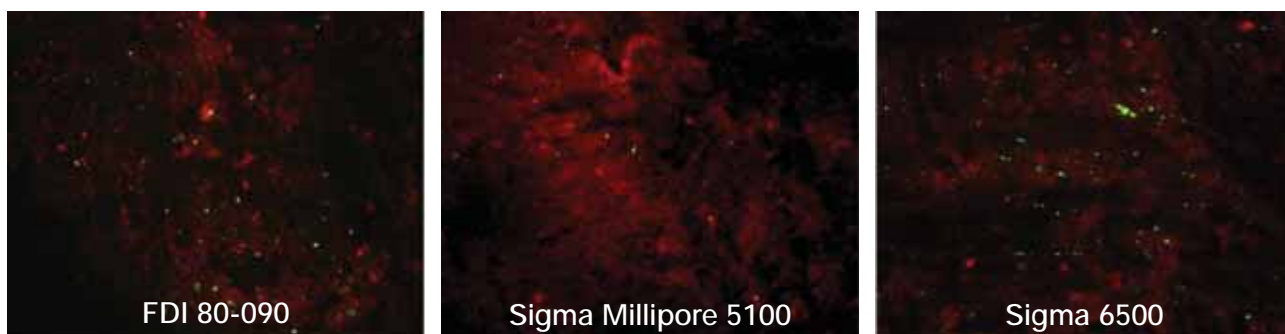
Broad titration

The broad titration of a new lot of anti-rabies conjugate is performed by preparing 2-fold dilutions for example: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 in PBS with optional Evans Blue counterstain and comparing them with the reference (current conjugate working dilution) to determine the end-point titre. The end-point titre is the dilution of conjugate, which demonstrates 4+ sparkling apple-green fluorescence and, ideally, detection of 100% of the RABV antigens present. Then from the end-point of the broad titration, a more precise optimal working dilution can be determined by performing a narrow titration.



By courtesy of Lillian Orciari, United States Centers for Disease Control and Prevention, and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 11.8A. The figure demonstrates the reactivity of three different anti-rabies conjugates at optimal working dilutions of each against the cosmopolitan RABV variant (4+ sparkling apple-green fluorescence and 4+ antigen distribution).



By courtesy of Lillian Orciari, United States Centers for Disease Control and Prevention, and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 11.8B. In contrast to Fig. 11.8A, differences are noted in the affinity of the same three conjugates at optimal working dilutions with a weakly positive bat sample infected with the Western *Epptesicus fuscus* RABV variant. The most prominent difference is the low level of detection of Sigma Millipore 5100 with that variant. A less than optimal working dilution might not detect antigen in a positive sample. This demonstrates the need to titrate conjugates against rabies virus variants endemic in a locality rather than the use of laboratory adapted viruses.

Narrow titration

If the end-point of the broad titration is at a 1:40 (slight decrease in fluorescence is detected in 1:80), the narrow titration should be performed using smaller increments, from between 1:40 and 1:80, for example: 1:40, 1:50, 1:60, 1:70, 1:80. However, to ensure that the end-point of the narrow titration is not missed, it is best to start with 1:30, which is one increment lower than 1:40, for example: 1:30, 1:40, 1:50, 1:60, 1:70 and 1:80. The diluted conjugates are added to a minimum of two different RABV variants and compared with the reference (current lot) conjugate. The working dilution of conjugate should be two steps more concentrated than the drop-off of the narrow titration. For example, if the amount of antigens observed or the intensity is diminished at a 1:60 dilution, the working dilution of the conjugate would be 1:40. After the working dilutions of conjugate are determined and tested for each reagent, aliquots of the stock (undiluted conjugate) should be stored according to the manufacturer's recommendations. Reconstituted lyophilized conjugates can be stored for up to 6 months at -70°C or below.

Working dilutions of conjugate

The working dilution of a conjugate is prepared in PBS with optional Evans Blue counterstain (the amount of counterstain will vary depending on the conjugate). To reduce nonspecific binding of protein aggregates, dissociated FITC and potential contaminants in the working dilution of conjugate, the working dilution of conjugate is filtered on to the brain impressions with syringe equipped with a 0.45 μ low protein binding filter (13 mm or 25 mm) depending on volume. The filters must be low protein binding (to prevent loss of antibody) and should be suitable to handle small volumes (13 mm filters for volumes up to 10 mL and 25 mm filters for volumes greater than 10 mL).

FITC-conjugated specificity control reagent

The specificity control for a hyperimmune serum reagent is a reagent produced in the same host as the rabies reagent (such as a horse or goat), but as normal serum or serum directed to an agent other than RABV. The specificity control for a reagent prepared from a mouse monoclonal antibody is a mouse monoclonal antibody of the same isotype as the rabies reagent, but directed to an agent other than RABV (8). The specificity control reagent should be diluted to the same concentration as the anti-rabies conjugate to which it is paired. (Currently, Sigma Millipore manufactures a specificity control reagent Catalogue #5102 paired with Sigma Millipore anti-rabies Conjugate Catalogue# 5100. Hyperimmune (polyclonal) anti-rabies conjugates are currently unavailable in the USA. However, internationally available from Biorad: http://www.emdmillipore.com/US/en/product/MM_NF-5102).

Protocol

1. Conjugate diluent (sterile 0.01 mol phosphate-buffered saline, pH 7.4–7.6) with optional Evans Blue (0.5–4.0 μ L/mL of 1% Evans Blue counterstain). See recipe for PBS below. Pre-filter and sterilize before use to dilute the conjugate.
2. Phosphate buffered saline, pH 7.4–7.6, for rinsing slides. Prepare a working solution of 0.01 mol phosphate buffer (pH 7.4) with 0.138 mol NaCl (sodium chloride) and 0.0027 mol KCl (potassium chloride). Large volume containers, such as carboys, work well for storing PBS but need to be sterilized regularly. Commercial packets of PBS salts are available and can be diluted in 1 L of distilled or deionized water if small quantities of PBS are used (Sigma-Aldrich P3813) in the laboratory. PBS is filtered and sterilized before use for conjugate diluent.
3. Mounting fluid. Use a 20% glycerol TRIS-buffered mounting medium to coverslip slides. Higher glycerol concentrations can adversely affect the avidity, stability of antigen-antibody binding capacity (fig. 4) (9). The TRIS buffer is prepared by combining 0.623 g of Trizma pre-set crystals and 0.85 g NaCl in 100 mL distilled water to prepare a 0.05 mol TRIS or 0.15 mol NaCl solution. The pH should be adjusted to 9.0, and the solution

filter sterilized through a 0.45 µm filter. The solution may be stored at room temperature for one year, but the pH should be checked quarterly. Prepare the mounting medium by mixing one part glycerol with four parts TRIS-saline solution. Store at room temperature for up to one month. Low (20%) glycerol mounting medium is commercially available (Sigma Millipore Cat# 5096 [<https://www.emdmillipore.com/>]).

4. Microscopes. Fluorescence microscopes for maximum antigen detection are ideally equipped with equipped with HBO 100W or metal halide illuminator (current LED – less suitable), Plan-Apochromat 20x lens (> 0.75 NA) and Plan-Fluorite 40x (> 0.75NA or oil). Inverted microscopes, although useful for cell culture techniques, are not recommended for routine DFA testing.

For general information about fluorescence microscopy and the equipment, microscope, objectives and illuminators refer to <http://micro.magnet.fsu.edu/primer/virtual/virtual.html>.

For more information regarding laboratory microscopes, emission lamps, objectives that are available for use in the DFAT, the reader is directed to several commercial sites listed in alphabetical order for comparison:

<http://www.leica-microsystems.com/sciencelab/topics/fluorescence-microscopy/>;

<http://www.microscopyu.com/articles/fluorescence/>;

<http://www.olympusmicro.com/primer/techniques/fluorescence/fluorhome.html>;

<http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html>.

Protocol for corrective action when controls fail (troubleshooting)

1. First, examine the positive control and negative control slides. The positive control should demonstrate 4+ sparkling apple-green fluorescence and detect 100% of antigen present when observed with a fluorescence microscope. The negative control should demonstrate no immunofluorescence.
 - a. If the controls demonstrate the expected values, the test is reportable based on the staining patterns observed in the test slides.
 - b. If the controls do not give the expected values, then the test is not reportable; a technical expert and supervisor should be notified. Troubleshooting methods should be employed and repeat testing is required.
 - c. The incident, troubleshooting process and resolution should be documented in the quality control records.
2. Positive control with < 3+ fluorescence (low intensity).
 - a. Check a previously tested positive control slide (stored in the freezer to maintain fluorescence) on the microscope.
 - i. If the intensity is 4+ then the problem is not related to the microscope equipment and is related to the reagents or implementation of the test procedure.
 - ii. If the intensity on the control slide previously observed as 4+ is only

- ≤ 3+ fluorescence, then most likely the problem is related to the microscope equipment. Proceed by examining the microscope.
- b. Microscope related intensity issues are usually related to light source, alignment, improper equipment settings or maintenance.
 - i. Check the hours on the light source; a less than optimal light source may cause this problem. The HBO 100W lamps (OSRAM 103/W2) purchased have an expected life of 300+ h (on and off cycles) and should be changed between 200 and 300 h. Check the transformer pad (if the microscope has one) each time the microscope is used for a message that the lamp is too old. Replace the lamp if necessary and realign the microscope.
 - ii. If the lamp has been changed recently, make sure the microscope settings are correct (alignment of light source, filters) and nothing is impeding the light such as a closed or partially opened aperture.
 - iii. In addition, the objective and ocular lens should be cleaned at this time.
 - iv. If the problem is not resolved, a microscope service representative may need to be consulted.
 - c. If the problem is not microscope related, reagents and test performance are suspect. First, check the equipment records for any potential problems. If there are no problems with incubator temperature or refrigeration of reagents, it is most likely a less than optimal reagent.
 - d. The most critical reagents used in the DFAT are the anti-rabies conjugates. Titres of new conjugate lots should be predetermined by titration before use with multiple known rabies virus variants. Working dilutions of the new lots need to be tested in parallel with the old lot before use in a test run.
 - i. If the lot of conjugate is not new, repeat dilution of the conjugate with freshly prepared PBS and Evans Blue.
 - ii. Also check the mounting medium used: it should be low glycerol mounting medium pH 9.0. The mounting medium should be stored refrigerated, since bacterial contamination will affect pH. Use of a new aliquot of mounting is recommended under these circumstances since it is an inexpensive reagent.
 - iii. Repeat DFA testing of the same positive control and a low positive control should demonstrate resolution if a reagent problem exists.
 - e. If the problem is not microscope, equipment or reagent related, it might be due to technical errors. Although rare, these can occur. Thorough training of new personnel, monitoring proficiency and strict adherence to the standard protocol are methods to eliminate these issues.

3. Negative control has fluorescent staining.

- a. Technical issues may be the cause of fluorescence on the negative control slide related to the improper washing of the slides, drying of the conjugate on the slide or over incubation.

- b. Nonspecific reactivity of the anti-rabies conjugates. Check the reaction with the specificity control.
 - c. Other issues may be contamination of a reagent with bacteria (PBS, conjugates, mounting medium)
4. Repeat testing of samples with strict adherence to the protocols should **resolve technical problems. Specificity related issues are not easily resolved**, especially with polyclonal or hyper-immune antibody conjugates. These problems need to be reported to the manufacturer of the reagents.
5. Observation by an experience laboratorian or the supervisor may be necessary to determine potential problems, especially when due to technical manipulations.

Chapter 12

The direct rapid immunohistochemistry test for the detection of lyssavirus antigens

Introduction

The direct rapid immunohistochemistry test (DRIT) is a streptavidin–biotin peroxidase staining technique for diagnosis of rabies by detecting lyssavirus antigens. During the past decade, the DRIT has been used extensively for enhanced, laboratory-based surveillance of rabies in Africa, Asia and throughout the Americas, with a sensitivity and specificity equivalent to that of the direct fluorescent antibody test (DFAT) as described in [Chapter 11](#) and elsewhere (1–13). All relevant biosafety precautions should be followed as discussed in [Chapter 3](#) of this manual.

Materials

Equipment

Light microscope with a 10x ocular and high-quality objective lens (e.g. 20x, 40x)

Supplies

- glass microscope slides
- Coplin jars or staining trays
- paper towels
- “humidity chamber” (a simple plastic cover placed over slides during benchtop staining)
- timer
- marking pens or pencils
- pipettes (including glass [or clear, low-thermal-expansion borosilicate, Pyrex])
- glass vials
- centrifuge tube (or similar)
- syringes
- syringe filters
- pipette tips
- pipetting device
- microscope slide coverslips
- positive and negative control tissue
- scalpels

Reagents

- formalin, 10% buffered
- phosphate buffered saline (PBS), pH 7.4
- hydrogen peroxide, 3%
- primary anti-rabies antibody conjugated to biotin: polyclonal anti-nucleo-protein or cocktail of anti-lyssavirus biotinylated monoclonal antibodies, stored at 4 °C
- streptavidin–peroxidase, stored at 4 °C
- 3-Amino-9-ethylcarbazole (AEC) substrate (note, other suitable chromogens may be used)
- N,N-dimethylformamide, solvent for AEC
- acetate buffer, 0.1M, pH 5.2
- Gill's haematoxylin formulation #2, diluted ~1:2 in distilled water, as a counterstain for AEC
- aqueous mounting media for coverslips
- polyethylene glycol (TWEEN 80)
- deionized or distilled water (dH₂O)

Protocol

1. Make touch impressions of suspect CNS tissues (e.g. brainstem and/or cerebellum) on labelled glass microscope slides (always include standard positive and negative controls). Note that antigen detection in any part of the CNS is indicative of a positive result, but a definitive negative diagnosis cannot be obtained without a full thickness cross-section of the brainstem, as with the DFAT.
2. Air-dry slides for ~ 5 min at room temperature (e.g. 25 °C).
3. Immerse slides in 10% buffered formalin at room temperature for 10 min in Coplin jars or other suitable containers to hold reagents (Fig. 12.1).

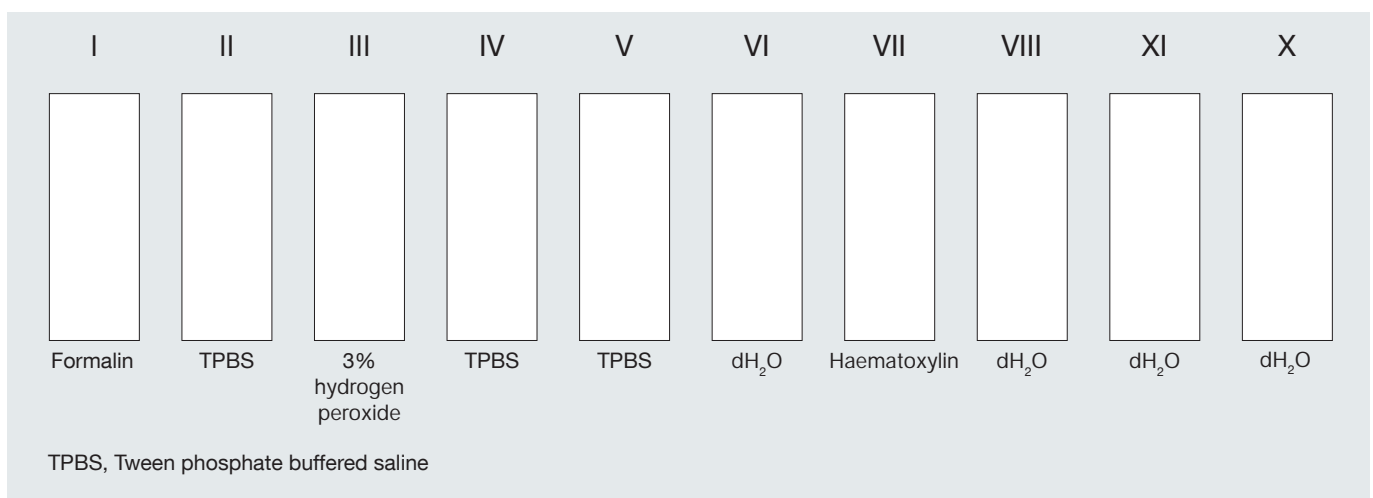


Fig. 12.1. Example of a staining dish array by reagents for use in the DRIT

4. Dip-rinse slides several times to wash off any excess fixative in wash buffer (PBS plus 1% Tween 80 [TPBS]).
5. Immerse slides in 3% H₂O₂ (hydrogen peroxide) for 10 min.
6. Remove excess hydrogen peroxide by dip-rinsing slides in TPBS. Transfer slides to another TPBS rinse. Work with one slide at a time (leave the remaining slides immersed within the TPBS), remove slide, shake off excess buffer, and blot excess buffer from slide edges surrounding the tissue impression.
7. Incubate slides at room temperature with primary antibody – e.g. biotinylated anti-rabies poly- or monoclonal antibodies – for 10 min (add enough of this primary antibody by drop to cover the impression) using a “humidity chamber” (e.g. place slides on a moistened paper towel and cover with the plastic top of a 96-well cell culture plate or another simple cover).
8. After incubation shake off excess conjugate. Dip-rinse slides with TPBS (shake off excess TPBS and blot buffer from slide edges surrounding the impression). Use this same wash buffer through step 10.
9. Incubate slides with streptavidin–peroxidase complex (add enough of this reagent to the slide to cover the impression) in the humidity chamber at room temperature for 10 min. After incubation, shake off excess liquid.
10. Dip-rinse slides with TPBS (shake off excess buffer and blot excess buffer from slide edges surrounding the impression).
11. Incubate slides with peroxidase substrate, e.g. AEC.
To prepare the AEC stock solution: dissolve one 20 mg tablet of 3-amino-9-ethylcarbazole in 5 mL of N,N-dimethylformamide in a glass vial or jar (label “AEC stock” and date). The AEC stock solution should be stored at 4 °C for ~ 1–2 months.
To prepare the AEC working dilution: add 7 mL of acetate buffer to a 15 mL centrifuge tube using a 10 mL plastic pipette. Add 0.5 mL of AEC stock solution (above) using a 1 mL glass or Pyrex pipette. Add 75 µL of 3% hydrogen peroxide. Filter using a 10 mL syringe with syringe nylon filter (0.45 µm) into a separate 15 mL tube. Once prepared, this mixture is only stable for 2–3 h, so it should be made just before use. Add enough of this reagent to the slide to cover the impression, then incubate in the humidity chamber at room temperature for 10 min. After incubation, shake off excess substrate.
12. Dip-rinse slides in deionized or distilled water.
13. Counterstain with diluted haematoxylin for 2 min.
14. Immediately dip-rinse stain from slides with deionized or distilled water. Make a second dip-rinse of slides with fresh deionized/distilled water to ensure removal of excess stain.
15. Transfer slides to fresh distilled water. Working with one slide at a time, shake off excess deionized/distilled water, blot excess from slide edges surrounding the impression, apply water-soluble mounting medium and cover-slip. Do not allow slides to air-dry before cover-slipping. If multiple slides are stained, they may stay in the deionized/distilled water rinse before cover-slipping.

16. View slides by light microscopy, using a 10x ocular and 20x objective lens to scan the field thoroughly, and a 40x (or higher) objective for inspection as needed. Negative CNS samples stain blue (Fig. 12.2). In positive CNS samples, lyssavirus antigens appear as reddish, intra-cytoplasmic inclusions against a blue neuronal background, using AEC and haematoxylin counterstain (Figs 12.3–4).

By courtesy of Charles E. Rupprecht, LYSSA LLSC, Atlanta, Georgia, USA

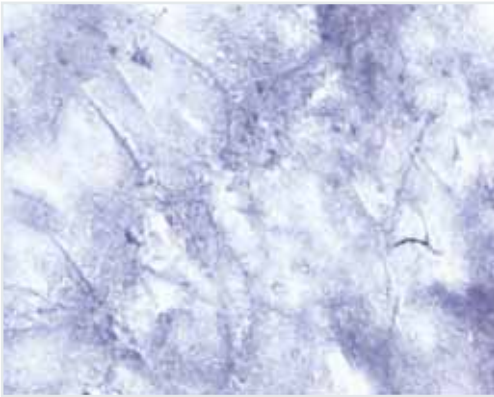


Fig. 12.2. Example of a Lyssavirus-negative CNS sample stained using the DRIT (~400x magnification)

By courtesy of Charles E. Rupprecht, LYSSA LLSC, Atlanta, Georgia, USA

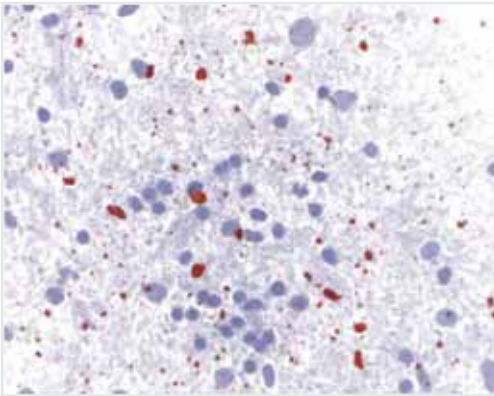


Fig. 12.3. Example of a Lyssavirus-positive CNS sample stained using the DRIT (~400x magnification)

By courtesy of Charles E. Rupprecht, LYSSA LLSC, Atlanta, Georgia, USA

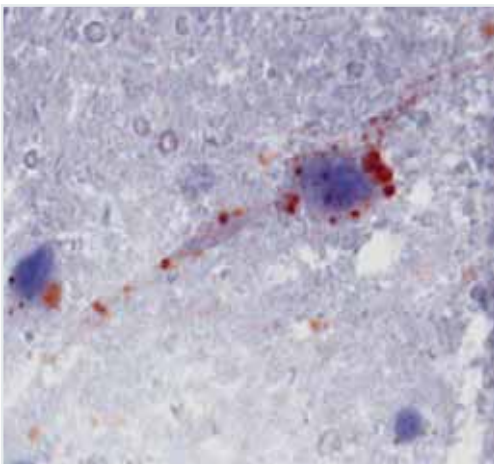


Fig. 12.4. Example of a Lyssavirus-positive CNS sample, displaying multiple stained inclusions within a neuron, using the DRIT (~600x magnification)

Discussion

Tissues may be selected for the DRIT in the same manner as that described in the chapters on brain removal ([Chapter 7](#)) and the DFAT ([Chapter 11](#)). Besides removal of the entire CNS or tissue selection via the foramen magnum using the straw technique, the brainstem can be accessed directly by a ventral approach, using a scalpel (Fig. 12.5). Lyssaviruses in the brains of infected animals produce intracytoplasmic inclusions of various shapes (Figs. 12.3–4). Negative samples do not have such specific inclusions (Fig. 12.2). A single microscopic field may contain numerous round or oval masses and irregular strings of antigen (Fig. 12.3). When stained specifically with biotinylated antibody, the substrate 3-amino-9-ethylcarbazole (AEC), upon oxidation, forms a rose-red end product and multiple inclusions may be observed within individual neurons (Fig. 12.4). Haematoxylin counterstain will produce a blue colour to CNS tissue and nuclear background (Fig. 12.2). The AEC-produced product is susceptible to deterioration in excessive light and will fade in intensity. Storage of DRIT-stained slides in the dark is recommended.

The DRIT detects the N antigens of all lyssaviruses for primary diagnosis. Either broadly reactive monoclonal or polyclonal antibodies may be conjugated to biotin for use in the DRIT. Alternatively, an indirect version of the DRIT may be utilized by using such primary, unconjugated antibodies, followed by an additional incubation of secondary antibodies (e.g. anti-mouse, anti-rabbit, anti-goat, etc. Ig), conjugated to biotin. When used with panels of monoclonal antibodies, characterization of antigenic variants is also possible (4). Conjugates for the DRIT may be obtained from WHO/OIE reference laboratories or they may be self-produced as described in the [chapter on conjugate production](#).



Fig. 12.5. Access to the brainstem of a dead suspect carnivore, directly from a full thickness ventral approach across the throat latch region using a scalpel only, for use in the DRIT or DFAT protocols (the caudal aspect of the carcass is at the top of the image and the ventral cranial aspect of the mandibles is at the bottom, partially concealed by a gloved hand).

References

1. Ang MJC, Llenaresas-Manalo D, Jarilla B, Tuason L, Avenido E, Demetria C, et al. Evaluation of direct rapid immunohistochemical test (DRIT) of *Canis lupus familiaris* hippocampal touch impression smears using a monospecific polyclonal antibody for rabies virus detection. *Acta Med Phil.* 2016; 50:1–5.
2. Coetzer A, Sabeta CT, Markotter W, Rupprecht CE, Nel LH. Comparison of biotinylated monoclonal and polyclonal antibodies in an evaluation of a direct rapid immunohistochemical test for the routine diagnosis of rabies in southern Africa. *PLoS Negl Trop Dis.* 2014;8:e3189. doi:10.1371/journal.pntd.0003189.
3. Coetzer A, Anahory I, Dias PT, Sabeta CT, Scott TP, Markotter W, et al. Enhanced diagnosis of rabies and molecular evidence for the transboundary spread of the disease in Mozambique. *J S Afr Vet Assoc.* 2017; 88:e1–e9. doi:10.4102/jsava.v88i0.1397.
4. Dyer JL, Niezgodna M, Orciari LA, Yager PA, Ellison JA, Rupprecht CE. Evaluation of an indirect rapid immunohistochemistry test for the differentiation of rabies virus variants. *J Virol Methods.* 2013; 190:29–33. doi:10.1016/j.jviromet.2013.03.009.
5. Dürr S, Naïssengar S, Mindekem R, Diguimbye C, Niezgodna M, Kuzmin I, et al. Rabies diagnosis for developing countries. *PLoS Negl Trop Dis.* 2008; 2(3):e206. doi:10.1371/journal.pntd.0000206.
6. Kirby JD, Chipman RB, Nelson KM, Rupprecht CE, Blanton JD, Algeo TP, Slate D. Enhanced rabies surveillance to support effective oral rabies vaccination of raccoons in the eastern United States. *Trop Med Infect Dis.* 2017; 2:34. doi:10.3390/tropicalmed2030034.
7. Lembo T, Niezgodna M, Velasco-Villa A, Cleaveland S, Ernest E, Rupprecht CE. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerg Infect Dis.* 2006; 12:310–3. doi:10.3201/eid1202.050812.
8. Madhusudana SN, Subha S, Thankappan U, Ashwin YB. Evaluation of a direct rapid immunohistochemical test (dRIT) for rapid diagnosis of rabies in animals and humans. *Virol Sin.* 2012; 27:299–302. doi:10.1007/s12250-012-3265-6.
9. Middel K, Fehlner-Gardiner C, Pulham N, Buchanan T. Incorporating direct rapid immunohistochemical testing into large-scale wildlife rabies surveillance. *Trop Med Infect Dis.* 2017; 2:21. doi:org/10.3390/tropicalmed2030021.
10. Rupprecht CE, Cliquet F, Fehlner-Gardiner C, Fooks, AR, Mueller T, Sabeta C, Slate D. Progress in the development of a direct rapid immunohistochemical test for diagnosing rabies. *Bulletin de l'OIE.* 2014; 3:87–95.
11. Saturday GA, King R, Fuhrmann L. Validation and operational application of a rapid method for rabies antigen detection. *US Army Med Dep J.* 2009;Jan–Mar:42–5. PMID:20088045.
12. Tao XY, Niezgodna M, Du JL, Li H, Wang XG, Huang Y, et al. [The primary application of direct rapid immunohistochemical test to rabies diagnosis in China]. *Chin J Exp Clin Virol.* 2008;22:168–70 (in Chinese). PMID:19031692.
13. Rupprecht CE, Xiang Z, Servat A, Franka R, Kirby J, Ertl HCJ. Additional progress in the development and application of a direct, rapid immunohistochemical test for rabies diagnosis. *Vet Sci.* 2018;5pii:E59. doi:10.3390/vetsci5020059.

Chapter 13

Immunohistochemistry

Introduction

Immunohistochemistry utilizes the principle of antigen–antibody reaction to identify specific antigens within tissue sections. Detection of antigen–antibody interactions is achieved by tagging the antibody with a substance that can be visualized, either by conjugation to a fluorescent marker or enzyme, followed by colourimetric detection using fluorescein or light microscopy. The technique of employing immunological detection of antigens dates back to the early 20th century, when Marrack demonstrated that anti-typhoid and anti-cholera sera labelled with diazotized benzidine-azo-R-salt imparted a red colour to bacteria (1). Although groundbreaking for immunological-based detection of antigens, Coons determined this labelling method to be relatively insensitive when applied to tissues, and subsequently described assays utilizing fluorescent labelled antibodies in fixed tissues, but interpretation was confounded by the enhanced endogenous fluorescent activity in formalin-fixed tissue (2–6).

In 1966, Nakane described a method of antigen detection in tissue using an antibody conjugated to an enzyme (horseradish peroxidase) and utilized a colourimetric substrate that could be detected by light microscopy, which is the theoretical basis of most modern tissue-based IHC assays (7). This chapter focuses on detection of rabies virus (RABV) antigens in formalin-fixed, paraffin-embedded (FFPE) tissues. The materials and methods describe a single protocol (8, 9). The reader should be aware of, and is encouraged to investigate, the myriad alternative nonproprietary and proprietary protocols available (10, 11). The detection of antigens in FFPE tissues presents a unique diagnostic challenge for the validation of the assay and interpretation of the results; the reader unfamiliar with this method should seek input from technologists and pathologists experienced with assay design and interpretation.

Materials

Reagents

- Mach 4 AP polymer system (Biocare Medical cat #M4U536 L)
- Mach 4 probe
- Mach 4 polymer
- background punisher (Biocare Medical cat#BP974 M)
- UltraClean diluent (Thermo Scientific cat#TA-125-UC)

- fast-red substrate system (Thermo Scientific cat#TA-125-AF)
- TRIS-buffered saline and Tween 20 (TST) (Thermo Scientific cat#TA-999-TT) or (Biocare Medical cat#TWB945M)
- modified Mayer's haematoxylin (Polyscientific cat#s216)
- lithium carbonate solution (Polysciences cat#44820-1)
- aqueous mounting medium (Polysciences cat#18606-500)
- charged glass slides and coverslips
- Reveal Decloaker 10X (Biocare Medical cat#RV1000 M)
- EDTA Decloaker 5X (Biocare Medical cat#CB917 M)
- Proteinase K (Sigma cat#03115879001)

Equipment

The staining procedure can be automated (Labvision, Biocare, Ventana, Dako) or performed manually. For automated staining, the reader should follow the manufacturer's recommended procedure. Manual staining requires basic histology equipment (such as slide racks, slide rack containers, Coplin jars), which is available from most scientific equipment suppliers.

Biological materials

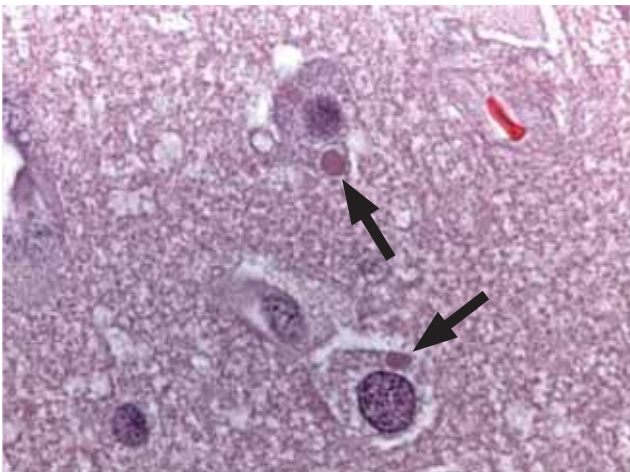
- FFPE test samples
- FFPE RABV-infected positive control tissue or infected cells
- FFPE non-infected negative control tissue
- additional FFPE tissue for assay validation (see critical parameters)
- miscellaneous material

Protocol

1. Place 3–5 μm FFPE tissue sections on charged slides in an oven and heat at 60 °C for 15 min.
2. Deparaffinize sections through two changes of xylene for 5 min, then 3 min and rehydrate through graded alcohols: absolute (2 min, then 1 min), 95% (1 min), 70% (1 min), then to distilled water.
3. Retrieve antigen (pre-treat slides/sections using Proteinase K Working Reagent (0.1 mg/mL in 0.6 mol TRIS/0.1% CaCl_2) for 15 min at room temperature or heat treatment optimized for antibody; see alternative materials and methods in discussion)
4. Rinse slides/sections in TST 20 twice.
5. Block in background punisher for 10 min at room temperature.
6. Blow excess background punisher off slides/sections (without rinse).
7. Apply primary antibody at designated dilution (in UltraClean Diluent) and incubate for 30 min at room temperature.

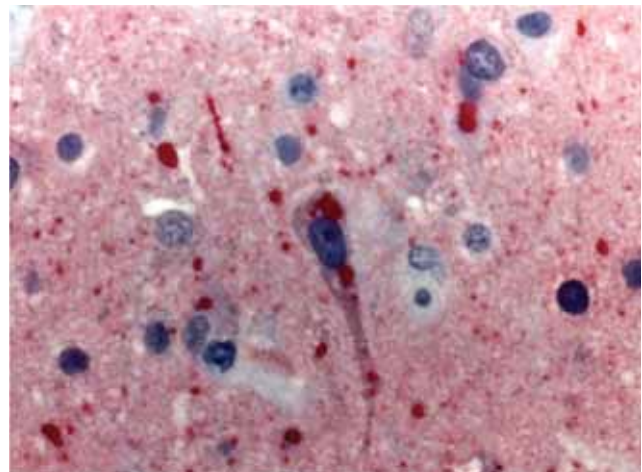
8. Rinse slides/sections in TST 20 three times.
9. Apply Mach 4 Universal AP Probe (for primary mouse antibodies) and incubate for 10 min at room temperature.
10. Rinse slides/sections in TST 20 three times.
11. Apply Mach 4 MR AP Polymer and incubate for 15 min at room temperature.
12. Rinse slides/sections in TST 20 twice.
13. Apply fast-red substrate working reagent and incubate for 20 min at room temperature.
14. Rinse slides/sections in deionized water.
15. Counterstain with modified Mayer's haematoxylin for 2 min at room temperature.
16. Wash in deionized water twice for 1 min each.
17. Immerse blue slides/sections in lithium carbonate solution for 1 min at room temperature.
18. Rinse in deionized water for 1 min.
19. Dry slides/sections in oven at 60 °C for a minimum of 5 min.
20. Mount slides/sections with a glass coverslip using aqueous mounting medium.

A positive result will show intense, granular or globular red cytoplasmic and axonal process staining of neurons and occasionally glial cells. Antigen will often co-localize with neuronal cytoplasmic inclusions (Negri bodies) or within the cytoplasm of neurons containing rabies viral antigen (Figs. 13.1– 2).



By courtesy of Anita Mahadevan, National Institute of Mental Health and Neurosciences, Bangalore, India and Wun-Ju Shieh, United States Centers for Disease Control and Prevention

Fig. 13.1. Haematoxylin and eosin stain of brain showing neurons with characteristic neuronal cytoplasmic inclusion (Negri) bodies (arrows)



By courtesy of Anita Mahadevan, National Institute of Mental Health and Neurosciences, Bangalore, India and Wun-Ju Shieh, United States Centers for Disease Control and Prevention

Fig. 13.2. Immunohistochemistry for RABV showing neuronal intracytoplasmic inclusion bodies co-localizing with RABV antigens (red)

Discussion

Critical parameters and troubleshooting

It is essential that the individual laboratory optimizes each step of the procedure with special attention paid to the primary antibody concentration, experimental conditions (e.g. antigen retrieval with enzyme vs heat) and the detection method employed (e.g. alkaline phosphatase vs horseradish peroxidase).

Primary antibody dilution testing is performed to determine the optimum dilution for the individual laboratory and experimental conditions. The primary antibody should be tested at dilutions more and less concentrated than the manufacturer's recommendation, and different antigen retrieval methods should be employed to determine the pre-treatment that provides intense staining with minimal, nonspecific background staining.

The optimized assay should be thoroughly characterized using normal and RABV-infected tissue, as well as tissue infected with non-rabies viral encephalitis (e.g. West Nile virus, Eastern equine virus) and non-infectious, inflammatory encephalitis. This is essential to determine the specificity, sensitivity and potential for nonspecific cross-reactivity.

Positive and negative controls should be performed for each experimental "run". Positive controls can be generated from: (i) FFPE RABV-infected cell pellets mixed with minced normal human tissues; or (ii) FFPE autopsy-derived tissues from RABV-infected patients. A single positive control for each run is adequate. The negative control is a replicate section of each FFPE block to be tested; treatment will be with an isotype matched antibody, non-sense hyperimmune mouse ascitic fluid, or matched non-immune sera, depending on the primary source of anti-RABV antibodies.

Precautions

Immunohistochemical staining must be interpreted in the context of the histopathology observed (Fig. 13.3) and characteristics or localization of the staining. Definitively identifying nonspecific staining is a critical point of interpretation and failure to do so will lead to false-positive results. For example, intense nonspecific cytoplasmic staining of plasma cells, ependymal lining cells and choroid plexus cells can be observed with some primary antibodies and staining may be misinterpreted as RABV antigen. Additionally, some prokaryotic (e.g. bacteria) and eukaryotic (e.g. protozoa, metazoan parasites) organisms can bind antibodies nonspecifically and show intense nonspecific staining.

Alternative materials and methods

Alternative protocols using various materials and methods are widely available and thoroughly described in reference (10). Notable differences in protocols revolve around different antigen retrieval techniques, antibody conjugated enzymes and substrates, and the accompanying modifications to materials and methods.

By courtesy of Anita Mahadevan, National Institute of Mental Health and Neurosciences, Bangalore, India and Wun-Ju Shieh, United States Centers for Disease Control and Prevention

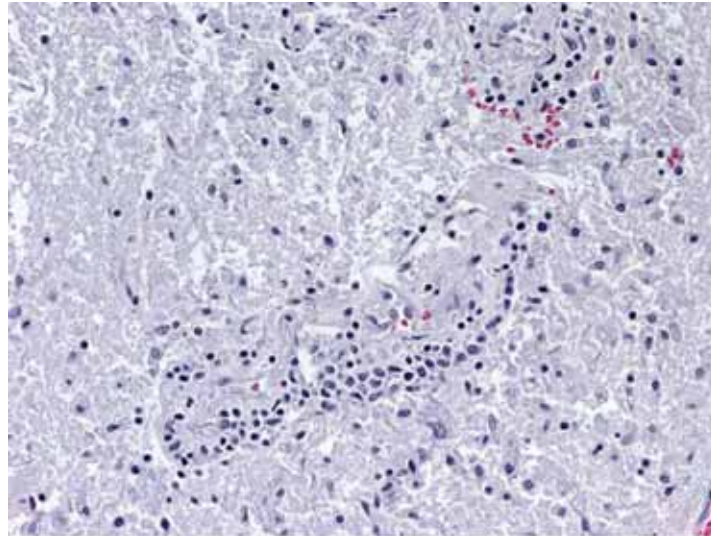


Fig. 13.3. Haematoxylin and eosin stain showing mixed mononuclear inflammation that is predominantly perivascular and gliosis in the brain of a patient with RABV infection

Time considerations

Immunohistochemical assays can generally be completed in a single day. The protocol can be modified to allow for overnight incubation of the primary antibody at 4 °C, usually with little modification to the protocol. It is essential to ensure that there is no drying out of sections if overnight incubation is performed.

Future considerations

Exposure to infectious agents and environmental contamination is a concern for diagnosticians and laboratories involved in testing tissue derived samples. RABV immunohistochemistry on FFPE tissues provides a safe mode of testing with essentially no risk of exposure to personnel.

Immunohistochemistry for RABV, coupled with histopathological evaluation and immunophenotyping, would together be a powerful tool to investigate the immunopathogenesis of RABV infection. Additionally, because these assays can be performed on archived tissue specimens, pathogenesis studies could be coupled with robust retrospective analysis, particularly if enzymatic methods of antibody detection are used, as these are more permanent than fluorescein-tagged preparations that tend to fade due to quenching of fluorescence preventing reviewing of slides.

Currently, a RABV immunohistochemistry diagnostic kit with fully characterized reagents is unavailable. Development and availability of such a kit would provide governmental and private health care laboratories with standardized reagents for screening biopsy and autopsy material from patients with suspect infectious encephalitides.

References

1. Marrack J. Nature of antibodies. *Nature*. 1934;133:292–93. doi:10.1038/133292b0.
2. Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. *Proc Soc Exp Biol Med*. 1941; 47:200–202. doi:10.3181/00379727-47-13084P.
3. Coons AH, Creech HJ, Jones RN, Berliner E. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J Immunol*. 1942; 45:159–70.
4. Coons AH, Kaplan MH. Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody. *J Exp Med*. 1950; 91:1–13. PMID:15395569.
5. Coons AH, Snyder JC, Cheever FS, Murray ES. Localization of antigen in tissue cells; antigens of rickettsiae and mumps virus. *J Exp Med*. 1950; 91:31–8.
6. Kaplan ME, Coons AH, Deane HW. Localization of antigen in tissue cells; cellular distribution of pneumococcal polysaccharides types II and III in the mouse. *J Exp Med*. 1950; 91:15–30.
7. Nakane PK, Pierce GB Jr. Enzyme-labeled antibodies: preparation and application for the localization of antigens. *J Histochem Cytochem*. 1966; 14:929–31.
8. Srinivasan A, Burton EC, Kuehnert MJ, Rupprecht C, Sutker WL, Ksiazek TG, et al. Transmission of rabies virus from an organ donor to four transplant recipients. *N Engl J Med*. 2005; 352:1103–11. doi:10.1056/NEJMoa043018.
9. Martines RB, Bhatnagar J, de Oliveira Ramos AM, Davi HP, Iglezias SD, Kanamura CT, et al. Pathology of congenital Zika syndrome in Brazil: a case series. *Lancet*. 2016; 388:898–904. doi:10.1016/S0140-6736(16)30883-2.
10. Kumar GL, Rudbeck L, editors. *Immunohistochemical staining methods*, 5th edition. Carpinteria: Dako; 2009.

Chapter 14

Antigenic typing of lyssaviruses by monoclonal antibodies

Introduction

Although rabies virus (RABV) is distributed globally with the exception of some insular territories, until the 1950s, when “rabies-related” lyssaviruses were discovered, the disease was considered a single antigenic entity. During the 1970s, other lyssaviruses were recognized by their reactivity with hyperimmune sera (1). After the discovery of hybridoma technology shortly thereafter and studies on the production of monoclonal antibodies (MAbs) against the nucleoprotein (N) of RABV, it became possible to identify viral variants via antigenic typing (2, 3). Such methods facilitated progress in epidemiological surveillance and research on lyssaviruses by using laboratory techniques applicable to both developed and developing countries.

The viral N protein has been the most commonly used antigen for serological typing of lyssaviruses, primarily because it is produced in high concentrations in infected brain tissue and it is the most conserved among the viral proteins. In combination with anti-N MAbs, Collaborating Centers of PAHO/WHO, such as the Canadian Food Inspection Agency and the Pasteur Institute in France, have used other proteins (such as the phosphoprotein [P] and glycoprotein [G]) as targets for MAb production and for antigenic typing (4–7).

In Africa and Eurasia, many studies were conducted with MAbs to recognize different RABV variants and improve the classification of the genus prior to molecular sequencing (4–6). In the Americas, different panels were used (7–10). Such MAbs were essential to improve diagnosis and recognition of the epidemiological situation for current control programmes in Latin America and the Caribbean. Recognition of the source of outbreaks of canine rabies and identification of wildlife species maintaining sylvatic cycles of RABV transmission allowed better utilization of targeted public health resources.

The importance of antigenic typing of RABV and other lyssaviruses by MAbs was incontestable and reflected in many studies in epidemiological concepts that have sometimes broken scientific “dogma” regarding the disease. Although modern genetic characterization and phylogenetic studies are well known today, antigenic typing by MAbs continues as a useful screening technique in several regions where rabies is still widespread and where alternative methods may not be readily available. In addition, the ease with which the test is performed, and the low cost of the technology used make it an attractive tool for epidemiological surveillance studies in developing countries.

Protocol

Indirect immunofluorescence is used to antigenically type RABV. The MAbs were applied to CNS impressions on slides or cultivated N2A cells that had been inoculated with street RABV isolates. The detection signal was obtained with FITC-labelled anti-mouse antibodies.

Preparation of the viral isolates for analysis with MAbs

Sample preparation in CNS tissue

A passage of street RABV isolates in mice or N2A cell culture can be performed before antigenic typing. The same procedures would be performed for fixed RABVs:

1. Weigh 0.5–1 g of the different fragments of the CNS samples, triturate in a sterile manner and add 2–4 mL of viral diluent, for preparing a 10–20% homogenate, as described.
2. Centrifuge the homogenate under cooling conditions at 3000 r/min (1400 *g*) for 30 min.
3. Remove the supernatant and keep it under refrigeration (2–8 °C) for intracerebral inoculation.
4. Perform the intracerebral inoculations with 3 µL per animal in suckling or weanling mice that weigh 11–14 g.
5. Prepare identification and readout cards of the inoculated samples.
6. Euthanize and collect brain from all the animals when they show signs of illness.

Slide preparation

1. Use marked slides suitable for immunofluorescence; identify slides with the sample number.
2. Gently place the CNS tissue on the slide, making two imprints of approximately 0.5 cm², being sure to include the brainstem and cerebellum, on the same slide, on the already demarcated end.
3. Prepare a sufficient number of slides to repeat the testing, if necessary.
4. Keep the slides at room temperature to air dry for 10–15 min.
5. Fix in acetone at –20 °C for 1–2 h or overnight.
6. Remove from acetone and dry for 10–15 min.
7. Store the slides at –20 °C until ready to use.

Sample preparation in cell culture

Preparation of CNS tissue homogenate

1. Prepare a 10–20% brain tissue homogenate – 0.6 g tissue and 2.4 mL of diluent.
2. Leave the selected antibiotic to be in contact with the homogenate for 1 h.

3. Centrifuge the tissue homogenate under refrigeration for 30 min at 3000 r/min (1400 *g*).
4. Separate the clarified tissue homogenate for inoculation into cell culture.

Preparation of cell suspension

5. Suspend N₂A cells at a concentration 5 x 10⁵ cells/mL in medium containing 10% fetal bovine serum (FBS), 30 µL of antibiotics (e.g. gentamycin) and 30 µL of essential amino acids.

Preparation of the cell culture plate

6. Inoculate 40 µL of sample suspension per well (for each sample use 2 or more wells).
7. Add 160 µL of culture medium (containing 30 µL of an antibiotic and 30µL of each amino acid to 10mL of medium prepared) and mix.
8. Add 100 µL of each cell suspension into each well.
9. Incubate the plate at 37 °C in a humidified chamber at 5% CO₂ incubator, for 48 h.
10. Remove the spent medium and fix cells in ice-cold 80% acetone, leaving for at least 15 min.
11. Discard the acetone appropriately.
12. Air dry the wells of the slide.

Both CNS tissue impressions and cell culture slides can be evaluated by the direct fluorescent antibody test (DFAT) to verify the concentration of antigen present. Ideally, the concentration of viral antigen should not cause 100% infectivity of cells or the tissue sections of the CNS because there can be reactions with the MAbs that interfere with the interpretation of the antigenic profile.

Titration of MAbs

- Typically, a panel of concentrated MAbs, diluted 1:10 in Eagle's minimum essential medium (EMEM), is received by the diagnostic laboratories. The working dilutions of these MAbs is approximately 1:1000, and it should be reassessed by each laboratory.
- Each of the MAbs is diluted 1:100 in EMEM (10% fetal bovine serum, 25 mmol HEPES buffer and sodium azide). From this stock solution, serial dilutions of each of the MAbs should be tested (e.g. 1:500; 1:1000 and 1:1500) by using slides prepared with fixed RABV (ERA [Evelyn Rokitniki Abelseth) or CVS [challenge virus standard) strains]) to determine the appropriate working dilution for each laboratory. This optimal working dilution should be established as the dilution at which the brightness is 3+ or 4+ for each MAb. Ideally, different street viruses should be used in place of laboratory strains once adequate experience is gained and the skill is obtained to differentiate local and regional antigenic variants.

Titration of conjugated anti-mouse immunoglobulin G

As with the MAb, the anti-mouse IgG conjugate should be titrated as well, using a guide based on the dilution recommended by the manufacturer.

Application of the technique

First step

1. Place 15 μL of the working dilution of each of the MAbs on each prepared section. The number of impressions needed will depend upon the number of MAbs in a panel.
2. Identify the slides with the sample number and each MAb (e.g. 1, 4, 9, 10, 12, 15, 18 and 19).
3. Incubate at 37 °C for 30 min in a humidified chamber (so that the impressions do not dry out).
4. Remove from the incubator and wash each slide twice using a wash bottle with buffered saline, 0.01 M, pH 7.6. This phase should be performed with utmost care to avoid transfer of a MAb of one section to another if using two or more per slide.
5. Keep slides submerged in buffered saline (0.01 M, pH 7.6) for 10 min and dry for the next step.

Second step

6. Place approximately 25–30 μL of anti-mouse IgG conjugate on each section, diluted according to the titre already established by the laboratory.
7. Incubate at 37 °C for 30 min.
8. Remove and wash as described above.
9. Remove the slides from the buffered saline and wash with distilled water.
10. Dry the slides and mount with buffered glycerol (pH 8.5) and coverslip.

The procedures for applying the IFA technique with CNS specimens can be used for cells grown in (Lab-Tek) chamber slides, Terasaki plates or microtitre plates, observing the same precautions for virus isolation in cell culture.

Interpretation of results

- Scoring is performed using a fluorescence microscope with 10 \times ocular and 40 \times objective lens, recording the reaction profiles.
- A positive (+) reaction is one that displays a fluorescence intensity at the working MAb dilution identical to that observed in the virus control.
- A negative (–) reaction is one that does not display any specific fluorescence with the working MAb dilution.
- A weak or weakly positive reaction is one in which the fluorescence intensity of the working MAb dilution is much lower than that observed in the homologous virus control. The reactions considered as weak or variable are indicated as a (v) (Table 14.1).

- When the antigenic profiles were established during the 1980s, the limited MAb panel was effective in the identification of more than 90% of the cases, because during this period there was a significant number of cases of canine rabies in Central and South America. Some viruses isolated from wild animals, despite presenting an antigenic profile named by many researchers as “non-compatible”, repeated frequently, when those isolated samples were analysed in a reduced MAb panel. For example, the variant isolated from most of the bats from the species *Histiotus velatus* presents a profile with positive reaction only for MAb C12. The RABV isolated from marmosets (*Callithrix jacchus*) presents a positive reaction profile only for MAbs C9 and C10.
- In Brazil, the wild canid variant (*Cerdocyon thous*) presents a profile similar to the variant 2, deriving from dogs; however, the MAb C1 does not react to these isolates or produces a weak reaction.
- Clearly, the reduced panel allows the identification of other RABV variants beyond the 11 identified initially (Table 14.2).

Table 14.1. Standard reaction profiles of different RABV antigenic variants with MAbs

Monoclonal antibodies	C1	C4	C9	C10	C12	C15	C18	C19	AgV
CVS/ERA-SAD/PAST	+	+	+	+	+	+	+	+	LabL Lab
Dog/mongoose	+	+	+	+	+	+	-	+	1
Dog	+	+	-	+	+	+	-	+	2
Vampire bat	-	+	+	+	+	-	-	+	3
<i>Tadarida brasiliensis</i>	-	+	+	+	+	-	-	-	4
Vampire bat (Venezuela)	-	+	v	+	+	v	-	v	5
<i>Lasiurus cinereus</i>	v	+	+	+	+	-	-	-	6
Arizona fox	+	+	+	-	+	+	-	+	7
Skunk southcentral	-	-	+	+	+	+	+	+	8
<i>Tadarida brasiliensis</i> (Mexico)	+	+	+	+	+	-	-	-	9
Skunk Baja SC	+	+	+	+	-	+	-	+	10
Vampire (other)	-	+	+	+	-	-	-	+	11

Table 14.2. Antigenic profiles established by the reduced panel of MAbs for RABV isolates of wild animals in Brazil

Monoclonal antibodies	C1	C4	C9	C10	C12	C15	C18	C19	AgV
CVS/ERA-SAD/PAST	+	+	+	+	+	+	+	+	Lab
<i>Cerdocyon thous</i>	-	+	-	+	+	+	-	+	?
<i>Histiotus velatus</i>	-	-	-	-	+	-	-	-	?
<i>Callithrix jacchus</i>	-	-	+	+	-	-	-	-	?

Discussion

Antigenic typing methods may be less technically demanding and less costly than molecular techniques and can thus be applied routinely to a large number of cases (9).

The sample slides can be used as impressions or prepared from N2A cells or from infected rodent brains. Use slides prepared with negative mouse brain, and brains of mice inoculated with a fixed RABV, as negative and positive profiles, respectively.

Slides prepared with original samples of the CNS, stored in good condition, may be used provided they show distribution of viral antigen in 75–100% of the fields surveyed, as assessed with the DFAT. Some MABs may produce variable results with original sections prepared from CNS samples, which interfere with the interpretation and definition of the antigenic profile.

The recommendation is that both stock and working MAB dilutions should be prepared using culture medium; however, if this medium is not available, dilutions can be prepared using buffered saline. If saline is used, the preservation of these reagents is at -20°C for the stock solution and between 4°C and 6°C for the working solution; these temperatures must be rigorously observed. Reagents prepared in buffered saline are less stable and must be prepared at least monthly.

Several studies have been conducted by the WHO/PAHO Collaborating Centers in an attempt to obtain a panel of MABs with increased discriminatory capacity (12, 13). Considering the host population and regional variants, a panel developed for studies in one area is not necessarily useful for application in other geographical areas.

Care must be taken when performing the antigenic typing tests with MABs. If the results of involving a new host suggest a variant with a known reaction profile or provide a profile incompatible with that defined by the standard panel, it is recommended that the reaction be repeated.

Proper preservation by refrigeration or freezing of fresh samples is necessary for antigenic typing.

Accurate species identification and recording of dates and location of collection related to each isolate is critical for a meaningful interpretation of resultant data.

Through its application to epidemiological research, classification based on MABs has brought many advances to the prevention and control of rabies. Among the major advances achieved by the use of MABs, several deserve special mention:

- a) The revelation of the diversity of lyssaviruses on the African continent and in Eurasia and their geographical distribution compared to the New World, where only RABV has been documented, was based initially on *Lyssavirus* MAB panels produced by North American and European reference laboratories working in collaboration. These results were confirmed much later by genetic sequencing (4, 6).

- b) The high variability of RABV in different species of bats, including vampires, was identified in a large number of retrospective surveys conducted in the Americas with panels of MAbs (14–19). These studies established the relevance of bats as reservoirs of RABV throughout both North and South America. Some panels continue to present limitations in characterizing isolates of insectivorous bats. In these cases, either additional MAbs or genetic typing should be obtained to identify possible new variants within a species or among species (20).
- c) Distinct species of wild carnivores, such as hoary foxes and crab-eating foxes, were recognized as reservoirs of RABV in the Americas (21, 22). These antigenic studies allowed differentiation from variants of canine origin. Of fundamental importance are the morphometric and/or genetic identifications of the species to which an isolate belongs to understand the epidemiological cycles which emerge and perpetuate.
- d) The presence of viral spillover from bats to domestic and wild carnivores was recognized, and knowledge was gained regarding the distribution of rabies on the African continent, Eurasia and the Americas. Moreover, a possible reservoir was identified in a non-human primate, the common marmoset (*Callithrix jacchus*) in Brazil (23). While the antigenic profile was not established easily by any single MAb or panel, the reactivity profile was maintained for more than 20 years (24).
- e) A *Desmodus rotundus* variant (AgV3) of vampire bats was identified. The reservoir is important throughout Latin America because of economic losses in livestock production and their impact on public health, especially in the Amazon Basin, as a transmitter to humans. A variant of the common vampire has also been found in domestic companion animals and wild animals; it is transmitted by *Desmodus rotundus* or fruit bats of the genus *Artibeus*, which have shown to host a similar variant (18, 25).
- f) Panels of MAbs and antigenic typing have been critical to the epidemiological investigation of areas free of canine rabies, by variants specific to this species, and also for the use of oral vaccines in the control of wildlife rabies by using such rapid techniques.
- g) Antigenic typing by MAbs has proven useful to identify human rabies by secondary cross-species transmission of bat → cat → human cases (26). It has been seen especially in Latin American countries such as Brazil (27), Colombia (28), Costa Rica (29) and Ecuador (30). The first cases of this transmission cycle occurred with identification of VA3 (Antigenic Variant 3) and more recently with antigenic variants of insectivorous bats in some countries, especially in the Andean region.
- h) The number of rabies cases in cats in some regions of the Americas has been greater than that in dogs and indicates the risk represented by wildlife, making it mandatory to monitor these animals and pets in rabies controlled urban areas, as well as through the practice of rapid diagnostic tests and the continuity of MAbs used for screening in control programmes. Evidently, the countries or regions that present the greatest difficulties in laboratory diagnosis should have reference laboratories that carry out deeper studies

on the identification of bats and other wildlife (morphometric or genetic) and viral strains (genetic studies).

In regions where dog rabies is in the final phase of control, it is imperative to identify the source of all cases of RABV infection in humans, dogs, cats and wildlife, based on laboratories that use standard techniques and reagents.

Clearly, many of our founding principles of rabies epidemiology were formulated based on antigenic typing studies (31).

To improve epidemiological surveillance in regions where further studies are needed to identify new reservoirs and lyssaviruses, production and selection of additional MAbs are necessary (32, 33). Most of the WHO Collaborating Centres have such MAbs for research collaboration.

Additional genetic studies should be performed via screening with MAbs, to identify new reservoirs and to understand the association of viral species (new or old) with new hosts, together with their distribution in time and space, and particularly the measure of similarity among virus isolates.

References

1. Shope RE, Murphy FA, Harrison AK, Causey OR, Kemp GE, Simpson DIH, et al. Two African viruses serologically and morphologically related to rabies virus. *J Virol.* 1970; 6:690–2. PMID:5530013.
2. Kohler G, Milstein C. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature.* 1975; 256:495–7.
3. Wiktor TJ, Koprowski H. Monoclonal antibodies against rabies virus produced by somatic cell hybridization: detection of antigenic variants. *Proc Natl Acad Sci U S A.* 1978; 75:3938–42. PMID:PMC392904.
4. Bingham J, Javangwe S, Sabeta CT, Wandeler AI, Nel LH. Report of isolations of unusual lyssaviruses (*rabies* and *Mokola virus*) identified retrospectively from Zimbabwe. *Tydskr S Afr vet Ver* 2001; 72:92–4.
5. King AA. Studies of the antigenic relationships of rabies and rabies-related viruses using antinucleocapsid monoclonal antibodies [dissertation]. Guildford (UK): University of Surrey; 1991.
6. Bourhy H, Kissi B, Lafon M, Sacramento D, Tordo N. Antigenic and molecular characterization of bat rabies in Europe. *J Clin Microbiol.* 1992; 30:2419–26. PMID:PMC265516.
7. Nadin-Davis SA, Huang W, Armstrong J, Casey GA, Bahloul C, Tordo N, et al. Antigenic and genetic divergence of rabies viruses from bat species indigenous to Canada. *Virus Res.* 2001; 74:139–56. PMID:11226582.
8. Smith JS, Reid Sanden FL, Roumillat LF, Trimarchi C, Clark K, Baer GM, et al. Demonstration of antigenic variation among rabies virus isolates by using monoclonal antibodies to nucleocapsid proteins. *J Clin Microbiol.* 1986; 24:573–80.
9. Nadin-Davis S. Molecular epidemiology. In: Jackson AC, Wunner WH, editors. *Rabies*, 2nd edition. San Diego: Academic Press; 2007:69–122.
10. Smith JS, Sumner JW, Roumillat LF. Enzyme immunoassay for rabies antibody in hybridoma culture fluids and its application to differentiation of street and laboratory strains of rabies virus. *J Clin Microbiol.* 1984;19:267–72 (<https://pdfs.semanticscholar.org/c695/90acf74e07d2360b5f5b3cf6a2be98eefff9.pdf>, accessed 27 July 2018).
11. Smith JS. Rabies virus epitopic variation: use in ecologic studies. *Adv Virus Res.* 1989; 36:215–53. doi:10.1016/S0065-3527(08)60586-2.
12. Diaz AM, Papo S, Rodriguez A, Smith JS. 1994. Antigenic analysis of rabies-virus isolates from Latin America and the Caribbean. *Zentralbl Veterinarmed B.* 1994; 41:153–60. PMID:7801717.
13. Smith JS, King AA. Monoclonal antibodies for the identification of rabies and non-rabies lyssaviruses. In: Meslin, F-X, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies*, 3rd edition. Geneva: World Health Organization; 1996:145–56.

14. Favoretto SR, Carrieri ML, Cunha EM, Aguiar EA, Silva LH, Sodre MM, et al. 2002. Antigenic typing of Brazilian rabies virus samples isolated from animals and humans, 1989–2000. *Rev Inst Med Trop São Paulo*. 2002; 44:91–5. PMID:12048546.
15. Cisterna D, Bonaventura R, Caillou S, Pozo O, Andreau ML, Fontana LD, et al. Antigenic and molecular characterization of rabies virus in Argentina. *Virus Res*. 2005; 109:139–47. doi:10.1016/j.virusres.2004.10.013.
16. Yung V, Favi M, Fernandez J. Genetic and antigenic typing of rabies virus in Chile. *Arch Virol*. 2002; 147:2197–205. doi:10.1007/s00705-002-0894-3.
17. Favi M, de Mattos CA, Yung V, Chala E, Lopez LR, de Mattos CC. First case of human rabies in Chile caused by an insectivorous bat virus variant. *Emerg Infect Dis*. 2002; 8:79–81. doi:10.3201/eid0801.010108.
18. Delpietro HA, Gury-Dhomen F, Larghi OP, Mena-Segura C, Abramo L. Monoclonal antibody characterization of rabies virus strains isolated in the River Plate Basin. *Zentralbl Veterinarmed B*. 1997; 44:477–83. doi:10.1111/j.1439-0450.1997.tb00998.
19. Velasco-Villa A, Gómez-Sierra M, Hernández-Rodríguez G, Juárez-Islas V, Meléndez-Félix A, Vargas-Pino F, et al. Antigenic diversity and distribution of rabies virus in Mexico. *J Clin Microbiol*. 2002; 40:951–8. PMID:PMC120240.
20. Kuzmin IV, Tordo N. Genus *Lyssavirus*. In: Dietzgen RG, Kuzmin IV editors. *Rhabdoviruses: molecular taxonomy, evolution, genomics, ecology, host-vector, interactions, cytopathology and control*. Norfolk (UK): Carster Academic Press; 2012:37–57.
21. Bernardi F, Nadin Davis S, Wandeler AI, Armstrong J, Gomes AAB, Lima FS, et al. Antigenic and genetic characterization of rabies viruses isolates from domestic and wild animals of Brazil identifies the hoary fox as a rabies reservoir. *J Gen Virol*. 2005; 86:3153–62. doi:10.1099/vir.0.81223-0.
22. Carnieli Jr P, Brandão PE, Carrieri ML, Castilho JG, Macedo CI, Machado LM, et al. Molecular epidemiology of rabies virus strains isolated from wild canids in Northeastern Brazil. *Virus Res*. 2006; 120:113–20. doi:10.1016/j.virusres.2006.02.007.
23. Favoretto SR, De Mattos CC, Morais NB, Araújo FAA, Mattos CA. Rabies in marmosets (*Callithrix jacchus*), Ceará, Brazil. *Emerg Infect Dis*. 2001; 7:1062–5.
24. Kotait I, Carrieri ML, Castilho JG, Montebello L, Oliveira RC. Emergence of rabies in non-human primates in Brazil. In: *Rabies in the Americas (RITA), 2008, Atlanta, USA. XIX International Conference on Rabies in the Americas*; 2008:72 (<https://drive.google.com/file/d/0B3qyU7XS47AjYmJkODVhNDQtZjEwMi-00Mzk1LWE2MjMtNDgxODMyMTI5NTM4/view?hl=en>, accessed 20 July 2018).
25. Fahl WO, Carnieli Jr P, Castilho JG, Carrieri ML, Kotait I, Iamamoto L, et al. *Desmodus rotundus* and *Artibeus* spp. bats might present distinct rabies virus lineages. *Braz J Infect Dis*. 2012; 16:545–51. doi:10.1016/j.bjid.2012.07.002.

26. Kotait I, Carrieri ML, Rupprecht CE. Secondary transmission of rabies in Latin America. In: Rabies in the Americas (RITA), 2012, São Paulo, Brasil. XXIII International Conference on Rabies in the Americas; 2012:32.
27. Kotait I, Carrieri ML, Favoretto S, Takaoka NY. [Human rabies caused by variant 3 *Desmodus rotundus* in the State of São Paulo, Brazil]. In: Rabies in the Americas (RIBA), 2002, Oaxaca, México. XIII International Conference on Rabies in the Americas; 2002. (in Portuguese).
28. Paez A, Polo L, Heredia D, Nuñez C, Rodriguez M, Agudelo C, et al. [An outbreak of human rabies transmitted by a cat in the town of Santander de Quilichao, Colombia]. Rev Salud Publica (Bogota). 2008;11:931–43 (in Spanish).
29. Badilla X, Pérez-Herra V, Quirós L, Morice A, Jiménez E, Sáenz E, et al. Human rabies: a reemerging disease in Costa Rica? Emerg Infect Dis. 2003; 9:72. doi:10.3201/eid0906.020632.
30. Mantilla A, Benevides W, Castilho JG, Carrieri ML, Kotait I. Human rabies in Ecuador transmission by felines infected by variant 3 of the rabies virus. In: Rabies in the Americas (RITA), 2010, Guadalajara, México. XXI International Meeting on Rabies in the Americas; 2010:39.
31. Rupprecht CE, Dietzschold B, Wunner WH, Koprowski H. Antigenic relationships of lyssavirus. In: Baer GM, editor. The natural history of rabies. Boca Raton: CRC Press; 1991:69–100.
32. Nadin-Davis SA, Fehlner-Gardiner C, Sheen M, Wandeler AI. Characterization of a panel of anti-phosphoprotein monoclonal antibodies generated against the raccoon strain of rabies virus. Virus Res. 2010; 152:126–36.
33. Nadin-Davis SA, Elmgren L, Sheen M, Sabeta C, Wandeler AI. Generation and characterization of a panel of anti-phosphoprotein monoclonal antibodies directed against Mokola virus. Virus Res. 2011; 160:238–45.

Annex. Materials and solutions

Materials

Reagents

Panel of MAbs, usually prepared against the N, G, or P proteins of rabies or other lyssaviruses; mouse anti-IgG antibody conjugated with fluorescein. This chapter describes a technique with a panel of eight MAbs (C1, C4, C9, C10, C12, C15, C18 and C19) that is used to differentiate existing variants in the Americas, as an example.

Equipment

- fluorescence microscope
- CO₂ incubator
- analytical balance
- refrigerator
- freezer –20 °C
- biological safety cabinets
- timer

Biological materials

- samples of CNS tissue from humans and several animal species that are positive for RABV, fixed virus strains of ERA or CVS (positive control in cell culture)
- negative samples of CNS tissues (negative control)
- laboratory mice cell lines, such as of murine neuroblastoma (N2A).

Solutions

Saline solution 0.85%

- | | | |
|--|--|---------|
| • NaCl | | 8.5 g |
| • distilled or deionized or purified water | | 1000 mL |

Saline buffered with sodium phosphate

- | | | |
|---|--|---------|
| • Na ₂ HPO ₄ · 12H ₂ O | | 2.65 g |
| • NaH ₂ PO ₄ | | 0.36 g |
| • NaCl | | 8.17 g |
| • distilled or deionized or purified water | | 1000 mL |

Saline buffered with potassium phosphate

- | | | |
|-----------------------------------|--|--------|
| • K ₂ HPO ₄ | | 1.45 g |
| • KH ₂ PO ₄ | | 0.22 g |

- NaCl 8.50 g
- distilled or deionized or purified water 1000 mL

Buffered carbonate/bicarbonate (pH 9.5)

- sodium carbonate 0.5 mol 10 mL
- sodium bicarbonate 0.5 mol 13 mL

Sodium carbonate 0.5 mol

- Na₂CO₃ anhydrous 5.3 g
- distilled or deionized or purified water 100 mL

Sodium bicarbonate 0.5 mol

- NaHCO₃ 4.2 g
- distilled or deionized or purified water 100 mL

Buffered glycerol

- glycerin PA 9 mL
- buffered carbonate/bicarbonate 0.5 mol 1 mL

Acetone 80%

- acetone PA 80 mL
- distilled or deionized or purified water 20 mL

Diluent for virus

- normal rabbit or equine serum 2 mL
- gentamicin sulfate 40 mg 1 mL
- saline solution 0.85% 97 mL

or

- normal rabbit or equine serum 2 mL
- penicillin 50 000 IU/streptomycin 200 mg 1 mL
- saline solution 0.85% 97 mL

Chapter 15

Use of a rapid skin biopsy technique for human rabies antemortem diagnosis

Introduction

Human rabies may be suspected after the onset of compatible clinical signs and confirmed by detection of agent-specific immunological and molecular biomarkers, using sensitive and specific diagnostic laboratory testing. A history of a suspect animal exposure is often a strong indicator of rabies in the differential diagnosis of acute progressive encephalitis, but the absence of a bite does not rule out the disease necessarily (1). Specific diagnostic algorithms have been developed for physicians in selecting priority differentials in encephalitis cases, but concomitant laboratory diagnosis remains critical for conclusive identification of the etiological cause (2).

Laboratory diagnosis may be conducted after death to confirm a suspect case, or while the person is ill. Antemortem testing may allow definitive identification of pathogens in the ill patient, while facilitating the identification of other persons who may have been exposed to the same source. Additionally, early diagnosis of rabies is critical for the institution of proper barrier methods to prevent secondary exposures to excreted virus (in saliva, respiratory secretions and tears for example); for proper risk assessment and prophylaxis of patient contacts, psychological resolution and closure for family and friends as to etiology, institution and monitoring of experimental therapeutic interventions as appropriate, initiation of grief counselling and preparations for a fatal aftermath of illness if confirmed; and for continued diagnostic evaluation, if the laboratory findings are negative after robust attempts.

Brain tissue is the ideal material for diagnosis of rabies in the laboratory. Often, collection of brain biopsy samples for antemortem testing is contraindicated, because of serious and unstable patient status. However, since rabies virus (RABV) and other lyssaviruses move centrifugally after replication of CNS, virus, antigens and nucleic acids may be detected in other areas and tissues, such as saliva and skin. Given the unique pathobiology of rabies, a combination of multiple tests conducted in parallel on different tissue samples such as serum, CSF, skin biopsy and saliva (and, if safe, to collect also brain biopsy), are necessary to increase specificity and negative predictive value of rabies laboratory testing and to provide definitive diagnosis. The objective of this chapter is to describe a rapid, sensitive and specific procedure for the detection of RABV (or other lyssavirus) antigens in a skin biopsy, using the direct fluorescent antibody test (DFAT).

Materials and methods

Equipment and supplies

Biosafety. Ideally, a Class 2 biosafety cabinet is recommended for testing of samples from an encephalitic patient. Universal precautions are also considered an essential safety measure when working with a patient suspected of an encephalitis of unknown etiology. Cryostat chambers should be regularly cleaned and disinfected as unused tissue sections are frozen and considered infectious. The knife may be disinfected by wetting the surface with absolute methanol. Knives that are removed from the cryostat should be housed in cases designed for safety. Before storing a cold knife, wet the blade with absolute ethanol or methanol to disinfect and remove any moisture that would accumulate on the cold metal.

Cryostat. Any commercial self-contained microtome suitable for the cutting of frozen histological specimens may be suitable.

Necropsy instruments. Two scalpels or, alternatively, a scalpel and forceps, should be used for preparing horizontal and vertical skin sections. It is recommended that one set of tools per sample is used to prevent cross-transfer of infected tissue between samples.

Autoclave and/or instrument sterilizer. All instruments should be cleaned and sterilized before re-use by using an autoclave or instrument sterilizer.

Specimen storage containers. Due to the risk of breakage, glass vials are unacceptable for storage of specimens. Wide mouth, screw cap, polypropylene jars (0.25–1.0 oz) or sample bottles are used in many laboratories.

Refrigerated storage. An explosion-proof freezer at $-20\text{ }^{\circ}\text{C}$ is required for fixation of slides and storage of acetone and other reagents. Long-term sample and control storage requires a freezer at $-70\text{ }^{\circ}\text{C}$. Frost-free freezers should not be used. Heat cycles in frost-free freezers can denature proteins in reagents and specimens and may compromise test results.

Microscope slides. These should be of the highest quality with a coverslip matched to the working distance of the microscope lens. The PTFE printed slides with two wells are preferable, allowing for determination of staining surface and conjugate conservation, as compared to slides without wells. Marking instruments that contact tissue (e.g. wax pencil or [Martex] pen) should not be used to denote stained regions of the slides, because this process can transfer infected tissue between slides.

Acetone fixation and post-stain rinse containers. These should be of adequate quality and in sufficient quantity. Plastic Coplin staining jars with lids are preferable.

Syringe filters (0.45 μm). Anti-RABV conjugates should be filtered to remove dissociated fluorescein isothiocyanate (FITC) and protein aggregates that may bind nonspecifically to tissue. Conjugates should be filtered only once and should be evaluated for the effect of filtration on the titre of the working dilution. Syringe filters allow the conjugates to be filtered as they are added to test slides. Similarly, syringe filters will remove contaminants and precipitates as mountants are added to slides. Filters for the conjugate must be low protein binding (e.g. cellu-

lose acetate) to prevent loss of FITC-labelled antibody from the conjugate. Filter materials that bind proteins with great avidity (e.g. mixed cellulose esters) should not be used for conjugate filtration. Both large volume, mid-volume and small volume filters are available for consideration. Smaller volume filters avoid dead volume loss to the filter membrane. An Evans Blue counterstain also binds to the membrane, but the filter is saturated quickly. To avoid slide to slide differences in counter stain, laboratories that use Evans Blue counterstain in the conjugate should discard the first three drops of conjugate expressed through the filter.

Incubator (37 °C) and humidified staining tray or chamber. Constant humidity must be maintained during the antibody staining process. Conjugate dried on the slides during the staining process may be mistaken for specific staining, resulting in a false-positive test, or may obscure specific staining, resulting in a false-negative test.

Fluorescence microscope. As discussed in [Chapter 11 on the DFAT](#), the quality of the fluorescence microscope is critical to the sensitivity of the skin biopsy test. Manufacturers offer many equipment options. At a minimum, all rabies diagnostic laboratories should have a reflected light (incident light) fluorescence microscope with high-quality objective lenses. Both magnification and numerical aperture (NA) must be considered in lens selection. Although image size increases with magnification, both resolution and image brightness are related to the NA of the objective lens, and brightness decreases with magnification. For example, a high quality 20x dry objective with a NA of 0.75 provides a brighter image over a larger field of view with no loss of resolution than a dry 40x objective with an NA of 0.75. The use of immersion oil increases image brightness by preventing the loss of emitted light in the airspace between coverslip and dry objective. Although not every slide must be observed with a 40x oil objective of high NA, resolution of very fine dust-like inclusions and recognition of some types of nonspecific staining is aided by examination with this type of lens. An oil immersion lens requires a high-quality immersion oil. An oil should be chosen that produces the least autofluorescence and thus the best contrast between FITC and tissue. The oil should have the same refractive index as glass.

Acetone. Only “reagent-grade” fixative should be employed, and the acetone should not be reused.

FITC-conjugated anti-RABV antibodies. Both polyclonal RABV-specific FITC-labelled and monoclonal antibody conjugates may be used. It should be predetermined that the conjugate is panspecific for any potential RABV variants or other lyssaviruses, especially if the suspect individual contracted rabies in an area foreign to the diagnostic laboratory. The conjugates are diluted in PBS + Evans Blue to prepare serial working dilutions of 1:5 and higher as appropriate per conjugate titrations on known positive and negative skin biopsy test material.

FITC-conjugated negative control conjugate. Although optional, a FITC-labelled conjugate that is specific to an antigen other than RABV (e.g. FITC-labelled anti-distemper reagent) should be considered. The specificity control reagent for an FITC-labelled polyclonal rabies reagent is an FITC labelled serum produced in the same animal host as the rabies reagent (i.e. typically goat or horse). The control reagent should be diluted to the same mg/ml protein concentration as the rabies reagent. Similarly, the specificity control for a rabies reagent prepared from a mouse monoclonal antibody is an FITC-labelled mouse monoclonal antibody of

the same isotype and protein concentration as the rabies reagent but directed to an antigen other than RABV.

Conjugate diluent. The conjugate diluent should be 0.01M phosphate buffered isotonic saline solution at pH 7.4–7.6. No protein stabilizer (e.g. bovine serum albumin) is needed in the diluent.

Counterstains. Counterstains are added to the working dilution of the conjugate to provide contrast and lower background and also to serve as a marker for accidental omission of the diagnostic reagent. Counterstain use is optional but valuable. A stock of Evans Blue counterstain (0.5% in PBS, for use in DFAT assays) can be aliquoted and stored at +4 °C for up to 6 months and indefinitely at –20 °C. The amount of counterstain added to a conjugate is determined by titration when the working dilution of the conjugate is determined. Due to counterstain the tissue, when examined under a fluorescence microscope, will be noticeably red, but should not be so strongly red as to diminish the specific apple-green fluorescence of RABV antigens. An Evans Blue concentration of 0.00125% works in many laboratories; it is prepared by adding 2.5 µL of 0.5% stock dye solution per mL of conjugate diluent.

Rinse–soak buffer. A PBS formula of the same pH and molarity as the conjugate diluent is used as the rinse–soak buffer (e.g. 0.01 mol phosphate buffer, pH 7.4, with 0.138 mol NaCl and 0.0027 mol KCl). Carboys or other large containers used for storage and dispensing of rinse buffers should be cleaned and disinfected by autoclaving on a regular schedule.

Mountant (0.05 mol TRIS-buffered saline pH 9.0 with 20% glycerol). Prepare 0.05 mol TRIS/0.15 mol NaCl solution by dissolving 0.623 g of pre-set crystals and 0.85 g NaCl in a total volume of 100 mL distilled water. Filter (0.45 µm) and store at 4 °C. Remake at least once per year and check pH quarterly. Prepare a one-month supply of mountant by mixing four parts TRIS–saline pH 9.0 with one part glycerol. Store at room temperature. Glycerol concentrations above 20% affect the antigen binding capacity of some antibodies and should not be used. Glycerol should be replaced at yearly intervals, as the pH changes slowly with time. The mountant should be remade or the pH tested monthly. The mountant is added to the coverslip by dispensing with a syringe fitted with a 0.45 µm syringe filter.

Immersion oil. This should be formulated specifically for fluorescence microscopy applications, as other immersion oils formulated for general microscopy may produce significant auto-fluorescence.

Protocol

Skin biopsy

For the DFAT of the skin, a biopsy of ~5–6 mm in diameter should be collected from the posterior region of the neck at the hairline. The biopsy specimen should contain a minimum of at least 10 hair follicles and be of sufficient depth to include the cutaneous nerves at the base of the follicle. Biopsies with a diameter of under less than 6 mm should be sent to the laboratory in pairs: one biopsy will be used for cryostat sectioning and the other used for RT-PCR and viral isolation. Rather

than requiring surgery the sample may be prepared as a punch biopsy, as is frequently used in dermatological settings (3). A punch biopsy is conducted using a circular blade ranging in size from 1 mm to 8 mm. The blade, which is attached to a pencil-like handle, is rotated down through the epidermis and dermis and into the subcutaneous fat, producing a cylindrical core of tissue (Fig. 15.1). Just before performing the biopsy, the lines of least skin tension are determined. The skin is stretched $\sim 90^\circ$ perpendicular to the lines of least skin tension using the non-dominant hand. The punch biopsy is removed. Following relaxation of the distending hand, the wound may have an elliptical shape that may heal by second intention or can be closed with sutures parallel to the lines of least skin tension. Some punch biopsies are shaped like an ellipse, although the same desired shape can be accomplished with a standard scalpel. Larger biopsies may require sutures.

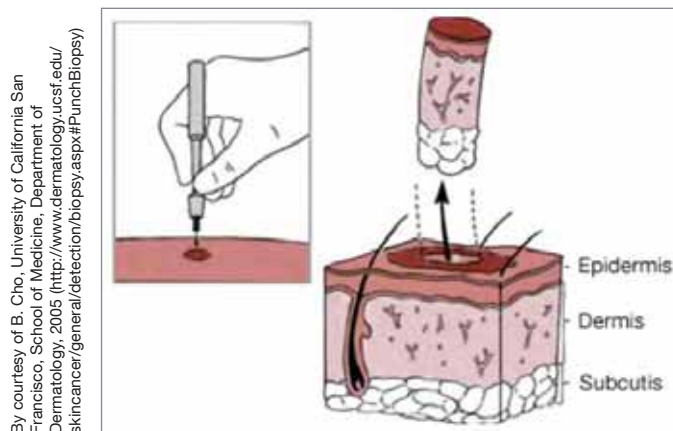


Fig. 15.1. Schematic representation of a full-thickness, circular punch skin biopsy

The specimen should be placed on a piece of sterile gauze moistened with sterile saline and deposited in a sealed container. No preservatives or additional fluids should be added. Fixatives such as formalin or alcohols may reduce or eliminate the avidity of the antigen–antibody reaction in the DFAT and this could potentially result in a false–negative result. Laboratory tests to be performed on this specimen will include the DFAT for viral antigens in frozen sections of the biopsy and in certain laboratories RT-PCR to detect viral nucleotide sequences.

All samples collected for rabies diagnosis should be considered as potentially infectious. Test tubes and other sample containers must be securely sealed (tape around the cap will ensure that the containers do not open during transit). If immediate shipment is not possible, samples should be stored frozen at -20°C or below. Ideally, samples should be shipped frozen on dry ice by an overnight courier in water-tight primary containers and leak-proof secondary containers that meet the guidelines of the International Air Transport Association and the OIE (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.03_TRANSPORT.pdf).

Tissue preparation

All processing of the skin biopsy sample is performed in a biosafety hood. Before processing of the skin biopsy sample for cryostat sectioning, adipose tissue (as well as a part of the dermis with follicles) must be carefully collected for other tests, such as RT-PCR (to avoid any cross-contamination). Once a sample

for amplification is collected, areas rich in hair follicles are selected for further processing. A sterile scalpel is used to cut blocks of tissue from the specimen.

Prepare a block on a horizontal plane of the skin biopsy (dermis and follicles face the skin surface) and vertical plane block (along hair follicles) of the tissue. Use all or as much of the specimen as needed. Selected tissue blocks should be ~3–5 mm in thickness. Once prepared, tissue blocks are placed into optimal cutting temperature (OCT) mounting media for approximately 30 s.

A cryostat implement or “chuck” is placed on dry ice in a plastic beaker, to avoid breakage. If the cryostat model contains a freezing shelf or specimen holder/deep freezing device (e.g. Peltier cooling element), this may be employed to freeze the OCT compound and tissue to the cryostat chuck. A small amount of OCT compound is poured on the cryostat chuck. It is important to avoid trapping gas bubbles in the OCT media, as these will interfere with section cutting. The OCT will start to freeze onto the chuck (turning white). Immediately thereafter, both horizontal and vertical cuts of tissue prepared from the specimen are placed on top of the rapidly freezing mounting media, next to each other. Wait for the OCT to begin turning white before placing the tissue blocks onto the chuck, as placing the tissue too close to the metal chuck may impair sectioning. Place the prepared specimen blocks on the partially frozen OCT, trying to keep the specimens on the same plane as the OCT (top) for optimum sectioning (if sections are below one other, they will not cut uniformly). Embed the tissue specimen in the OCT by adding additional OCT to the tissue as it freezes, making certain the dermal–epidermal junction is parallel with the chuck surface for the horizontal cut and at a 90-degree angle to the chuck surface (vertical cut through hair follicles) for a secondary cut.

Once the OCT is allowed to freeze entirely, the chuck/specimen is placed in the cryostat object clamp. For sectioning skin the cryostat should be maintained at a temperature between -21°C and -25°C . If the specimen is placed on the chuck using the dry ice technique, it is important to allow the chuck/specimen to equilibrate to the cryostat temperature before cutting sections as the block at -80°C may be too brittle for sectioning. It is highly recommended that this technique be practised numerous times on negative tissue to ensure success with a valuable diagnostic sample. Practise with positive animal skin is suggested to gain experience with visualization of rabies antigen in situ. Vibrissae from the snout of rabid animals are excellent specimens for visualizing rabies antigen associated with hair follicles and dermal nervous tissue.

Sectioning the embedded specimen

The specimen chuck is secured to the object clamp of the cryostat and the knife adjusted and squared with the specimen for uniformity in sectioning. The specimen should be faced to expose the tissue by manually advancing the specimen through the cutting plane of the knife in gradual increments that will remove unwanted tissue/OCT. Removing increments of OCT or specimen that are too large may dislodge them from the chuck. Serial frozen sections are cut at 6–8 μm and placed on red or white PTFE printed slides with two wells. Cut sections adhere by flash–thaw to the warm slide. As the knife separates the section from the OCT block the section will curl away from the OCT block. Position the warm slide so that the frozen section contacts the well of the microscope slide. Consi-

derable caution is required at this step as the operator's hand will be close to the sharp cutting blade. Sections that expose the root hair plexus (Fig. 15.2) are optimal. Slides should be numbered consecutively and properly labelled with the identification number, date of testing and origin of the sample. Approximately 16 slides (each with two sections) are prepared from each sample. Slides need to be air-dried for 10–15 min before fixation. Two of 16 slides (the last two) are stored typically as a backup, or as a negative control for future tests (if the sample tests negative).

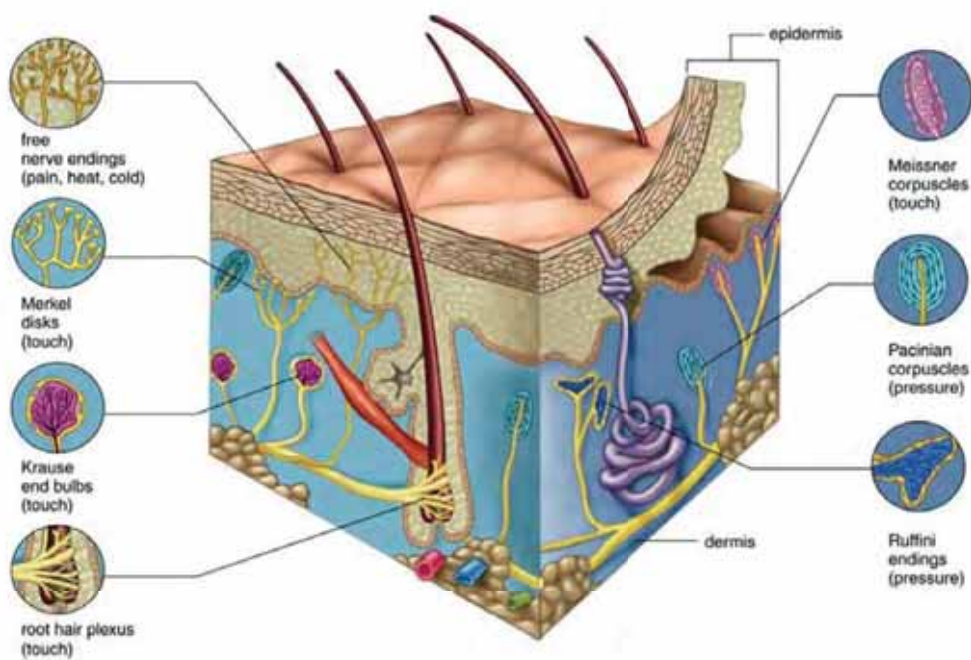


Fig. 15.2. Schematic representation of skin, identifying nerves

Control slides

Control slides are preferably either skin samples from the posterior region of the neck at the hairline from human rabies cases or skin with tactile whiskers from the facial area from rabid animals (e.g. nonhuman primates, foxes, skunks, raccoons, hamsters, mice, rats, ferrets from other rabies experiments). Control slides are prepared in the same manner as test slides. Positive and negative control slides are fixed in acetone at the same time as test slides to control for the effect of acetone fixation on test performance. Control skin material for slide preparation should be retained for this purpose from animals naturally infected with the most common variant of RABV. Control slides are stored unfixed and frozen at $-20\text{ }^{\circ}\text{C}$ for up to one month or at $-70\text{ }^{\circ}\text{C}$ for up to one year.

Acetone fixation

After air-drying the slides are fixed in acetone for 60 min at $-20\text{ }^{\circ}\text{C}$.

The slides are air dried for 5–10 min to remove the acetone. Approximately 14 slides are placed into a humidity chamber (two rows, each seven slides plus a positive control) ordered with slides numbered 1, 3, 5, 7, 9, 11 and 13 for one

conjugate (polyclonal) staining and 2, 4, 6, 8, 10, 12 and 14 for a second conjugate (monoclonal) staining. A positive control slide has one section (one well) for each conjugate.

Staining

Based on prior conjugate titration and working dilution determination, FITC anti-rabies monoclonal, polyclonal antibodies and negative control reagents are diluted in PBS + Evans Blue to prepare working dilutions. The FITC-labelled antibodies are filtered through a 0.45µm filter and applied onto the fixed slides (one or two drops per well). Slides are covered in a humid chamber and incubated for 30 min at 37 °C in the incubator. Thereafter, slides are rinsed twice for 5 min each in PBS and covered with coverslips using glycerol/saline medium.

Conjugate titration

Conjugate titration should be performed according to standard protocol for the DFAT (see [Chapter 11](#)).

Briefly:

1. Prepare 2-fold dilutions (e.g. 1:5, 1:10, 1:20, 1:40) of the conjugates and react two or more positive and negative skin biopsy sections with each dilution. At least two technologists should independently read the titration. The last dilution providing crisp 4+, apple-green fluorescence with minimal background fluorescence is the end-point dilution of the reagent.
2. Determine more precise working dilutions by preparing limited dilutions of the conjugate (around the end-point dilution from step 1) and react two or more positive and negative skin biopsy sections with each dilution. For example, if the end-point dilution of the titration is 1:80, the conjugate should be retested at dilutions of 1:50, 1:60, 1:70, 1:80, 1:90 and 1:100. At least two technologists should independently read the titration. The last dilution demonstrating crisp 4+ fluorescence and detecting 100% of the RABV antigens is the end-point dilution of the reagent. The working dilution of conjugate should be two steps more concentrated than the end-point. For example, if the end-point dilution of the conjugate is 1:80, the working dilution should be 1:60. This is to ensure that a conjugate of maximum avidity to all RABV variants is employed.

Because antigen presentation and antibody avidity and affinity vary with different lyssaviruses, and viral particles may appear quite different when stained with different reagents, titration results should be confirmed by reactions with skin sections prepared from the most frequently occurring RABV variants in the area, especially variants implicated in human cases. Although positive control slides made from a single source are adequate for day-to-day test controls, an accurate reagent evaluation requires observations on multiple variants. Control material for conjugate titrations should be also retained for this purpose from animals infected naturally or experimentally with a variant that is genetically diverse from the most common local variant. For example, canine RABV should be used in countries enzootic for rabies of dogs. Conversely, laboratories in the eastern United States might want to titrate conjugates for recognition of RABV-infected raccoons and retest the working dilution by comparison to staining of a RABV bat variant.

If adequate tissue is unavailable locally, material may be obtained from either a WHO or OIE reference laboratory. Control material should be stored as aliquots to avoid repeated freeze–thaw cycles and maintained at -70°C or below.

Microscopic interpretation

Each slide and each section are thoroughly read for the presence of apple-green bright fluorescent staining of inclusions. Localization in the root hair plexus around hair follicles (see Figs. 15.2–3) is characteristic of RABV antigens, but antigens could also be detected in the skin as numerous sensory nerves are found in dermal tissues (Fig. 15.2). Any specific fluorescent staining of dust-like particles is considered positive for the presence of RABV antigens. Additional sections from specimens presenting staining that appear as positive should be stained with a negative control to confirm the specificity of the diagnostic conjugates to RABV antigens as opposed to possible artifacts that could produce a false-positive interpretation. Examination of consecutive sections from the same tissue is valuable when validating the presence of RABV antigens in nervous tissue.

By courtesy of Michael Niezgoda, United States Centers for Disease Control and Prevention

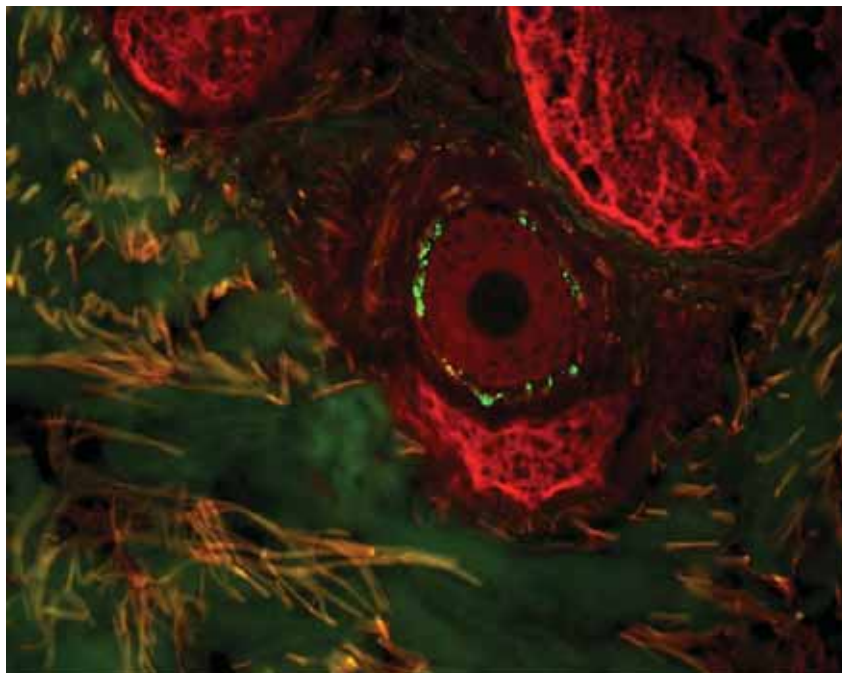


Fig. 15.3. Cryostat section and detection of rabies virus antigens in a skin biopsy from a rabid human patient (400x magnification)

The nerve path can be traced through contiguous sections by visualizing rabies antigen in the same nerve trunk seen in these sections.

Discussion

The DFAT on skin biopsy samples is one of the most important tests in the antemortem diagnosis of rabies in encephalitic human patients (Figs. 15.4–14). Its basis is rooted in the essential pathobiological nature of RABV and its tissue tropisms (4–7). The technique was developed initially in several experimental animal rabies models (8, 9). In the United States, it became a routine laboratory method included in human rabies antemortem tests in the later quarter of the 20th century, to date (10, 11). Over the years, it has been implemented around the globe for the rapid detection of lyssavirus antigens in suspect human rabies patients (12–22).

Although useful, especially for antemortem testing, the skin biopsy technique remains less reliable than the use of CNS tissue. After the consideration of brain tissue as a biopsy in the DFAT, the skin biopsy procedure is a rapid technique, allowing results within a few hours on the same day of sample acquisition. The selected skin area at the hairline of the nape of the neck must have several follicles included. A full thickness biopsy is necessary to contain all of the innervated tissues. No surgery is necessary to obtain a sample. Rather, as in routine dermatological procedures, a punch biopsy may be used in a shaven area above the hairline at the nape of the neck. The sample is snap frozen at -80°C or colder in a sterile container, with no fluid or fixative added before laboratory shipment. Care is needed in the sectioning of the biopsy and its particular orientation in the cryostat. The goal is to examine cross sections of hair follicles at the root hair plexus. Incidentally, numerous other sensory nerves may be transected and in positive cases RABV antigens can also be visualized by the DFAT in these nerves.

It is recommended that individuals lacking experience with cryostat sectioning establish experience on RABV negative tissues. A punch biopsy for rabies diagnosis can easily be wasted on errors. Issues that complicate success in cryostat sectioning include:

- a) Improperly sharpened microtome blades (consider purchase of a microtome blade sharpener or have the blade professionally sharpened). Knives that are not sharp enough will tear the specimen, resulting in poor sections.
- b) Embedded tissues that have been left in the cryostat for days, allowing the OCT to desiccate. Sectioning freshly prepared specimens is essential, as partially desiccated OCT does not allow proper sectioning of the specimen.
- c) Maintain proper cutting temperature of the specimen. If the cryostat is left open for extended periods of time during sectioning (more than 5 min) the specimen block will warm up, reducing the success rate for obtaining proper sections.
- d) Cutting to the proper depth in the tissue. It is recommended to check if you have cut to the correct depth by observing with a microscope the slide containing a section you have cut. Air-dry the section and, without mountant, view the section on low magnification. Examine for the presence of hair shafts



By courtesy of Robert Ruedd, New York Department of Health, USA
and Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 15.4. Suitable biopsy tool



By courtesy of Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 15.5. Nuchal skin and biopsy tool



By courtesy of Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 15.6. Three adequate sized biopsies



By courtesy of Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 15.7. Cryostat interior with freezing shelf



By courtesy of Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 15.8. Tissue freezing medium



By courtesy of Charles E. Rupprecht, LYSSA LLC, Atlanta, Georgia, USA

Fig. 15.9. Applying freezing medium to the cryostat chuck



Fig. 15.10. Partially frozen medium (correct time to add biopsy)



Fig. 15.11. Biopsies placed into the partially frozen medium



Fig. 15.12. Thoroughly frozen specimen on the chuck



Fig. 15.13. Advancing the specimen through the cryostat blade while teasing the sections from the frozen block to position the section onto the microscope slide



Fig. 15.14. Transferring the frozen section onto the room temperature microscope slide

and, more specifically, the thickness of the hair follicle. Ideally, the section will be through the hair bulb at the base of the hair follicle where the root hair nerve plexus is located.

The presence and detectability of RABV antigens in peripheral tissues follows prior viral replication in the CNS. However, generation of robust humoral immune responses following centrifugal virus spread, especially in cases of implementation of aggressive therapies and extended morbidity period, leads over time to an increase in circulating RABV-specific antibodies, with subsequent clearance from some peripheral tissues. Hence, over time, a skin biopsy sample may become negative, even in a patient with rabies. Additionally, not all biopsy samples will be positive for viral antigens, based upon viral tropisms. Serial sampling may be necessary.

Given the unique pathobiology of rabies, it is recommended that in addition to the DFAT testing of skin biopsy samples, additional laboratory methods are used to detect viral amplicons in saliva, skin and other tissues, as well as detection of viral antibodies in serum and CSF. Such techniques performed in parallel will increase diagnostic sensitivity and specificity, and the collective negative predictive values obtained thereof will provide more conclusive interpretation of laboratory results in the antemortem diagnosis of human rabies.

References

1. Feder HM Jr, Petersen BW, Robertson KL, Rupprecht CE. Rabies: still a uniformly fatal disease? Historical occurrence, epidemiological trends, and paradigm shifts. *Curr Infect Dis Rep.* 2012; 14:408–22. doi:10.1007/s11908-012-0268-2.
2. Meynard JB, Flamand C, Dupuy C, Mahamat A, Eltges F, Queuche F, et al. International encephalitis consortium. Case definitions, diagnostic algorithms, and priorities in encephalitis: consensus statement of the international encephalitis consortium. *Clin Infect Dis.* 2013; 57:1114–28.
3. Thomas JZ. Punch biopsy of the skin. Saginaw Cooperative Hospital, Saginaw, Michigan. *Am Fam Physician.* 2002; 65:1155–8.
4. Schneider LG, Hamann I. [The rabies pathogenesis in mice. 3. Centrifugal virus spread and virus generalisation in the organism]. *Zentralbl Bakteriolog Orig.* 1969; 212:13–41 (in German).
5. Fischman HR, Schaeffer M. Pathogenesis of experimental rabies as revealed by immunofluorescence. *Ann N Y Acad Sci.* 1971; 177:78–97.
6. Murphy FA, Harrison AK, Winn WC, Bauer SP. Comparative pathogenesis of rabies and rabies-like viruses: infection of the central nervous system and centrifugal spread of virus to peripheral tissues. *Lab Invest.* 1973; 29:1–16. PMID:4580585.
7. Lafon M. Subversive neuroinvasive strategy of rabies virus. *Arch Virol Suppl.* 2004; 18:149–59. PMID:15119770.

8. Smith WB, Blenden DC, Fuh TH, Hiler L. Diagnosis of rabies by immunofluorescent staining of frozen sections of skin. *J Am Vet Med Assoc.* 1972; 161:1495–501.
9. Blenden DC, Bell JF, Tsao AT, Umoh JU. Immunofluorescent examination of the skin of rabies-infected animals as a means of early detection of rabies virus antigen. *J Clin Microbiol.* 1983; 18:631–6.
10. Anderson LJ, Nicholson KG, Tauxe RV, Winkler WG. Human rabies in the United States, 1960 to 1979: epidemiology, diagnosis, and prevention. *Ann Intern Med.* 1984; 100:728–35.
11. Noah DL, Drenzek CL, Smith JS, Krebs JW, Orciari L, Shaddock J, et al. Epidemiology of human rabies in the United States, 1980 to 1996. *Ann Intern Med.* 1998; 128:922–30. PMID:9634432.
12. Bryceson AD, Greenwood BM, Warrell DA, Davidson NM, Pope HM, Lawrie JH, et al. Demonstration during life of rabies antigen in humans. *J Infect Dis.* 1975; 131:71–4. PMID:1089129.
13. Warrell MJ, Looareesuwan S, Manatsathit S, White NJ, Phuapradit P, Vejajiva A, et al. Rapid diagnosis of rabies and post-vaccinal encephalitides. *Clin Exp Immunol.* 1988;71:229–34. PMID:2894908. Erratum in: *Clin Exp Immunol.* 1988; 72:521.
14. Bingham J, Mlambo P. Ante-mortem diagnosis of human rabies by the skin biopsy technique: three case reports from Zimbabwe. *Cent Afr J Med.* 1995; 41:258–60. PMID:7585915.
15. Gdalevich M, Mimouni D, Ashkenazi I, Shemer J. Rabies in Israel: decades of prevention and a human case. *Public Health.* 2000; 114:484–7.
16. Soun VV, Eidson M, Wallace BJ, Drabkin PD, Jones G, Leach R, et al. Ante-mortem diagnosis of New York human rabies case and review of U.S. cases. *Int J Biomed Sci.* 2006; 2:434–45.
17. Perez AL, Werchniak AE. Antemortem diagnosis of rabies via nuchal skin biopsy. *Arch Dermatol.* 2007; 143:663.
18. Dacheux L, Reynes JM, Buchy P, Sivuth O, Diop BM, Rousset D, et al. A reliable diagnosis of human rabies based on analysis of skin biopsy specimens. *Clin Infect Dis.* 2008; 47:1410–7. doi:10.1086/592969.
19. Krieger N, Bourhy H, Spiegel A. First human rabies case in French Guiana, 2008: epidemiological investigation and control. *PLoS Negl Trop Dis.* 2012; 6:e1537. doi:10.1371/journal.pntd.0001537.
20. Imported human rabies in a U.S. Army soldier – New York, 2011. *MMWR.* 2012; 61:302–5.
21. Schupbach A, Zhang L, Thomas B, Ellerbroek J, Leone D. Fatal human rabies infection confirmed with skin biopsy specimen. *J Am Acad Dermatol.* 2012; 67:321–3.
22. Human rabies – Wyoming and Utah, 2015. *MMWR.* 2016; 65:529–33.

Chapter 16

Demonstration of Lyssavirus antigens by flow cytometry

Introduction

The nature of infection with rabies virus (RABV) dictates a rapid, sensitive, specific and efficient diagnosis, which is of utmost relevance to the guidance of decision-makers to administer PEP to the patient and the execution of epidemiological measures to control potential epidemics.

Different techniques are available for diagnosis of rabies in animal and human samples (1, 2). Among the techniques, immunofluorescence and immunohistochemistry (i.e. the identification of antigens and inclusions in the samples) are the first choices due to their feasibility and cost, although both methods may present a number of false-negative results that necessitate the use of confirmatory tests (3). The mouse inoculation test (MIT), virus isolation in cell culture and reverse transcriptase polymerase chain reaction (RT-PCR) are some of the other techniques that may be used for this purpose (4, 5). However, ethical issues and the time required to obtain results have greatly reduced the use of MIT. Otherwise, RT-PCR has the advantage of being fast, inexpensive and exhibiting a sensitivity comparable to that of the MIT (2-6). Moreover, RT-PCR does not allow for the isolation of the viral strain, which can be achieved with techniques that employ cell cultures. Thus, viral isolation in cell culture has been demonstrated to be an appropriate alternative for the confirmation of RABV infection (2).

Several cell lines have already been described as susceptible to RABV infection, which makes them suitable for use in diagnosis (7-10). However, isolation of RABV in cell culture usually requires a few days (3-5 days) to allow for the detection of viral antigens by immunofluorescence or any other technique (2, 4). Fluorescence-activated cell sorting (FACS) is an automated technique that is used for the analyses of cells in suspension (11). This method can substantially reduce the time required for diagnosis (12). Through a system using laser beams, optical means, software and fluorescent dyes, this technique allows for the detection and quantification of multiple antigens in cells. This technique is fast, easy to perform and reproducible, and it allows for the simultaneous analyses of many samples, which represents an important advantage for routine laboratories. Additionally, because the analysis of the fluorescence is automated, the inter-human variation in the results is drastically reduced.

The use of FACS in virology is well established, and the quantifications of infected cells or viral antigens in infected cells have already been shown for several viruses including dengue, rotavirus and HIV (13-18). FACS allows the detection and quantification of RABV antigens in infected cells using a laser that excites the fluorophore conjugated to the RABV antibody and thus enables the detection of different RABV antigens, such as ribonucleoproteins or glycoproteins, depending

on the specificity of the conjugate used (12, 19). FACS has additionally been used for the detection and quantification of RABV-neutralizing antibodies (20, 21).

A protocol similar to that described below can be easily adapted to perform the quantification of antibodies by simply adding some steps and using a control serum with a predefined neutralizing antibody titre. Recently, a Vero cell line expressing the RABV glycoprotein (G) on the cell membrane was used as an alternative method for the detection and quantification of antibodies targeting the G protein with the advantage of not requiring the handling of the infectious RABV (19). Because the FACS reduces the time required for a confirmatory rabies diagnosis, the routine use of this technique could help to guide both medical and epidemiological measurements, prevent the use of unnecessary human vaccinations and help public health authorities in the establishment of actions to avoid epidemics.

Protocol

The detection of lyssavirus antigens in cell cultures by FACS is similar to a traditional immunofluorescence assay using fluorescence microscopy.

Briefly:

1. The cells are infected with a sample of a virus stock or a suspension of tissue samples suspected of infection and, after a period of incubation, intracellular staining for the virus antigens is performed.
2. Different cells lines, such as BHK-21 (C13) (ATCC CCL10), C6 (ATCC, CCL107) and murine neuroblastoma cells (MNA), have already been used for rabies virus detection by FACS (12, 19).
3. BHK-21 and C6 cell lines were cultured in Dulbecco's modified Eagle's medium (F-12; Sigma-Aldrich) supplemented with glutamine (2 mmol; Thermo Fisher), fetal calf serum (10%; Sigma-Aldrich), penicillin/streptomycin (100 IU/ μ g/mL; Thermo Fisher) and amphotericin B (0.25/ μ g per mL).
4. To determine the sensitivity of the technique, it is imperative that the kinetics of infection be examined for the selected cell line, preferentially using a laboratory strain (in our case, Pasteur virus, PV) and a street rabies virus (WRS; in our case, one isolated from a naturally infected cow).
5. The cells were plated in 24-well dishes overnight (3.0×10^5 cells/well) and infected with 1 CCID₅₀ of PV or WRS for 1 h at 37 °C in a 5% CO₂ atmosphere. The cells were then washed once with PBS, and 1 mL of fresh medium was added to each well.
6. At the indicated time points (Fig. 16.1), the cells were harvested for detection of intracellular nucleoprotein rabies virus antigen.
7. For intracellular staining, the cell culture medium was discarded, the wells were washed once with 1x PBS, and the cells were detached using trypsin-EDTA (0.05%; Sigma-Aldrich). The cell suspension of each well was washed twice (with PBS and 0.05% Tween-20), and the cells were permeabilized using a 1:1 (v/v) ratio of 4% p-formaldehyde and FACS lysis solution (BD Biosciences) for 10 min. The cell suspensions were washed again as previously described and incubated with anti-rabies virus nucleo-

protein polyclonal FITC-conjugated antibodies in PBS for 45 min at 37 °C in the dark. The cells were then washed again, recovered in Coulter Isoton II, and stored at 4 °C in the dark until flow cytometric analysis (12).

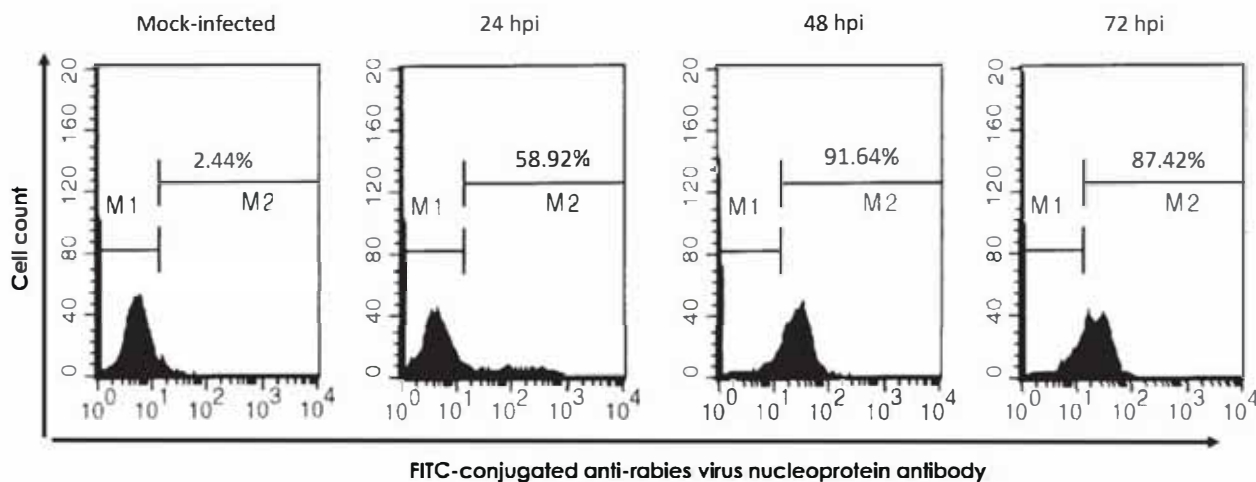


Fig. 16.1. Histograms showing the kinetics of the infection of rat glioma cells (C6) by a rabies virus isolated from a naturally infected cow. The cells were infected with 1 CCID₅₀ of the sample, and the quantification of the C6-infected cells was performed with the flow cytometry described in the protocol. A FITC-conjugated anti-rabies virus nucleoprotein (polyclonal antibody) was used for antigen detection in a FACS Calibur (12)

The protocol described herein has proven effective for detection of intracellular RABV antigen in the BHK-21 and C6 cell lines. However, some changes and new commercial reagents that have lately become available could improve the technique and results. For example, in the protocol described for the detection of RABV antigens in MNA cells, the authors of the study adopted some changes that could be advantageous (20). The number of cells was adjusted to 2.5×10^6 cells/mL before staining, and the cells were washed twice with cold PBS (1.000 r/min for 5 min at 4 °C). The cells were then incubated for 20 min at 4 °C with 100 μ L of Cytofix/Cytoperm solution (BD Biosciences, USA). After new washings (twice) in the staining solution (1% bovine serum albumin, 0.01% sodium azide and 0.1% saponin in PBS), the cells were incubated with RABV anti-nucleocapsid antibody that was FITC-conjugated (in the staining solution) for 20 min in the dark. Finally, the cells were washed again, and the final pellet was suspended in 500 μ L of FACS flow buffer (BD Biosciences) and stored at 4 °C until flow cytometry was performed. When the samples were to be analysed immediately, PBS could be used to recover the cell pellets. Otherwise, when the FACS analyses were performed later (3–5 days), a buffer containing 1.5% p-formaldehyde was used, and the samples needed to be protected from light and maintained at 4 °C.

Researchers interested in the use of FACS to detect and quantify intracellular lyssavirus antigens should always be aware that the sensitivity to the virus differs not only between different cell lineages but also with the number of passages of each lineage (7, 8, 10, 22). Standardization in local conditions is advisable.

Data analysis after cell acquisition on the flow cytometer may be performed by software that integrates the equipment (such as CellQuest on the FACS Calibur or BD FACSDiva™ Software-BD Biosciences) or commercially available software, (such as WinList™, FlowJo, LLC, and Cyflogic). Using the software of choice, a gate containing the cells should be defined based on the size (forward scatter) and complexity (side scatter) of the cell population. After defining the cell population to be analysed, controls for cell auto-fluorescence (using non-stained cells) and isotype controls (using unrelated antibodies conjugated with the same fluorophore of the specific antibody) should be performed. Both of these controls are essential for excluding the cell auto-fluorescence and the unspecific binding of antibodies to the cell surface. After these steps, which are necessary for the equipment set-up, are performed, the infected and non-infected cells can be acquired to determine a gate that differentiates the two cell populations (at least 10 000 events should be counted). Once all of these steps have been completed, FACS can now be used for the different possibilities, i.e. aiding the diagnostic routine or basic research purposes. It is important to emphasize that the FACS analysis detects not only infected cells but can determine also the number of infected cells and estimate the viral load in each cell using mean fluorescence intensity analysis.

References

1. Diagnosis in animals and humans. In: Rabies [website]. Atlanta (GA): United States Centers for Diseases Control and Prevention; 2018 (<https://www.cdc.gov/rabies/diagnosis/animals-humans.html>, accessed 22 May 2018).
2. Doung V, Tarantola A, Ong S, Mey C, Choueng R, Ly S, et al. Laboratory diagnostics in dog-mediated rabies: an overview of performance and a proposal strategy for various settings. *Int J Infect Dis*. 2016; 46:107–14. doi:10.1016/j.ijid.2016.03.016.
3. Sing R, Sing KP, Cherian S, Saminathan M, Kapoor S, Reddy GBM, et al. Rabies – epidemiology, pathogenesis, public health concerns and advances in diagnosis and control: a comprehensive review. *Vet Quart*. 2017; 37:212–51. doi:10.1080/01652176.2017.1343516.
4. Woldehiwet Z. Clinical laboratory advances in the detection of rabies virus. *Clinica Chimica Acta*. 2005; 351:49–63. doi:10.1016/j.cccn.2004.09.018.
5. Sacramento D, Bourhy H, Tordo N. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. *Mol Cell Probes*. 1991; 5:229–40. PMID:1714538.
6. Araújo DB, Langoni H, Almeida MF, Megid J. Heminested reverse-transcriptase polymerase chain reaction (hnRT-PCR) as a tool for rabies virus detection in stored and decomposed samples. *BMC Res Notes*. 2008;1:17. doi:10.1186/1756-0500-1-17.
7. Bordignon J, Piza AT, Alvarez-Silva M, Caporale GM, Carrieri ML, Kotait I, et al. Isolation and replication of rabies virus in C6 rat glioma cells (clone CCI-107). *Biol*. 2001; 29:67–73. doi:10.1006/biol.2001.0278.
8. Rudd RJ, Trimarchi CV. Comparison of sensitivity of BHK-21 and murine neuroblastoma cells in the isolation of a street strain rabies virus. *Journal of Clin Microbiol*. 1987; 25:1456–8. PMID:3305560.
9. Consales CA, Mendonca RZ, Gallina NM, Pereira CA. Cytophatic effect induced by rabies virus in McCoy cells. *J Virol Meth*. 1990; 27:277–85. PMID:1691201.
10. Smith AL, Tignor GH, Emmons RW, Woodie JD. Isolation of field rabies virus strains in CER and murine neuroblastoma cell cultures. *Intervirol*. 1978; 9:359–61. PMID:344267.
11. Jensen BD, Horan PK. Flow cytometry: rapid isolation and analysis of single cells. *Meth Enzym*. 1989; 171:549–81. PMID:2480506.
12. Bordignon J, Pires SC, Caporale GMM, Carrieri ML, Kotait I, Lima HC, et al. Flow cytometry assay for intracellular rabies virus detection. *J Virol Meth*. 2002; 105:181–6. PMID:12176155.
13. Abad FX, Bosh A, Comas J, Villalba D, Pinto RM. Evaluation of flow cytometry for the monitoring of infectious human rotavirus in water. *Water Sci Technol*. 1997; 35:451–53. doi:10.1016/S0273-1223(97)00302-8.

14. Barardi CR, Emsile KR, Vesey G, Williams KL. Development of a rapid and sensitive quantitative assay for rotavirus based on flow cytometry. *J Virol Meth.* 1998; 74:31–8. PMID:9763126.
15. McSharry JJ. Analysis of virus-infected cells by flow cytometry. *Methods.* 2000; 21:249–57. doi:10.1006/mth.2000.1005.
16. Kao CL, Wu MC, Chiu YH, Lin JL, Wu YC, Yueh YY, et al. Flow cytometry compared with indirect immunofluorescence for rapid detection of dengue virus type 1 after amplification in tissue culture. *J Clin Microbiol.* 2001; 39:3672–7. doi:10.1128/JCM.39.10.3672-3677.2001.
17. McSharry JJ, Costantino R, Robbiano E, Echols R, Stevens R, Lehman JM. Detection and quantification of human immunodeficiency virus-infected peripheral blood mononuclear cells by flow cytometry. *J Clin Microbiol.* 1990; 28:724–33. PMID:1970576.
18. Yang G, Olson JC, Pu R, Vyas GN. Flow cytometric detection of human immunodeficiency virus type 1 proviral DNA by the polymerase chain reaction incorporating digoxigenin- or fluorescein-labeled dUTP. *Cytometry.* 1995; 21:197–202. doi:10.1002/cyto.990210212.
19. Fontana D, Prieto C, Kratje R, Etcheverrigaray M. Target cells for antibodies detection in rabies vaccine control. *Vaccine.* 2014; 32:2805–7. doi:10.1016/j.vaccine.2014.02.030.
20. Vengatesan D, Raj GD, Raja A, Ramadass P, Gunaseelan L. Detection of rabies virus antigen or antibody using flow cytometry. *Cytometry.* 2006; 70B:335–43. doi:10.1002/cyto.b.20104.
21. Bordignon J, Comin F, Fereira SC, Caporale GM, Lima Filho JH, Zanetti CR. Calculating rabies virus neutralizing antibodies titers by flow cytometry. *Rev Inst Med Tropic de São Paulo.* 2002; 44:151–4. PMID:12163908.
22. Pouliott C, Dupuis M, Appler K, Brunt S, Rudd R, Davis A. Susceptibility of neuroblastoma cells to rabies virus may be affected by passage number. *J Virol Meth.* 2017; 247:28–31. doi:10.1016/j.jviromet.2017.05.005.

Annex. Equipment, reagents and supplies

- **Equipment.** Several companies offer flow cytometers, including BD (www.bd.com), Bio-Rad (www.bio-rad.com), Thermo Fisher Scientific (www.thermofisher.com), and Beckman Coulter (www.beckman.com).
- **Software.** For the analysis of the FACS data, the equipment had available software. For example, the BD Biosciences flow cytometers have software for analyses such as the CellQuest on the FACSCalibur and, most recently, the BD FACSDiva™. Additionally, some software is available free of charge on the internet; see the Purdue University Cytometry Laboratories catalog for examples (<http://www.cyto.purdue.edu/flowcyt/software/Catalog.htm>).
- **Reagents.** For cell fixation and permeabilization, the use of Cytofix/Cytoperm solution (BD Biosciences, USA; catalog number 554714) is recommended. The intracellular staining of the cells with antibodies can be performed with the perm and wash solution provided in the Cytofix/Cytoperm kit. Reagents: antibody titration research can use the BD™ CompBeads (BD Biosciences, USA; catalog number 552843).

Chapter 17

Rapid immunochromatographic tests for the detection of rabies virus antigens in brain material

Introduction

Decentralized, or point-of-care, testing has the potential to improve the surveillance of many human and animal diseases in low-resource settings (1). Among those tests that can be used under field conditions, lateral flow devices (LFDs) – also referred to as rapid immunochromatographic diagnostic tests (RIDTs), lateral flow immunoassays (LFAs) or immunochromatographic strip tests – present interesting characteristics. They are rapid and easy to use without special training needed for implementation and evaluation. Another advantage is that the tests have no special storage requirements in terms of temperature; they can be shipped and stored at room temperature. LFDs were initially described in the 1960s, and the first commercially available LFD in 1988 was a home pregnancy test (2). Thereafter, this test method was introduced into many different areas (3). This expansion included the diagnosis of viral diseases, such as foot-and-mouth disease (4), avian influenza (5) and Ebola virus disease (6).

The LFDs for detection of RABV were developed and proof of principle studies yielded encouraging results regarding sensitivity and specificity (7, 8). Further studies concentrated on the Anigen/BioNote LFD, showing its potential to detect lyssaviruses, essentially from Africa, the Middle East, Asia and Europe. Reported sensitivities compared with the DFAT ranged between 93% and 100% in field samples (9–13). However, in a comparative study assessing the diagnostic sensitivity of six different LFDs strictly following manufacturers' instructions, insufficient test characteristics prompted a careful consideration on the selection and use of LFDs for detection of RABV (14). This chapter summarizes the general principle alongside the various components and discusses the use, limitations and further needs for improvement.

Principle

Once a liquid sample is applied to the sample pad, gold conjugated antibodies bind specifically to the antigens in the sample. This antigen–antibody complex then travels along a nitrocellulose membrane by liquid migration and is eventually immobilized by a second antibody, which is fixed on the test strip (15). This aggregation of the conjugate forms a visible line in the test area. Unbound conjugated antibodies are later captured downstream at a second line representing the control line (Fig. 17.1). Excess reagents move past the capture lines and are entrapped in the wick or absorbent pad. Usually, the test is read visually after 5–10 min and is valid if the control line appears.

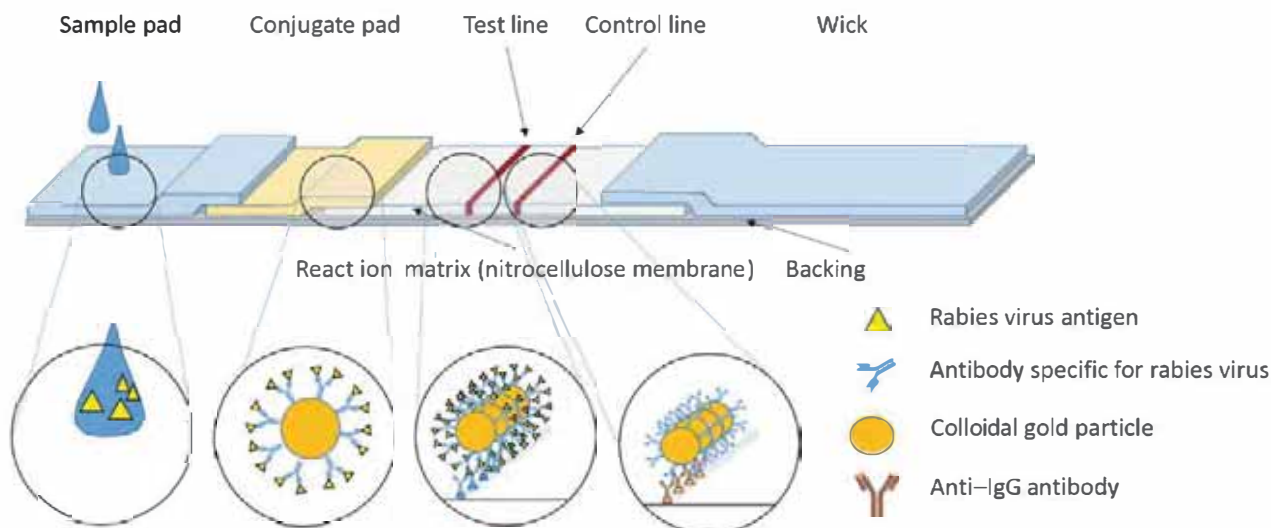


Fig. 17.1. General structure of a sandwich lateral flow device (modified from reference 15, with permission)

Materials

Membranes

The materials of choice for the sample pad or conjugate pad are glass fibres, polyesters, or rayons. Pre-treated conjugate pads that allow rapid flow rates are commercially available (e.g. Whatman, Kent, UK). For the reaction matrix, nitrocellulose membranes are commonly used as the basis for the test due to their relatively low cost, flow characteristics, high protein-binding affinity, relative ease of handling and the wide range of products available (15). Other membrane materials developed for use in LFDs include nylon (e.g. Millipore, Pall Biosciences), polyethersulfone (e.g. Pall Biosciences or Nalgene-Nunc) and polyethylene or fused silica (e.g. Fusion5, Whatman). However, these are less frequently used (1). Nitrocellulose membranes are manufactured by dissolving the raw materials in a mixture of organic solvents and water. When the organic solvents evaporate, and the water concentration increases, nitrocellulose fibres precipitate and form a random mesh- or sponge-like structure. The consistency and reproducibility of this structure is key to the reproducibility of any LFD manufactured using this type of membrane, such as the FF120HP membrane (GE Healthcare Life Sciences).

Antibodies

The most critical part of the LFD is the selection of the antibody (16). Like the other diagnostic tests for rabies that make use of either monoclonal or polyclonal antibodies, such as the direct rapid immunochemistry test (DRIT) and the direct fluorescent antibody test (DFAT), the antibody must be as broad as possible to allow for detection of all circulating RABV variants, and eventually other lyssavirus species. At the same time, the antibody needs to be highly specific and have high affinity for the target antigens, such as the nucleoprotein (N) or glycoprotein (G). Screening for potential candidates may be performed using the ELISA or dot-blot formats. Both monoclonal antibodies as well as polyclonal antibodies can be used. However, monoclonal antibodies have the advantage of better standardization and eternal availability (17).

Once the most appropriate antibody is selected, it must be conjugated for visualization, usually with colloidal gold particles. Either this process is performed internally using available colloidal gold (e.g. Sigma-Aldrich, UK) or kits are employed (e.g. Abcam, UK). Preferentially, however, companies specialized in this area are used to performing this process. Conjugate is then added onto the pad followed by drying at high temperature for fixation.

The second place where the antigens are detected is at the test line. Here, the antigen capture antibody needs to be immobilized onto the membrane without losing its affinity for the RABV antigens. Another antibody downstream of the test line is the capture antibody to unbound conjugated antibodies. To this end, depending on the species source of the primary test RABV antibody, commercially available anti-Mouse-IgG are commonly used. Generally, the consistent immobilization of immunologically active and specific antibodies to test and control lines is a cornerstone of sensitive and reproducible LFDs (16). The LFDs on the market are targeting RABV with one test also detecting other lyssaviruses (9, 12). At present, there is only one test line, but future optimizations may include another line or a separate strip for lyssaviruses others than those belonging to phylogroup I, as exemplified for other applications (17).

Assembly

All pre-treated membranes, pads and the backing are assembled into larger cards. These cards are then cut into strips of an appropriate size. Presently for RABV, all LFDs on the market are assembled into plastic cassettes. This is not essential, as other LFDs only use material such as cardboard as a backing for the membranes.

Manufacturer's instructions

The proper use of the test must have clear, understandable instructions that not only reflect the test itself but also consider how to obtain the sample of choice (e.g. brain material). Also, the user of the tests should be made aware of the potential risk when sampling rabies suspect animals and appropriate personal protective equipment should be recommended.

Principally, any test for viral antigens should use brain material from those anatomical regions where the content has been shown to be highest (e.g. medulla oblongata). Using a straw or scalpel via the foramen magnum provides good results without the need to open the skull (see [Chapter 7](#) on brain removal and reference 18). If opened, similar to recommendations for the DFAT, pieces from different brain regions should be homogenized before being further processed and applied to the LFD. A critical point is the optimal amount of brain material to be applied to the LFD, as generation of signals on the test line may be compromised when the concentration of target analyte exceeds a certain critical value (19). Conversely, excessive dilution can lead to false-negative results due to a suspension with viral loads below the detection limit (10). In some cases, modifications of manufacturer's instructions have led to an improved sensitivity of the LFD from the initial protocol. In fact, the modified LFD protocol has demonstrated higher sensitivity than locally performed DFAT (10). Such modifications require appropriate evaluation and validation; however, they are the responsibility of the manufacturers, as those tests are commercially marketed and should contain clear guiding instructions.

Regulatory issues

LFDs for rabies are veterinary and/or medical devices, and as such require special attention to regulatory issues when being commercialized. Also, many patents are to be considered. Negligence may prevent the device from being marketed, no matter the quality of the product (20).

Generally, before market release, the LFD must go through a process of validation according to the recommendations of international or national organizations. The primary test characteristics, such as sensitivity and specificity as well as test agreement with other standard laboratory tests, must be determined (21). For reliable rabies diagnostic tests, very high values for specificity and sensitivity are essential. Manufacturers should contact WHO or OIE reference laboratories for rabies very early to allow for a broad assessment of the diagnostic value. To harmonize diagnostic methods, the OIE has finalized a formal procedure for the validation and certification of diagnostic kits open to both public and private laboratories, which are desirous of having approval and registration (21).

All manufacturers must have implemented good manufacturing practices to have a stringent quality control management system. This should also encompass batch release testing. On the customer side, the LFD selected for use should also be tested beforehand by either using reference material or by collaborating with national or international laboratories, which could do this assessment upon request. Alternatively, and depending on the prevailing diagnostic capacities, both the LFDs and the standard diagnostic test may be performed in parallel by sending brain samples to a laboratory capable of doing this. In addition, batch-to-batch comparison should be performed to ensure ongoing quality of the device and hence provide reproducible results.

Discussion

Given that LFDs have demonstrated high sensitivity and specificity when proper sampling procedures are followed, these tests can be used primarily to extend rabies diagnostic capacity in resource-limited and remote settings. As such, the implementation of these tests could substantially increase rabies surveillance by offering both rapid and reliable diagnosis of rabies in animals. Particularly, the logistic challenges of shipping samples to national reference laboratories would be overcome. Furthermore, through the incentive of gaining a rapid preliminary result on a suspect case, this technique could enhance participation of field technicians involved in rabies surveillance and increase reporting. Also, monitoring efforts alongside mass dog vaccination campaigns could use these tests under field conditions.

Depending on product specific sensitivity and specificity, confirmation of results using gold standard methods should be considered. Therefore, standard techniques should be established or reinforced in parallel to LFD use at a central laboratory. The use of LFDs for rabies diagnosis in suspect animals involved in human exposure is not recommended when recommended primary tests (e.g. DFAT) are available. Conversely, in settings without timely access to those tests, positive LFD results can constitute a strong motivation for bite victims to rapidly seek

post-exposure prophylaxis (PEP). Thus far, negative LFD test results should never be relied upon for PEP recommendations.

The use of LFDs for the detection of rabies virus in saliva is not recommended, even if claimed by manufacturers. Per se, intermittent shedding of virus in saliva diminishes the diagnostic value. Additionally, the limit of detection of these tests is likely above the antigen level in saliva (10, 14). So far, LFDs have not been tested for human diagnosis on postmortem brain biopsies and therefore their reliability in this context is unknown, even if such samples should not be particularly different from brain samples collected in animals at the postmortem stage of the disease.

The viral RNA from the test strip can be extracted and tested using reverse transcriptase polymerase chain reaction (RT-PCR) after use of the LFDs and storage at room temperature (10, 14). If samples are tested by RT-PCR, this allows not only for a confirmation of the LFD results but also for further characterization of virus isolates and phylogenetic studies after sequencing. Thus, strips could be shipped easily by regular mail to a specialized laboratory, such as a national reference laboratory, or to an internationally approved laboratory for this purpose, further adding value to the application of LFDs.

References

1. Posthuma-Trumpie GA, Korf J, van Amerongen A. Lateral flow (immuno) assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Anal Bioanal Chem.* 2009; 393:569–82. doi:10.1007/s00216-008-2287-2.
2. Tiplady S. Lateral flow and consumer diagnostics. In: Wild D, editor. *The immunoassay handbook: theory and applications of ligand binding, ELISA and related techniques*, 4th edition. Oxford: Elsevier; 2013.
3. O'Farrell B. Lateral flow technology for field-based applications – basics and advanced developments. *Top Companion Anim Med.* 2015; 30:139–47. doi:10.1053/j.tcam.2015.12.003.
4. Ferris NP, Clavijo A, Yang M, Velazquez-Salinas L, Nordengrahn A, Hutchings GH, et al. Development and laboratory evaluation of two lateral flow devices for the detection of vesicular stomatitis virus in clinical samples. *J Virol Methods.* 2012; 180:96–100. doi:10.1016/j.jviromet.2011.12.010.
5. Slomka MJ, To TL, Tong HH, Coward VJ, Mawhinney IC, Banks J, et al. Evaluation of lateral flow devices for identification of infected poultry by testing swab and feather specimens during H5N1 highly pathogenic avian influenza outbreaks in Vietnam. *Influenza Other Respir Viruses.* 2012; 6:318–27. doi:10.1111/j.1750-2659.2011.00317.
6. Walker NF, Brown CS, Youkee D, Baker P, Williams N, Kalawa A, et al. Evaluation of a point-of-care blood test for identification of Ebola virus disease at Ebola holding units, Western Area, Sierra Leone, January to February 2015. *Euro Surveill.* 2015; 20:21073. PMID:25846490.
7. Kang B, Oh J, Lee C, Park BK, Park Y, Hong K, et al. Evaluation of a rapid immunodiagnostic test kit for rabies virus. *J Virol Methods.* 2007; 145:30–6. doi:10.1016/j.jviromet.2007.05.005.
8. Nishizono A, Khawplod P, Ahmed K, Goto K, Shiota S, Mifune K, et al. A simple and rapid immunochromatographic test kit for rabies diagnosis. *Microbiol Immunol.* 2008; 52:243–9. doi:10.1111/j.1348-0421.2008.00031.
9. Markotter W, York D, Sabeta CT, Shumba W, Zulu G, Roux Le K, et al. Evaluation of a rapid immunodiagnostic test kit for detection of African lyssaviruses from brain material. *Onderstepoort J Vet Res.* 2009; 76:257–62. doi:10.4102/ojvr.v76i2.50.
10. Lechenne M, Naissengar K, Lepelletier A, Alfaroukh IO, Bourhy H, Zinsstag J, et al. Validation of a rapid rabies diagnostic tool for field surveillance in developing countries. *PLoS Negl Trop Dis.* 2016; 10:e0005010.
11. Reta T, Teshale S, Deresa A, Ali A, Getahun G, Baumann MPO, et al. Evaluation of rapid immunodiagnostic test for rabies diagnosis using clinical brain samples in Ethiopia. *J Vet Sci Med Diagn.* 2013; 2:3. doi:10.4172/2325-9590.1000117.

12. Servat A, Picard-Meyer E, Robardet E, Muzniece Z, Must K, Cliquet F. Evaluation of a rapid immunochromatographic diagnostic test for the detection of rabies from brain material of European mammals. *Biologicals*. 2012; 40:61–6. doi:10.1016/j.biologicals.2011.12.011.
13. Voehl KM, Saturday GA. Evaluation of a rapid immunodiagnostic rabies field surveillance test on samples collected from military operations in Africa, Europe, and the Middle East. *US Army Med Dep J*. 2014:27–32. PMID:25074599.
14. Eggerbauer E, de Benedictis P, Hoffmann B, Mettenleiter TC, Schlottau K, Ngoepe EC, et al. Evaluation of six commercially available rapid immunochromatographic tests for the diagnosis of rabies in brain material. *PLoS Negl Trop Dis*. 2016; 10:e0004776. doi:10.1371/journal.pntd.0004776.
15. O'Farrell B. Evolution in lateral flow-based immunoassay systems. In: Wong R, Tse H, editors. *Lateral flow immunoassay*. Totowa (NJ): Humana Press; 2009:1–33.
16. Brown MC. Antibodies: Key to a robust lateral flow immunoassay. In: Wong R, Tse H, editors. *Lateral flow immunoassay*. Totowa (NJ): Humana Press; 2009:1–16.
17. O'Farrell B. Lateral flow immunoassay systems: evolution from the current state of the art to the next generation of highly sensitive, quantitative rapid assays. In: Wild D, editor. *The immunoassay handbook*, 4th edition. Oxford: Elsevier; 2013:89–107.
18. Montaña Hirose JA, Bourhy H, Sureau P. Retro-orbital route for the collection of brain specimens for rabies diagnosis. *Vet Rec*. 1991:291–2.
19. Qian S, Bau HH. Analysis of lateral flow biodetectors: competitive format. *Anal Biochem*. 2004; 326:211–24.
20. Wong RC. Regulatory issues in the development and marketing of lateral flow immunoassays. In: Wong R, Tse H, editors. *Lateral flow immunoassay*. Totowa (NJ): Humana Press; 2009:1–13.
21. Chapter 1.1.6. Principles and methods of validation of diagnostic assays for infectious diseases. In: *Manual of diagnostic tests and vaccines for terrestrial animals*. Paris: World Organisation for Animal Health; 2013:1–16 (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf).

Chapter 18

Mass spectrometry-based proteomic approaches for the detection of rabies virus peptides

Introduction

Proteomics techniques based on mass spectrometry (MS) have emerged as one method of choice for the characterization of multiple proteins expressed in complex biological systems (1, 2). Such an approach may be useful for the accurate detection of rabies virus (RABV) peptides. During the past several decades, MS instrumentation has expanded, offering many opportunities for diagnostic and research laboratories. Basically, MS equipment comprises instruments capable of producing and separating ions according to their mass-to-charge ratio (m/z). Many different configurations or types of MS instruments are possible. Each is suited to the analysis of particular types of specimens. However, these instruments share three basic components: an ion source, a mass analyzer, and a detector (3).

The most common types of ionization used in the analysis of peptides and proteins are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). The MALDI technique involves mixing the sample with a matrix compound that absorbs in the ultraviolet (UV) light range (4). Conversely, ESI generates a continuous supply of ions from a liquid solution, where a liquid sample is emitted from a capillary, nozzle or needle, in the presence of an electric field. The ESI is often connected with online liquid chromatography (LC) to separate the peptides in complex mixtures. The MALDI is more tolerant to sample contaminants such as buffers and salts, when these are present at low concentrations, than the ESI (5, 6).

Time-of-flight (TOF), the quadrupole and the ion trap are the commonest types of mass analyzers. In MALDI, ionization frequently generates singly charged species, and is very commonly coupled with TOF, which can detect high masses (7, 8). Ion traps are restricted to smaller mass ranges and are well suited to ESI, which generates multiple charged species. Generally, ion traps associated with Orbitrap break this rule and offer mass resolution up to 100 000 Da [dalton] (9, 10).

Shotgun proteomics refers to the use of bottom-up proteomics techniques, in which peptide detection is used to infer protein presence in complex samples. In this case, protein fractionation and separation occur prior to protein digestion. This is followed by direct peptide analysis by peptide mass fingerprinting (PMF) or peptide separation by LC, interfaced to tandem mass spectrometry (MS/MS) (11, 12).

As the proteome is complex, there is no standard method for preparing protein samples. Selecting the appropriate instrumentation and software is essential for analysis by MS. Thus, protocols differ depending on the sample type and the analytical method used. For complex protein samples, the most common MS

workflows analyse peptides, which are previously fractionated by LC and ionize, thereby fragmenting more efficiently than proteins, resulting in spectra easier to interpret and analyse.

Protocol

The overall schematic approach employed for analysis of RABV proteins is illustrated in Fig. 18.1.

Viral replication assay

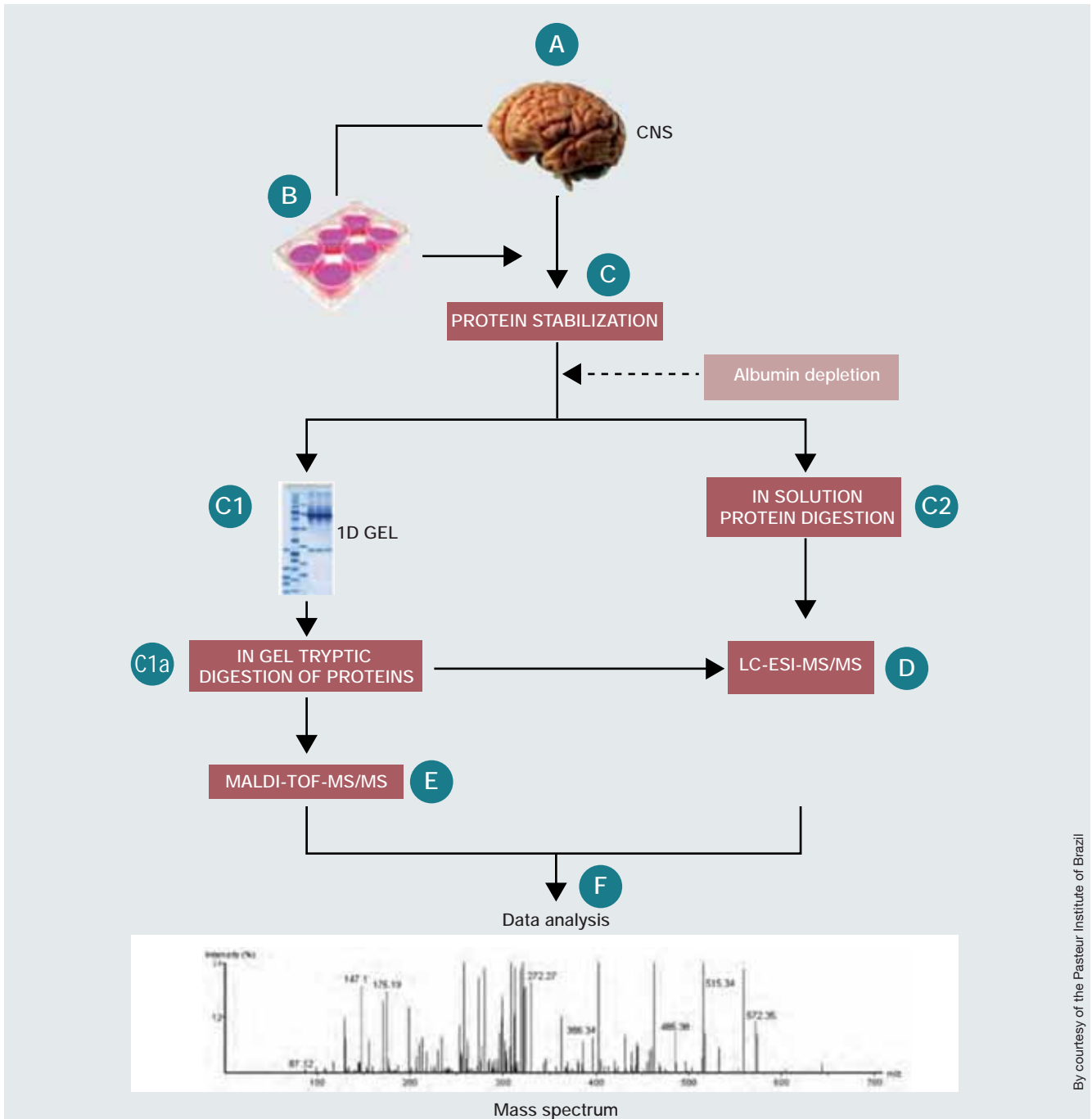
Street RABV can be isolated from animal brains. The tissue is macerated, homogenized manually on ice and diluted with cell culture medium (modified Eagle's medium [MEM], supplemented with 10% fetal bovine serum [FBS], 3% of gentamicin and 3% nonessential amino acids, at 20% [w/v] concentrations). This is used as the primary virus inoculum.

The virus inoculum containing 20% brain tissue is propagated to the third-passage in N₂a cells in the presence of cell culture medium (13). This material is incubated for 48–72 h. Each virus passage in N₂a cells has the purpose of promoting the reinfection of cells and the increase in virus concentration. For each well, 1 mL of N₂a cell (1×10⁶ cells/mL) suspension, 0.5 mL of the tissue culture suspension and 2.5 mL of culture are added in a 6-well plate (14, 15).

Protein extraction

The FBS is a complex mixture of proteins, composed of a high concentration of albumin commonly used to supplement cell culture media. This reagent alone could be challenging for analytical capabilities of traditional proteomic methods. Reduction of sample complexity is thus an important first step in the analysis of a viral protein. Therefore, a protein depletion step is suggested when using traditional proteomic methods. In this case, the step depletion of highly abundant proteins, such as the albumin and IgG, may be carried out according to manufacturer's instructions (e.g. ProteoPrep Blue Albumin & IgG Depletion kit, Sigma, St Louis (MO), USA).

Cell lysis using detergents is a milder and easier alternative to physical disruption of cell membranes. One study using the detergent 3-(3-cholamidopropyl) dimethylammonium 1-propanesulfonate (CHAPS) showed that this could yield the largest amount of soluble RABV glycoprotein, when compared with other detergents (16). Additionally, the non-denaturing zwitterionic CHAPS detergent is fully dialyzable, which makes the removal process easy. Addition of protease or phosphatase inhibitors in buffered detergent solution is necessary to limit proteolysis in cell extracts, just before making the complete cell extraction buffer.



By courtesy of the Pasteur Institute of Brazil

Fig. 18.1. Schematic diagram of a pathogen microarray process and analysis; experimental procedure for detection of RABV proteins by mass spectrometry

- A: Brain suspension infected with RABV
 - B: Viral replication in N2A cells using a 6-well tissue culture plate
 - C: Detergent solubilization of membrane protein
 - C1: SDS-PAGE electrophoresis for protein separation
 - C1a: In-gel tryptic digestion of proteins
 - C2: In-solution protein digestion
 - D: LC-ESI MS/MS analysis
 - E: MALDI-TOF-MS/MS analysis
 - F: Data analysis by software
- The dotted arrow indicates an optional step: depletion of albumin.

Assay procedure

1. Harvest the supernatant and scraped cells after RABV replication in a 6-well plate.
2. Clarify the mixture by centrifugation at 12 000 r/min for 15 min at 4 °C.
3. Add 500 µL of ice-cold buffered detergent solution (100 mmol Tris-HCl, 300 mmol NaCl, 20% DMSO, 8 mmol EDTA, 1.2% CHAPS) with protease/phosphatase inhibitors into a 1.5 mL microcentrifuge tube, over the 500 µL clarified solution. It is important to note that these volumes may require optimization for each specific application.
4. Incubate for 30 min at room temperature, with occasional vortexing.
5. Determine the total protein concentration of the sample using an applicable method, such as the Bradford (a spectroscopic analytical procedure used to measure the concentration of protein in a solution) assay or a related method.
6. Store the extracts at –80 °C until ready for analysis. Avoid repeated freeze–thaw cycles. In preparation for performing the assay, allow the samples to thaw on ice.
7. Mix well prior to analysis.
Cell extracts prepared by this method routinely have a protein concentration of ~1 up to ~10 mg/mL.

Protein digestion in solution

If you do not perform the “pre-run the gel step” (2.4), after protein extraction, digest the sample. This protocol is optimal for 1 mg of total protein. Volumes can be adjusted to lower amounts of protein, down to 300 µg. All reagents should be prepared fresh.

Assay procedure

1. Add urea 8 mol (1:1) in protein extraction solution.
2. Add dithiothreitol (DTT) 5 mmol for 25 min at 56 °C.
3. Discard the DTT and add iodoacetamide (IAA), 14 mmol, for 30 min at room temperature.
4. Discard the IAA and add DTT 5 mM for 15 min at room temperature.
5. Reduce the urea concentration by diluting the reaction mixture with ammonium bicarbonate, 50 mmol (1:5).
6. Digest the protein with 20 ng of trypsin gold (Promega) overnight at room temperature.
7. *Add 0.4% trifluoroacetic acid (final concentration).
8. Stop the reaction and adjust the pH of the solution to below 2.0.
9. Remove small, unwanted compounds from macromolecules by dialysis. Sample cleanup can be performed using a trap column or filter prior to the analytical column.
10. Freeze–dry to dryness.
11. Store samples at –20 °C.

- * When samples will be analysed by electrospray/nanospray-type MS, on step 7, add 10 μL of extraction solution 1 for 10 min at room temperature. Then add 12 μL of extraction solution 2 for 10 min at room temperature and evaporate in a rotatory evaporator for 40 min or until 1 μL of sample remains.

“Pre-run the gel”

Protein electrophoresis is a laboratory technique used to separate proteins in complex biological samples. Protein is separated based on net charge, size and shape within an electric field. A protein separation step by one-dimensional separation in polyacrylamide gel electrophoresis (PAGE) could be important when analysing complex mixtures. “Pre-running the gel” is optional but can result in a higher quality separation.

Assay procedure

Prepare the protein sample

SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) is used to analyse the proteins in complex extracts. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (17). Laemmli sample buffer is especially formulated for preparation of protein samples in the Laemmli SDS-PAGE system. When a Coomassie blue stain is used (depending on the well size and gel thickness), the amount of protein loaded should range between 0.5–4.0 μg for purified samples and between 40–60 μg for crude samples. Silver staining methods are approximately 100-fold more sensitive, and therefore require less protein per sample.

1. Dilute the protein sample (20 μg) directly in 2x Laemmli sample buffer at a 1:1 ratio.
2. Incubate the protein sample at 95 °C for 5 min (or at 70 °C for 10 min) after addition of sample buffer for a complete disruption of molecular interactions.
3. After heat treatment in SDS sample buffer, insoluble material should be removed by brief centrifugation. This is easily accomplished by a 2 min spin in a microcentrifuge at 17 000 *g*.
4. The supernatant of the treated sample is now ready to load.

Prepare the gel electrophoresis

1. Prepare a glass cassette on a casting stand assembly according to the manufacturer’s instructions.
2. Prepare the gel solution (12% gel concentration) in a separate small beaker.
3. Vortex the solution gently.
4. Add 10 mL of separating gel solution into the gap between the glass plates.
5. To make the top of the separating gel horizontal, fill in approximately 0.3 mL of *n*-butanol into the gap until an overflow occurs.
6. Wait for 30 min to allow polymerization.

7. Discard the n-butanol.
8. Add stacking gel (5%) until overflow occurs.
9. Insert the well-forming comb, taking care not to trap any bubbles below the teeth of the comb.
10. Wait for 20–30 min for polymerization.

Load the gels

1. Slowly remove the combs from the gels.
2. Fill each well with tank buffer.
3. Gently load 10 μ L of sample and loading protein marker.

Run the gel

1. Fill the chamber with tank buffer.
2. Put the lid on the gel unit and connect it to the power supply according to the manufacturer's instructions.
3. Set an appropriate voltage and run the electrophoresis.
4. As for the total running time, stop the electrophoresis run when the protein marker almost reaches below the glass plate. Generally, this requires about 1 h for a 120V voltage and a 12% separating gel.

Stain the protein gel with Coomassie brilliant blue

1. Use the fixing solution for 1 h to overnight with gentle agitation.
2. Stain the gel in staining solution for 20–30 min with gentle agitation.
3. Destain the gel in destaining solution. Refill the solution several times until the background of the gel is fully destained.
4. If necessary, store the destained gel in storage solution.

In-gel tryptic digestion of proteins

One of the most important steps in sample preparation for proteomics is the conversion of proteins to peptides; in most cases, trypsin is used as enzyme. Trypsin is a protease that specifically cleaves the proteins, creating peptides both in the preferred mass range for MS sequencing and with a basic residue at the carboxyl terminus of the peptide, producing information-rich, easily interpretable, peptide fragmentation mass spectra.

Assay procedure

1. Excise protein bands from the gel.
2. Destain the gel in destaining solution for 1 h. Repeat this step three times.
3. Discard the destaining solution and add 200 μ L of acetonitrile for 5 min. Repeat this step twice.
Evaporate the remaining acetonitrile in the rotatory evaporator for 3 min.
4. Add 30 μ L DTT 10 mM and incubate for 30 min at room temperature.
5. Discard the DTT and add 30 μ L of IAA 50 mmol for 30 min at room temperature.

6. Discard the IAA and wash the gel in 100 μL of ammonium bicarbonate 100 mmol for 10 min.
7. Discard the ammonium bicarbonate and dehydrate the gel in 200 μL acetonitrile for 5 min.
8. Discard the acetonitrile and rehydrate the gel in 200 μL of ammonium bicarbonate 100 mmol for 10 min.
9. Repeat steps 7 and 8 twice.
10. Digest the spots with 20 ng of trypsin gold (Promega) overnight at room temperature.
11. Add 10 μL of extraction solution 1 for 10 min at room temperature.
12. Discard the extraction solution 1 and add 12 μL of extraction solution 2 for 10 min at room temperature.
13. Evaporate in the rotatory evaporator for 40 min or until 1 μL of sample remains.
14. Remove small, unwanted compounds from macromolecules by dialysis. Sample cleanup can be performed using a trap column or filter prior to the analytical column.
15. Freeze-dry to dryness.
16. Store samples at $-20\text{ }^{\circ}\text{C}$.

Mass spectrometry analysis

The final peptide mixture prior to MS is analysed by either MALDI-TOF-MS/MS or LC-ESI-MS/MS. It is often necessary to desalt peptide mixtures prior to analysis by MS, because excess trace amounts of detergents or salts could interfere with peptide ionization or background in the mass spectra. Single-step desalting is most often carried out with C18 resin, which concentrates and purifies the samples for sensitive downstream analysis. The sample is desalted and is concentrated in 4 μL of solution appropriate for analysis by MALDI-TOF and ESI-Q-TOF.

If nanospray or electrospray is performed, it is desirable to rinse three times with 200 μL water to remove the TFA, which may interfere with ionization in nanospray/electrospray.

Assay procedure

1. For LC-ESI-MS/MS analysis: Elute peptides from the precolumn using 30 μL of LC-ESI-MS/MS elution solution.
For MALDI-TOF-MS/MS analysis: Elute peptides from the precolumn using 30 μL of MALDI-TOF-MS/MS elution solution.
2. Collect the eluate in a clean tube.
3. The samples are now ready for MS analysis.

Peptide database search

The protein database is typically a collection of amino acid sequences (e.g. FASTA bioinformatics format) which is used to generate the in silico peptide database against which the MS data will be searched. Swiss-Prot (<ftp://ftp.ebi.ac.uk/pub/databases/>) and NCBI nr (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz>) are protein databases commonly used for searching proteomic MS data (15).

Assay procedure

1. Include instrumentation-specific parameters.
2. MS/MS spectra are searched against a RABV protein (NCBI Taxonomy ID 11292) database.
3. Insert cleavage specificity of the trypsin enzyme: a maximum of two missed cleavages with carbamidomethylation of cysteine included as a fixed modification.
4. Add specific parameters. Generally, set precursor mass tolerance to 10 ppm for Orbitrap or QTOF MS, and 0.5 Da [Daltons] for MS/MS fragment ions.
5. The confidence interval for protein identification is set to 95% and only peptides with an individual ion score above the identity threshold are considered correctly identified. Other parameters that may be included are the charge states and fragmentation expected. For example, the type of fragmentation used and the type of mass spectrometer selected will affect the types of fragment ions expected.

References

1. Altelaar AF, Munoz J, Heck AJ. Next-generation proteomics: towards an integrative view of proteome dynamics. *Nat Rev Genet.* 2013; 14:35–48. doi:10.1038/nrg3356.
2. Mann M, Kulak NA, Nagaraj N, Cox J. The coming age of complete, accurate, and ubiquitous proteomes. *Mol Cell.* 2013; 49:583–90. doi:10.1016/j.molcel.2013.01.029.
3. Han X, Aslanian A, Yates JR. Mass spectrometry for proteomics. *Curr Opin Chem Biol.* 2008; 12:483–90. doi:10.1016/j.cbpa.2008.07.024.
4. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10000 daltons. *Anal Chem.* 1988; 60:2299–301. doi:10.1021/ac00171a028.
5. Kjellstrom S, Jensen ON. In situ liquid–liquid extraction as a sample preparation method for matrix-assisted laser desorption/ionization MS analysis of polypeptide mixtures. *Anal Chem.* 2003; 75:2362–9. doi:10.1021/ac026297w.
6. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. *Science.* 1989; 246:64–71. doi:10.1126/science.2675315.
7. Yates III JR. Mass spectral analysis in proteomics. *Annu Rev Biophys Biomol Struct.* 2004; 33:297–316. doi:10.1146/annurev.biophys.33.111502.082538.
8. Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science.* 2006; 312:212–7. doi:10.1126/science.1124619.
9. Makarov A. Electrostatic axially harmonic orbital trapping: a high-performance technique of mass analysis. *Anal Chem.* 2000; 72:1156–62. doi:10.1021/ac991131p.
10. Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Cooks RG. The Orbitrap: a new mass spectrometer. *J Mass Spectrom.* 2005; 40:430–43. doi:10.1002/jms.856.
11. Henzel WJ, Billeci TM, Stults JT, Wong SC, Grimley C, Watanabe C. Identifying proteins from two dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. *Proc Natl Acad Sci U S A.* 1993;90:5011–5.
12. Ogorzalek Loo RR, Hayes R, Yang Y, Hung F, Ramachandran P, Kim N, et al. Top down, bottom-up, and side-to-side proteomics with virtual 2-D gels. *Int J Mass Spectrom.* 2005; 240:317–25. doi:10.1016/j.ijms.2004.10.013.
13. Castilho JG, Iamamoto K, Lima JYO, Scheffer KC, Carnieli Jr P, Oliveira RN, et al. [Patterning and appliance of virus isolation techniques in mice neuroblastoma cells (N2A)]. *BEPA.* 2007; 4:12–18 (in Portuguese); <http://periodicos.ses.sp.bvs.br/pdf/bepa/v4n47/v4n47a02.pdf>, accessed 22 May 2018.
14. Costa BA, Fuoco NL, Chaves LB, Rodrigues AC, Ribeiro OG, Nogi KI, et al. Rabies lyssavirus isolates from Brazilian different reservoirs species present distinct pattern of propagation in N2a cell. *Virology.* 2016; 5:1–5. doi:10.4172/2161-0517.1000159.

15. Katz IS, Dias MH, Lima IF, Chaves LB, Ribeiro OG, Scheffer KC, et al. Large protein as a potential target for use in rabies diagnostic. *Acta Virol.* 2017; 61(3). doi:10.4149/av_2017_306.
16. Ramya R, Mohana Subramanian B, Sivakumar V, Senthilkumar RL, Sambasiva Rao KR, Srinivasan VA. Expression and solubilization of insect cell-based rabies virus glycoprotein and assessment of its immunogenicity and protective efficacy in mice. *Clin Vaccine Immunol.* 2011; 18:1673–9. doi:10.1128/CVI.05258-11.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–5.

Annex 1. Preparation of buffers and reagents

2x Laemmli sample buffer pH 6.8

4% SDS
20% glycerol
10% 2-mercaptoethanol
0.004% bromophenol blue
0.125 mol Tris HCl
Dissolve in ddH₂O

Polyacrylamide solution

Solution A: 30% acrylamide and 0.8% bis-acrylamide dissolved in water

Polyacrylamide solution (10 mL)	12%	5%
Solution A (mL)	4.0	1.7
Solution B (mL)	2.5	2.5
ddH ₂ O	3.5	5.8
10% ammonium persulphate (μL)	120	20
TEMED* (μL)	120	20

Solution B: Tris Base 1.5 mol (pH=8.8) and 20% SDS 2 dissolved in water

*Added just before pouring gel.

Running buffer

3 g Tris base (25 mM)
14.4 g glycine (192 mmol)
1 g SDS (0.1%)
Bring the total volume up to 1 L with ddH₂O

Fixing solution

50% methanol
10% glacial acetic acid

Staining solution

0.1% Coomassie brilliant blue
50% methanol
10% glacial acetic acid

Destaining solution

40% methanol
10% glacial acetic acid

Storage solution

5% glacial acetic acid

Dithiothreitol (DTT) 10 mmol

1.5 mg DTT in 1 mL 100 mmol ammonium bicarbonate

Iodoacetamide (IAA) 50 mmol

10 mg IAA in 1 mL 100 mmol ammonium bicarbonate

Trypsin solution

20 ng/μL (Promega, sequence grade) in 50 mmol ammonium bicarbonate

Extraction solution 1

5% formic acid in ddH₂O

Extraction solution 2

5% formic acid in 50% acetonitrile

LC-ESI-MS/MS elution solution

50% acetonitrile
0.1% formic acid
49.9% ddH₂O

MALDI-TOF-MS/MS elution solution

50% acetonitrile
0.1% trifluoroacetic acid
49.9% ddH₂O

Annex 2. Recommendations regarding protein for mass spectrometry analysis

1. Wear powder-free gloves.
2. Use head caps and facial masks when possible.
3. Be aware that any surfaces, glassware or chemicals exposed to the laboratory atmosphere for more than a few minutes will be contaminated with keratin as dust from the atmosphere settles.
4. Do not autoclave tips and tubes.
5. Use tips and tubes from unopened boxes.
6. Do not wash the materials with detergents.

Part 4. Demonstration of viral antibodies

Chapter 19

The rapid fluorescent focus inhibition test

Introduction

The classical rapid fluorescent focus inhibition test (RFFIT) was developed at the Communicable Disease Center (now the United States Centers for Disease Control and Prevention [CDC]) in 1973 by J. Smith, P. Yager and G. Baer to provide an alternative to the *in vivo* mouse neutralization test (MNT) for demonstrating the presence or absence of rabies virus neutralizing antibodies (RVNA) in serum (1). Several steps of the MNT procedure were preserved or used as the basis of the RFFIT. In essence, the *in vitro* (RFFIT) method was developed to mimic the *in vivo* (MNT) method. Use of live rabies virus (RABV) in the method necessitates performance in a Biosafety Level (BSL) 2 or 3 laboratory (2). The RFFIT results are primarily used:

- to determine the immunization status of individuals who have undergone pre- or post-exposure prophylaxis against RABV;
- for the evaluation of vaccines or new schedules of rabies pre- or post-exposure prophylaxis;
- as demonstration of response to rabies vaccination for animals scheduled for importation to so-called rabies-free countries;
- for the management of plasma donors used in the production of rabies immunoglobulin (RIG); and
- for the evaluation and calibration of newly developed serological tests (3).

The RFFIT is rarely used for the diagnosis of rabies in humans, as neutralizing antibodies appear in the cerebrospinal fluid (CSF) and in the serum of infected patients very late in the course of the disease (4). Modifications of the classical RFFIT described in this chapter led to the development of such methods as the fluorescent antibody neutralization test (FAVN) (5) and the European Pharmacopeia method for rabies immunoglobulin product potency (6).

Samples evaluated by RFFIT are typically serum but can include human or animal CSF and plasma (only acid citrate dextrose [ACD], or heparin plasma, not ethylenediaminetetraacetic acid [EDTA] plasma). Samples are serially diluted, exposed to RABV and incubated to allow reaction between any RVNA present and for RABV to occur. Subsequently, a suspension of freshly trypsinized cells is added to the serum–RABV mixture. After cells are incubated with the mixture, the resulting monolayer is stained with an anti-rabies fluorescein isothiocyanate (FITC) conjugate and evaluated microscopically for presence or absence of RABV infection. The level of RVNA in the sample is determined by counting the microscopic fields positive for RABV and using a formula to calculate the 50% end-point (ED_{50}) titre. Titre results can be standardized to International Units per millilitre (IU/mL) by comparison to a reference serum of known titre. Described below are

generally accepted procedures for performance of the RFFIT; specific accepted ranges (such as CO₂ levels and incubation time or temperature) in the procedures must be validated for optimal performance in individual laboratories.

Methods

Sample preparation

Samples are evaluated for haemolysis and lipaemia, and if necessary more suitable samples are obtained prior to testing. Serum is heat-inactivated in a water bath or heat block at 56 °C for 30 min to inactivate complement, which can interfere in antibody measurement (7).

Sample dilution

Serial dilutions of samples are made using EMEM10 (Annex 1.1.1) as the diluent in 8-well (Lab-Tek) slides (Annex 1.2.2). Typical dilutions use half (4-wells) of a slide and are 1:2.5, 12.5, 62.5 and 312.5 resulting in a final volume of 0.1 mL (a screening test can be performed by making two dilutions, e.g. 1:10 and 1:50). Alternatively, the dilutions can be prepared in 96-well plates and 0.1 mL transferred to the Lab-Tek slides. These samples are then further diluted by adding 0.1 mL of RABV yielding final dilutions of 1:5, 1:25, 1:125 and 1:625 (see Fig. 19.1 for a diagram of the procedure, and Fig. 19.2 for a picture of a slide).

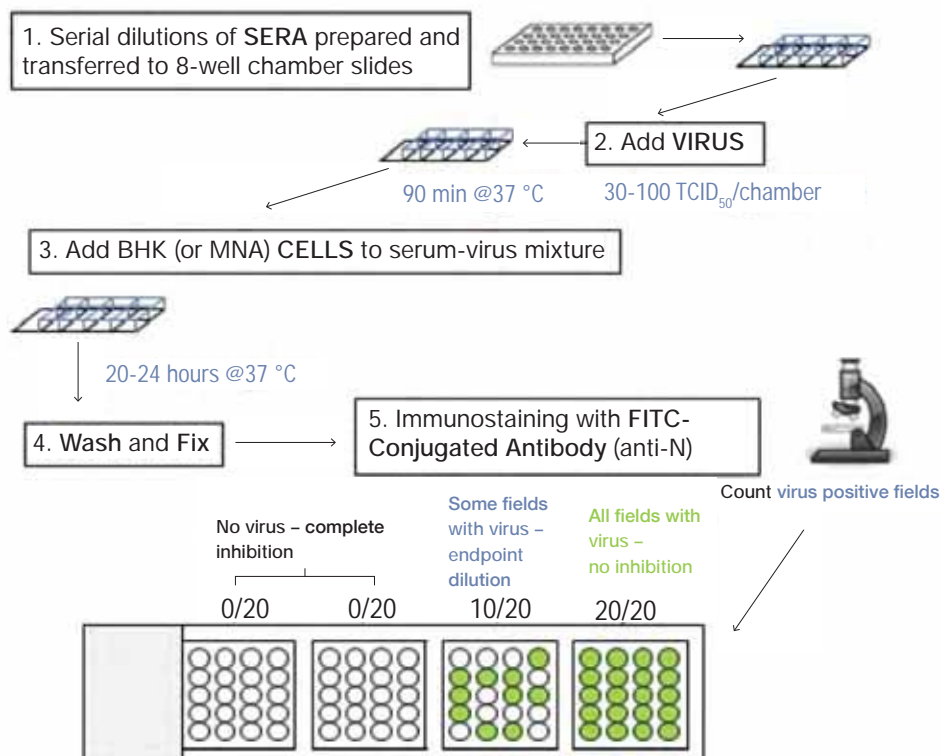


Fig. 19.1 Steps of the classical RFFIT procedure¹

BHK, baby hamster kidney cell; FITC, fluorescein isothiocyanate; MNA, murine neuroblastoma cell; TCID, tissue culture infectious dose
¹ Reproduced with permission from: Yager ML, Moore SM. The rapid fluorescent focus inhibition test. In: Rupprecht C, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention (Volume 2). Elsevier; 2015:199-215.

By courtesy of Susan Moore, Kansas State University,
Manhattan, USA

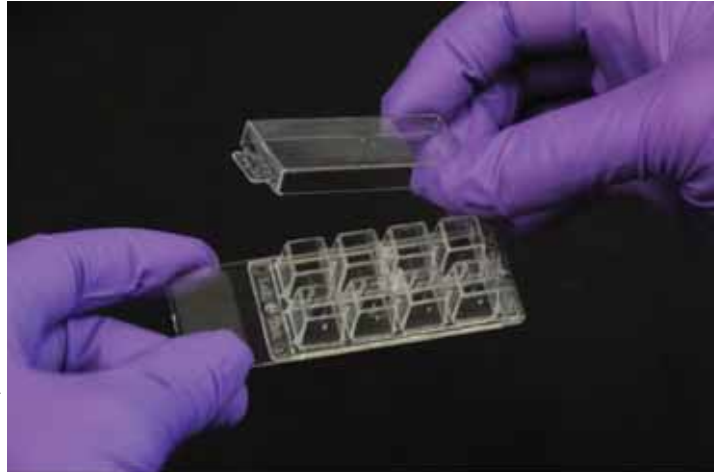


Fig. 19.2. The 8-well (Lab-Tek) chamber slide

RFFIT control systems

Each test should contain controls to identify errors and to document acceptable test performance. Large sets of test sera benefit from having controls on the first and last slides. Results of tested serum samples are validated only after all acceptance criteria are satisfied.

Reference serum

To standardize titre results, test samples of unknown antibody titres are compared with the titre of a standard rabies immunoglobulin (SRIG) (Annex 1.3.3) or an in-house (internal) reference human serum of known potency value (IU/mL) tested in the same set of testing, in the same manner as the test samples. Samples of reference serum prepared in-house should be regularly qualified against an international SRIG according to a defined interval.

Cell control

A cell suspension unexposed to the challenge RABV is used as a control for evaluation of cell cultures and growth media. One well receives only the cell suspension and does not receive an inoculation of RABV.

RABV back titration

Titration of the challenge (or working dilution) RABV (without addition of antibody) determines the actual RABV dose (as the 50% fluorescent focus infectious dose [FFID₅₀] or 50% tissue culture infectious dose [TCID₅₀]) used in each test. The challenge RABV dilution and a 1:10 and a 1:100 dilution of the challenge RABV are added to one well each.

Internal standards

A negative control sample and at least one positive control sample of known RVNA level similar in matrix and titre level to the test samples are useful in determining the validity of results. When testing samples on adjacent slides that are expected to be negative or to show ED₅₀ titres greater than the highest dilution

tested, a positive or negative sample included among these slides is a useful check that both RABV and sample have been added to the test slides.

Acceptance criteria for the controls and standards must be set by each laboratory. Ideally, internal standards should include a high RVNA positive, a low RVNA positive and a negative RVNA sera at minimum. For optimum assay performance the SRIG range should be determined by the average titre \pm 1 standard deviation (8). This range is best determined after sufficient SRIG titre measurements taken over a representative period of time, for example 30 times over several months. The acceptable virus dose is between 30 and 100 FFID₅₀, optimally not varying more than 2-fold (8). Internal standards should produce results \pm two standard deviations of the average value determined from several replicate tests over several assay runs. The cell control should not have any virus growth present. Test sets are accepted only if all acceptance criteria are met. Results on sets with failed acceptance criteria are not valid and require retesting.

Challenge RABV dilution preparation and inoculation

The stock RABV (Annex 1.3.2) is diluted to obtain 50 FFID₅₀/0.1 mL using EMEM10 as the diluent (Annex 1.3.2.4). Prepare a back titration by diluting the challenge RABV dilution 1:10 and 1:100. Add 0.1 mL of each (challenge dilution, 1:10, and 1:100) to wells containing 0.1 mL EMEM10. The cell control well does not receive RABV inoculation. To all remaining wells for the controls, standards and test samples add 0.1 mL challenge dilution. The final volume in each of the wells is 0.2 mL. Shake the slides gently to mix the contents of the wells. The slides containing the serum dilution/RABV mixture are incubated at 35–37 °C in a CO₂ controlled humidity incubator (Annex 1.2.3) for 90 min.

Cell suspension preparation and addition

Approximately 30 min before the end of the 90 min incubation, prepare a cell suspension from a cell culture aged 3 days (Annex 1.3.1). Cells should have a final concentration of 5 x 10⁵ cells/mL (or 1 x 10⁵ cells/0.2 mL).

It may be necessary to perform a viable cell count using 0.5% trypan blue; for example 0.5 mL cell suspension in 1.5 mL of 0.5% trypan blue (dilution factor of 4).

Calculate the dilution necessary to give 5 x 10⁵ cells/mL.

For example: If the initial cell suspension has 3 x 10⁶ cells/mL, the dilution factor necessary to give 5 x 10⁵ cells/mL is calculated by dividing the number of cells in the cell suspension by the desired number of cells.

$$\frac{3 \times 10^6}{5 \times 10^5} = 6$$

The dilution necessary to obtain 5 x 10⁵ cells/mL is: 1:6 (cell suspension: EMEM10).

Approximately 2 mL of suspension are needed for each slide. For eight slides, the total volume needed is 8 x 2 = 16 mL. **Note:** if using baby hamster kidney

(BHK) cells, 0.01% diethylaminoethyl (DEAE)-Dextran is added 15 min before the cells are added to the wells at the ratio of 1 mL/100 mL of cell suspension to enhance RABV infectivity of the cells (9).

Following the 90 min incubation period, add 0.2 mL of the cell suspension to all wells. Shake the slides gently to mix the contents. The serum–RABV–cell mixture is incubated at the appropriate temperature and CO₂ level for 20–24 h.

Acetone fixation of slides and staining

After cell growth is achieved, the chamber slides are inverted to discard the liquid (which can contain substantial RABV) into a virucidal solution. The chamber structure is disassembled from the slide at this point or, if using plastic slides, retained until after the conjugate incubation step (see following step). The cell monolayer in the slide wells is first rinsed in phosphate buffered saline, known as PBS (Annex 1.1.2) and/or cold acetone (Annex 1.1.4), then fixed in cold acetone for 10–30 min. The slide is then air dried. Anti-rabies FITC conjugate (see Annex 1.3.4 to determine concentration) is added to each well sufficiently to cover the entire well surface; generally, 0.16 mL is sufficient. The slide is incubated for 30 min at 35–37 °C in a humid incubator. After incubation the slide is rinsed, first in PBS and then in water and air dried.

Microscopic evaluation

The slide is evaluated under a fluorescence microscope with high-quality objective lenses for sufficient magnification (typically with 100–200x) and numerical aperture sufficient to read at least 20 fields per well and distinguish specific from nonspecific staining. It is important that the 20 fields read are representative of the total area of the well for best accuracy. RABV that has not been neutralized by RVNA during the virus–antibody incubation step infects the cells and is identified microscopically by the anti-rabies FITC conjugate. Each serum dilution well is observed for the presence of fluorescing foci by viewing 20 microscopic fields containing a good monolayer of cells and determining if any RABV is present (see Fig. 19.3 for examples of positive and negative fields). The number of fields with RABV present is recorded to be used in the calculation of the ED₅₀ titre.

1 Reproduced with permission from: Yager ML, Moore SM. The rapid fluorescent focus inhibition test. In: Rupprecht C, Nagaratnam T, editors. Current laboratory techniques in rabies diagnosis, research and prevention (Volume 2). Elsevier; 2015:199–215.

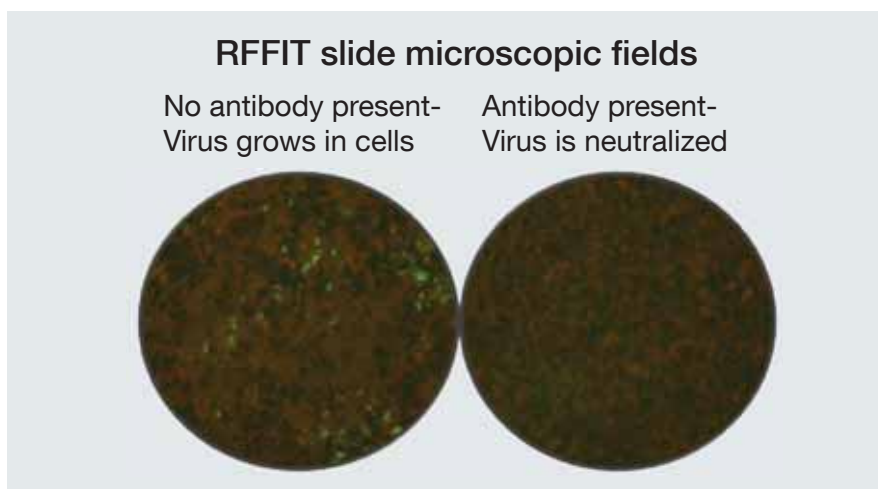


Fig. 19.3 Examples of positive (virus present) and negative (no virus present) RFFIT slide microscopic fields¹

Determination of RVNA ED₅₀ titres

The Reed–Muench method is used to calculate the ED₅₀ titre in the RFFIT assay. Fig. 19.4 gives instructions and an example of an ED₅₀ calculation using this formula (10). The ED₅₀ titre can be converted to the IU/mL value by comparing the sample titre with the reference serum titre at 2.0 IU/mL by use of the following formula: IU/mL = ED₅₀ reciprocal titre of test sample/ED₅₀ reciprocal titre of the SRIG x potency of the SRIG in IU/mL.

Example of a test serum with ED₅₀ titre of 1:540 and ED₅₀ titre of the SRIG of 1:200: (540/200) x 2.0 = 5.4 IU/mL.

A chart similar to Fig. 19.5 with calculated sample serum ED₅₀ titres and a known specific SRIG ED₅₀ titre is useful for the commonly used 5-fold serial dilution scheme when the typical reading pattern occurs: the dilution next lower than the end-point dilution has complete neutralization of RABV and the dilution next higher has no neutralization. Owing to possible specificity or efficiency of neutralization issues, partial neutralization will sometimes occur in more than one dilution. This reading pattern is also more commonly seen when dilutions schemes of 2-fold or 3-fold are used due to the smaller difference between dilutions. Unexpected reading patterns, such as greater or equal neutralization in progressively higher dilutions, may indicate an error in testing (serum dilution or RABV addition) that requires investigation.

Test interpretation

The Advisory Committee on Immunization Practices (ACIP) recommends using a level defined as complete RABV neutralization at a 1:5 serum dilution by the RFFIT as an indicator of an adequate adaptive humoral immune response to vaccination (11). WHO is more conservative and recommends that the response be at least 0.5 IU/mL (12), a level which corresponds to some RVNA neutralization in the 1:25 dilution.

For diagnostic purposes, RFFIT serology results demonstrating the absence of RVNA of an unvaccinated subject are inconclusive since neutralizing antibody often is not present until the later clinical stages of disease. Conversely, the presence of RVNA in an unvaccinated subject may indicate natural exposure to RABV antigens. Cross-reactive antibodies or nonspecific inhibition of RABV or cell growth can be misinterpreted as very low levels of RVNA. Typically, these are detected at levels below the 1:25 serum dilution (e.g. in the 1:5 serum dilution) or below 0.1 IU/mL; rarely are they observed into the 0.5 IU/mL range.

Determination of Rabies Virus Neutralizing Antibody 50% End Point Titers Calculated by the Reed-Muench Method
Color coding is used for tracking steps

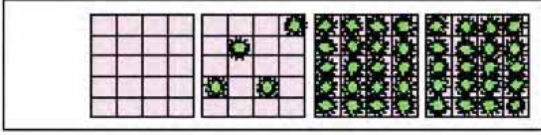
INSTRUCTIONS	SAMPLE PROBLEM										
Serum Dilution	1:5 1:25 1:125 1:625										
Example RFFIT Lab Tek Slide											
Fields Containing Infected Foci	0 4 20 20										
Fields Containing Uninfected Foci	20 16 0 0										
<p>1 Determine the cumulative number of infected fields for each dilution as follows: Beginning with the lowest dilution, add the number of infected fields counted to the number counted in the next lowest dilution. Repeat for all dilutions</p> <p style="text-align: center;">Example of Cumulative Infected Fields</p>	$0 \quad - \quad 0+4=4 \quad - \quad 4+20=24 \quad - \quad 24+20=44 \rightarrow$										
<p>2 Determine the cumulative number of uninfected fields for each dilution as follows: Beginning with the highest dilution, add the number of uninfected fields to the number counted in the next highest dilution. Repeat for all dilutions</p> <p style="text-align: center;">Example of Cumulative Uninfected Fields</p>	$\leftarrow 16+20=36 \quad - \quad 0+16=16 \quad - \quad 0+0=0 \quad - \quad 0$										
<p>3 Determine the ratio of infected fields for each dilution using the formula:</p> <p style="text-align: center;">Example of Ratio of Infected Fields</p>	<table border="1"> <tr> <td colspan="2" style="text-align: center;">Cumulative infected fields</td> </tr> <tr> <td colspan="2" style="text-align: center;">Cumulative infected fields + Cumulative uninfected fields</td> </tr> <tr> <td style="text-align: center;">0/36=0</td> <td style="text-align: center;">4/20=0.20</td> </tr> <tr> <td style="text-align: center;">24/24=1</td> <td style="text-align: center;">44/44=1</td> </tr> </table>	Cumulative infected fields		Cumulative infected fields + Cumulative uninfected fields		0/36=0	4/20=0.20	24/24=1	44/44=1		
Cumulative infected fields											
Cumulative infected fields + Cumulative uninfected fields											
0/36=0	4/20=0.20										
24/24=1	44/44=1										
<p>4 Determine the percentage of infected fields for each dilution using the formula:</p> <p style="text-align: center;">Example of Percentage of Infected Fields</p>	<table border="1"> <tr> <td colspan="4" style="text-align: center;">Ratio of infected fields x 100</td> </tr> <tr> <td style="text-align: center;">0x100=0%</td> <td style="text-align: center;">0.2x100=20%</td> <td style="text-align: center;">1x100=100%</td> <td style="text-align: center;">1x100=100%</td> </tr> </table>	Ratio of infected fields x 100				0x100=0%	0.2x100=20%	1x100=100%	1x100=100%		
Ratio of infected fields x 100											
0x100=0%	0.2x100=20%	1x100=100%	1x100=100%								
<p>5 Determine the highest dilution that contains less than 50% infected fields, then determine the lowest dilution that contains greater than 50% infected fields.</p> <p style="text-align: center;">Example of % Infected Fields Next < 50%</p> <p style="text-align: center;">Example of % Infected Fields Next > 50%</p>	<table border="1"> <tr> <td style="text-align: center;">20%</td> <td style="text-align: center;">100%</td> </tr> <tr> <td colspan="2" style="text-align: center;">50% - % infected fields next < 50%</td> </tr> <tr> <td colspan="2" style="text-align: center;">% infected fields next > 50% - % infected fields next < 50%</td> </tr> <tr> <td style="text-align: center;">50 - 20</td> <td style="text-align: center;">= 0.3750</td> </tr> <tr> <td style="text-align: center;">100 - 20</td> <td></td> </tr> </table>	20%	100%	50% - % infected fields next < 50%		% infected fields next > 50% - % infected fields next < 50%		50 - 20	= 0.3750	100 - 20	
20%	100%										
50% - % infected fields next < 50%											
% infected fields next > 50% - % infected fields next < 50%											
50 - 20	= 0.3750										
100 - 20											
<p>6 Determine the interpolative value using the formula:</p> <p style="text-align: center;">Example of Interpolative Value</p>	$\frac{50 - 20}{100 - 20} = 0.3750$										
<p>7 Determine Log₁₀ of the dilution factor. In this example 5-fold dilutions are used.</p> <p style="text-align: center;">Example of Log₁₀ Dilution Factor</p>	<table border="1"> <tr> <td style="text-align: center;">Log₁₀ 5 = 0.7</td> </tr> </table>	Log ₁₀ 5 = 0.7									
Log ₁₀ 5 = 0.7											
<p>8 Determine the corrected interpolative value ("difference of logarithms") using the formula:</p> <p style="text-align: center;">Example of Corrected Interpolative Value</p>	<table border="1"> <tr> <td style="text-align: center;">interpolative value x Log₁₀ dilution factor</td> </tr> <tr> <td style="text-align: center;">0.375 x 0.7 = 0.2625</td> </tr> </table>	interpolative value x Log ₁₀ dilution factor	0.375 x 0.7 = 0.2625								
interpolative value x Log ₁₀ dilution factor											
0.375 x 0.7 = 0.2625											
<p>9 Identify the highest dilution that contains < 50% infected fields and determine its Log₁₀.</p> <p style="text-align: center;">Example of Dilution Next < 50% Infected Fields and its Log₁₀</p>	<table border="1"> <tr> <td style="text-align: center;">1:25</td> <td style="text-align: center;">Log₁₀ 25 = 1.40</td> </tr> </table>	1:25	Log ₁₀ 25 = 1.40								
1:25	Log ₁₀ 25 = 1.40										
<p>10 Determine the log reciprocal of the 50% endpoint dilution (log ED₅₀) using the formula:</p> <p style="text-align: center;">Example of Log ED₅₀</p>	<table border="1"> <tr> <td colspan="2" style="text-align: center;">Corrected interpolative value + Log₁₀ dilution next < 50% infected fields</td> </tr> <tr> <td style="text-align: center;">0.2625 + 1.40</td> <td style="text-align: center;">= 1.6625</td> </tr> </table>	Corrected interpolative value + Log ₁₀ dilution next < 50% infected fields		0.2625 + 1.40	= 1.6625						
Corrected interpolative value + Log ₁₀ dilution next < 50% infected fields											
0.2625 + 1.40	= 1.6625										
<p>11 Determine the 50% endpoint dilution (ED₅₀) by taking the inverse log of log ED₅₀</p> <p style="text-align: center;">Example of Log ED₅₀</p>	<table border="1"> <tr> <td style="text-align: center;">10^{1.6625} = 46</td> <td style="text-align: center;">ED₅₀ = 1:46</td> </tr> </table>	10 ^{1.6625} = 46	ED ₅₀ = 1:46								
10 ^{1.6625} = 46	ED ₅₀ = 1:46										

Fig. 19.4. Example of rabies virus neutralizing antibody 50% end-point titre calculation by Reed-Muench method¹

¹ Reproduced with permission from: Yager ML, Moore SM. The rapid fluorescent focus inhibition test. In: Rupprecht C, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention (Volume 2). Elsevier; 2015:199-215.

ED₅₀ Titers Corresponding to the Number of Fluorescing Foci (calculated by the Reed and Muench Method)
IU/mL Calculated from SRIG reading 6/20 in 1:125 serum dilution / titer=1:198

Infected Fields		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 1:5	IU/mL	0.11	0.11	0.10	0.10	0.09	0.09	0.08	0.07	0.07	0.06	0.05	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02
	Titer	11	11	10	10	9	9	8	7	7	6	5	4	4	3	3	3	3	3	2	2	2
Infected Fields		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2 1:25	IU/mL	0.57	0.55	0.52	0.49	0.46	0.43	0.4	0.36	0.33	0.29	0.25	0.22	0.19	0.17	0.16	0.15	0.14	0.13	0.12	0.12	
	Titer	56	54	51	49	46	43	40	36	33	29	25	22	19	17	16	15	14	13	12	12	
Infected Fields		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
3 1:125	IU/mL	2.83	2.71	2.59	2.45	2.31	2.16	2.00	1.83	1.65	1.46	1.26	1.09	0.97	0.87	0.8	0.74	0.69	0.65	0.62	0.59	
	Titer	280	268	256	243	229	214	198	181	163	145	125	108	96	86	79	73	68	64	61	58	
Infected Fields		0 / 20	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
4 1:125	IU/mL	14.12	13.5	12.9	12.3	11.5	10.8	10	9.15	8.25	7.3	6.31	5.45	4.83	4.35	3.99	3.7	3.45	3.25	3.09	2.95	
	Titer	1:198	1340	1278	1213	1143	1069	990	906	817	723	625	540	478	431	395	366	342	322	306	292	

Fig. 19.5. Calculation chart for 50% end-point titre by Reed–Muench method and associated IU/mL calculation by comparison against a standard rabies immunoglobulin serum (titre of 1:198)¹

¹ Reproduced with permission from: Yager ML, Moore SM. The rapid fluorescent focus inhibition test. In: Rupprecht C, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention (Volume 2). Elsevier; 2015:199–215.

Quality control and quality assurance

Quality control (QC) and quality assurance (QA) practices are essential to maintain consistently adequate performance of assays, to give meaning to the results and to allow comparison of results over time and among laboratories (13). Establishment and control of reagents, equipment and personnel training are the basis of a good QA programme. It is important to identify problems early because intervention is generally easier the sooner a problem is identified and limits the scope of reporting inaccuracies. Below is a general list of QC and QA practices to guide a RFFIT programme.

Note: The assay should be validated within the laboratory before use to define performance characteristics (precision, accuracy, linear range, sensitivity and specificity).

- 1.1 Expiration dates of reagents prepared in-house, such as EMEM10, must be determined in each laboratory by evaluation of effects on cell growth and RABV titre. This involves comparison of results from assays prepared with fresh and stored reagents. New batches of reagents impacting the cell culture such as fetal bovine serum (FBS) should also be similarly tested before use. Additionally, absence of RVNA in new batches of FBS must be confirmed by RFFIT testing before use.
- 1.2 Critical key components (RABV, cells, SRIG (or in-house reference serum), anti-rabies FITC conjugate) must be controlled and qualified before use; routine checks for accepted performance are vital for obtaining accurate and precise results.
 - 1.2.1 Use only qualified and defined challenge RABV seed and stock preparation; check RABV titre and (if possible) strain identity by sequencing.
 - 1.2.2 Routinely evaluate cell stocks for infection, including mycoplasma, and use only within a defined passage number.
 - 1.2.3 Qualify new SRIG lots (preparations) by comparing them with previously prepared lots; if not using an international SRIG, the in-house reference serum must be regularly qualified against an international SRIG.
 - 1.2.4 FITC-conjugate must be evaluated for specificity and quality of fluorescence.
- 1.3 Equipment used in the test performance should also be qualified and routinely checked, especially CO₂ and temperature control of the incubators and accuracy/precision of pipettes; as well as, class II biosafety cabinets. Storage equipment (refrigerators and freezers) must be monitored to detect out of range temperature levels and lapses in temperature that may affect the quality of samples or reagents. Proper functioning of microscopes is critical; routine checks and cleaning are recommended.
- 1.4 Best results are obtained by strictly controlling the key components and establishing a validated standard performance. This includes adequate training and evaluation of staff for each step and periodic qualifica-

- tion through proficiency testing. Of particular note is the importance of consistent slide reading between technicians.
- 1.5 Internal and external proficiency testing are the best tools to evaluate consistent assay performance, to identify any shifts or drifts and to enhance agreement between laboratories. Contacting another RFFIT laboratory to exchange samples for comparison is also an acceptable way to monitor performance if no established RFFIT proficiency programme is available.
 - 1.6 Performance level requirements (acceptance criteria for test results) should be within the performance criteria defined by internal validation.
 - 1.7 Documentation is critical in providing proof of high-quality results, troubleshooting and reconstruction of the assay.
 - 1.7.1 Assay steps should be clearly recorded (what, when, how and by whom).
 - 1.7.2 Personnel training and competency assessment; reagent receipt, storage, preparation and evaluation; and equipment performance should be recorded.
 - 1.7.3 Evaluation of documentation should be routinely performed to identify errors before results are reported.
 - 1.7.4 Records storage plan should be established and maintained.
 - 1.8 For troubleshooting, the following are suggested items to investigate:
 - 1.8.1 Serum sample quality (integrity and conservation of quality in storage).
 - 1.8.2 Water quality.
 - 1.8.3 Cell or RABV stock contamination.
 - 1.8.3.1 Increased ratio of unviable to viable RABV particles can cause inaccuracy of RVNA measurements.
 - 1.8.3.2 Cross-contamination of cells or RABV with other cell lines or virus strains.
 - 1.8.4 Errors in sample or reagent labelling/identity.
 - 1.8.5 Person to person variation in test performance steps.
 - 1.8.6 Changes in control serum, FBS or other reagents; variation in lots, effects of storage/shipping conditions.
 - 1.8.7 Changes in equipment performance.
 - 1.8.8 Changes in environment (pH, temperature, humidity, CO₂).
 - 1.9 Adaptions and changes in the assays for different purposes require investigation, development and validation (14–17).

Discussion

The RFFIT provides information regarding the RABV neutralization capabilities of rabies-specific antibodies circulating in the blood. As with other serum neutralization assays, it requires a significant amount of time and labour and, if all controls are not within parameters, more time must be spent investigating the test failure. Common testing problems can be traced to the health of cell and RABV cultures. Cell-based serological assays such as the RFFIT are expected to have less precision than other ligand-based assays due to dependence on biological processes involving interaction between (and within) cells and virus, and because both RABV and RVNA are affected by changes in environmental conditions, such as pH and temperature (18). Though challenging, control of the assay can be achieved by ongoing QA measures including equipment and reagent qualification and monitoring, personnel training, and routine assay QC performance and review. Of greatest importance is strict adherence to RABV dose and SRIG ranges, as many problems with drift in assay conditions can be detected by attention to ongoing tracking of these values. Detection in drifts and shifts in sensitivity, accuracy and precision of RVNA measurement can best be achieved through routine monitoring of controls and standards as well as proficiency test results. Establishment of assay performance parameters by method validation within each laboratory is essential to determine acceptance criteria and reportable range, and individual set performance of controls and standards will identify results not meeting these criteria (14). Although the RFFIT is the primary method for measuring RVNA, the requirements for performance in a BSL2 or BSL3 facility, high standardization and stringent controls limit its use in some areas. Although the method was developed and is most commonly used to measure and monitor RVNA in response to rabies vaccination and as determination of booster administration, it can be adapted for other purposes or to other platforms, such as tissue cultures plates. Annex 2 provides a method to perform the RFFIT on 96-well plates.

Other adaptations have been described and include use of recombinant RABV with green fluorescent protein (GFP) in place of CVS-11 to enhance the fluorescence emitted and eliminate the cost of time of the conjugate step; as well as, automated reading (13, 19, 20). Use of small sample sizes can be accommodated in various adaptations which have noted a change in linear range as compared with the classical RFFIT (21, 22). Adaptation for other purposes, which can range from product potency to research into RVNA effectiveness against specific RABV variants or new rabies biologicals, involves differing levels of performance characteristics. Although full coverage of this topic is beyond the scope of this chapter, suggestions are given in the QC and QA section above for validation for defining performance characteristics and ongoing quality control of modified RFFIT assays (23). Performance characteristics as defined by assay validation cannot be assumed to remain the same after modification of the assay; therefore, assays that have not been shown to produce correlated results are not comparable. The classical RFFIT is a robust method to measure the clinically significant component of rabies vaccination response: RABV neutralizing antibodies. Historically used for rabies vaccine evaluation and approval, it remains the gold standard assay for this purpose and provides information for the development of new rabies biologicals. Knowledge of the assay and its limitation and critical components is beneficial to the routine performance in the laboratory and to those utilizing the results.

References

1. Smith JS, Yager PA, Baer GM. A rapid reproducible test for determining rabies neutralizing antibody. *Bull World Health Organ.* 1973; 48:535–41.
2. Biosafety in microbiological and biomedical Laboratories. In: Chosewood LC, Wilson DE, editors. Atlanta (GA): Centers for Disease Control and Prevention; 2009.
3. Feysaguet M, Dacheux L, Audry L, Compoin A, Morize JL, Blanchard I, et al. Multicenter comparative study of a new ELISA, PLATELIA RABIES II, for the detection and titration of anti-rabies glycoprotein antibodies and comparison with the rapid fluorescent focus inhibition test (RFFIT) on human samples from vaccinated and non-vaccinated people. *Vaccine.* 2007; 25:2244–51. doi:S0264-410X(06)01329-6 [pii];10.1016/j.vaccine.2006.12.012 [doi].
4. Dacheux L, Wacharapluesadee S, Hemachudha T, Meslin FX, Buchy P, Reynes JM, et al. More accurate insight into the incidence of human rabies in developing countries through validated laboratory techniques. *PLoS Negl Trop Dis.* 2010; 4:e765. doi:10.1371/journal.pntd.0000765.
5. Cliquet F, Aubert M, Sagne L. Development of fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *J Immunol Methods.* 1998; 212:79–87. doi:10.1016/S0022-1759(97)00212-3.
6. European Pharmacopoeia. Human rabies immunoglobulin. Strasbourg: European Directorate for the Quality of Medicines and HealthCare; 2005:1750–1.
7. Namekar M, Kumar M, O’Connell M, Nerurkar VR. Effect of serum heat-inactivation and dilution on detection of anti-WNV antibodies in mice by West Nile virus E-protein microsphere immunoassay. *PLoS One.* 2012; 7:e45851. doi:10.1371/journal.pone.0045851.
8. Smith J. Rabies Serology. In: Baer GM, editor. *The natural history of rabies.* 2nd edition. Boca Raton (FL): CRC Press; 1991:235–52.
9. Kaplan MM, Wiktor TJ, Maes RF, Campbell JB, Koprowski H. Effect of polyions on the infectivity of rabies virus in tissue culture: construction of a single-cycle growth curve. *J Virol.* 1967; 1:145–51. PMID:4990035.
10. Habel K. Habel test for potency. In: Meslin FX, Kaplan MM, Koprowski H, editors. *Laboratory techniques in rabies, 4th edition.* Geneva: World Health Organization; 1996:369–73.
11. Manning SE, Rupprecht CE, Fishbein D, Hanlon CA, Lumlerdacha B, Guerra M, et al. Human rabies prevention – United States, 2008: recommendations of the Advisory Committee on Immunization Practices. *MMWR Recomm Rep.* 2008; 57(RR-3):1–28. doi:rr5703a1 [pii].
12. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012; http://apps.who.int/iris/bitstream/handle/10665/85346/9789240690943_eng.pdf, accessed 24 May 2018).

13. Moore SM, Gordon CR, Hanlon CA. Measures of rabies immunity. In: Jackson AC, editor. Rabies, 3rd edition. San Diego (CA): Elsevier; 2013:461–96.
14. Kostense S, Moore S, Companjen A, Bakker AB, Marissen WE, von ER, et al. Validation of the rapid fluorescent focus inhibition test (RFFIT) for rabies virus neutralizing antibodies in clinical samples. *Antimicrob Agents Chemother.* 2012; 56:3524–30. doi:AAC.06179-11.
15. Validation of analytical procedures: text and methodology Q2(R1), (1995, 1995). Geneva: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Health.
16. Guidelines for validation of analytical procedures: methodology Q(2b), (5/19/1997, 1997). Geneva: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Health.
17. Chaloner-Larsson G, Anderson R, Egan A. A WHO guide to good manufacturing practice (GMP) requirements. Part: Validation. Geneva: World Health Organization; 1997 (http://apps.who.int/iris/bitstream/handle/10665/64465/WHO_VSQ_97.02.pdf, accessed 24 May 2018).
18. Kostense S, Hendriks J. Challenges of immunogenicity assays for vaccines. *Bioanalysis.* 2012; 4:397–406. doi:10.4155/bio.11.327.
19. Khawplod P, Inoue K, Shoji Y, Wilde H, Ubol S, Nishizono A, et al. A novel rapid fluorescent focus inhibition test for rabies virus using a recombinant rabies virus visualizing a green fluorescent protein. *J Virol Methods.* 2005; 125:35–40. doi:10.1016/j.jviromet.2004.12.003.
20. Peharpre D, Cliquet F, Sagne E, Renders C, Costy F, Aubert M. Comparison of visual microscopic and computer-automated fluorescence detection of rabies virus neutralizing antibodies. *J Vet Diagn Invest.* 1999; 11:330–3.
21. Jackson FR, Turmelle AS, Farino DM, Franka R, McCracken GF, Rupprecht CE. Experimental rabies virus infection of big brown bats (*Eptesicus fuscus*). *J Wildl Dis.* 2008; 44:612–21. doi:10.7589/0090-3558-44.3.612.
22. Turmelle AS, Allen LC, Schmidt-French BA, Jackson FR, Kunz TH, McCracken GF, et al. Response to vaccination with a commercial inactivated rabies vaccine in a captive colony of Brazilian free-tailed bats (*Tadarida brasiliensis*). *J Zoo Wildl Med.* 2010; 41:140–3.
23. Moore SM, Hanlon CA. Rabies-specific antibodies: measuring surrogates of protection against a fatal disease. *PLoS Negl Trop Dis.* 2010; 4:e595. doi:10.1371/journal.pntd.0000595.

Annex 1

A1. Reagents, equipment and biological material

1.1 Reagents

1.1.1 Growth media (EMEM10) preparation

1.1.1.1 Eagle's minimum essential medium (EMEM) with Earle's salts 10x is diluted to 1x with sterile, distilled, cell culture grade water. The diluted EMEM can be stored refrigerated before supplementation and used as growth media.

1.1.1.2 EMEM10 is prepared from 1x EMEM supplemented with the following to be used in the RFFIT procedures.

1.1.1.2.1 10% FBS

1.1.1.2.2 2% MEM non-essential vitamins (100x)

1.1.1.2.3 2% L-glutamine 200 mmol (100x)

1.1.1.2.4 1% sodium bicarbonate solution (7.5%)

1.1.1.2.5 1% antibiotic antimycotic (100x) (optional)

1.1.2 PBS free of magnesium and calcium, sterile, cell culture grade

1.1.3 Trypsin: either 0.25% trypsin or 0.5% trypsin EDTA may be used.

1.1.4 Acetone (100%) is used to fix the tissue culture monolayer to the glass slide surface before staining. If plastic slides are used, the acetone is diluted with water to an 80% stock.

1.2 Equipment

1.2.1 Work involving RABV and cell culture is performed under a Class II Biosafety Cabinet using standard rabies safety measures (1).

1.2.2 Multi-chambered tissue culture slides such as the 8-chamber (Lab-Tek) slides are used in the RFFIT. Microtitre titre plates can be used to pre-dilute samples of high titre before addition to Lab-Tek slides for final dilution. Modified RFFITs can be performed in 96-well plates (2), 384-well plates, or Terasaki plates; 4-well, 6 mm PTFE-coated slides can also be used with supplemental humidity and covers such as microtitre plate covers.

1.2.3 A humid, CO₂ controlled incubator is used for routine cell maintenance and for the RFFIT procedure. The recommended conditions are a 0.5–2.0% CO₂ and 35–37 °C for MNA cells, and 2–5% CO₂ and 35–37 °C for BHK cells.

1.2.4 A fluorescence microscope appropriately equipped to evaluate slides stained with anti-rabies FITC conjugate is used to detect presence or absence of RABV-infected cells. In particular, the microscope should be equipped by a longer focal lens if using RFFIT adapted to a 96-well plate to allow the plate to be read in an inverted position.

- 1.2.5 **Pipettes.** Automated pipettes can be used both to add diluent and to serially dilute specimens in both microtitre plates and in multi-chamber slides.
- 1.2.6 A water bath or heat block set at 56 °C for heat inactivation of serum samples.

1.3 Biological material

1.3.1 Cell cultures

- 1.3.1.1 Both murine neuroblastoma cells (MNA or Neuro-2) and baby hamster kidney cells (BHK-21 clone 13) cell lines have been used successfully in the RFFIT. Neuro-2 (N2A) and BHK-21 cell lines are available from American Type Culture Collection (ATCC) (Neuro-2a; <http://www.atcc.org/products/all/CCL-131.aspx>. BHK-21; <http://www.atcc.org/en/Global/Products/5/8/C/6/CCL-10.aspx>). MNA cell lines are available from Diagnostic Hybrids/Quidel (<https://www.quidel.com/cultures-fluorescent-tests/cell-cultures/continuous-cell-lines/continuous-freshcells>);
- 1.3.1.2 Cell cultures are subcultured on a schedule per the supplier's specifications (e.g. every 3–4 days) by rinsing the monolayer of a T75 cm² flask with PBS and then adding 2 mL of room temperature trypsin. The flask is incubated at room temperature or 35–37 °C. After the cell layer detaches from the flask surface, sufficient EMEM10 is added to the flask to result in a suspension containing 15–20 x 10⁶ cells. The suspension is carefully mixed by pipetting to disperse cell clumping. It may be necessary to perform a viable cell count using trypan blue at a dilution of 1:4 (0.5 mL cell suspension; 1.5 mL of 0.5% trypan blue); 3 x 10⁶ cells are seeded into a number of new T75 flasks containing enough fresh EMEM10 to total 25 mL including the cell suspension and trypsin. These flasks are incubated at the appropriate temperature and CO₂ levels recommended by the supplier, until ready for use in RFFIT procedures (e.g. 3–5 days). These procedures can be modified according to laboratory conditions and cell lines in order to obtain a suspension of 15–20 x 10⁶ cells/flask on the day of use. T150 cm² flasks can be used by doubling all reagent volumes except trypsin, which is increased to 2.5 mL. These flasks should yield 30–40 x 10⁶ cells. After cell cultures are established, seed and stock RABV are grown in the cells and evaluated for further use in testing.

1.3.2 RABV

A supply of “seed” RABV Challenge Virus Standard (CVS-11) is first produced and tested for sufficient infectivity; from this “seed”, “stock” virus is grown and used in the RFFIT procedure. CVS-11 RABV is available from multiple sources, such as ATCC (<http://www.atcc.org/Products/All/VR-959.aspx#generalinformation>) as well as WHO or OIE collaborating centres.

1.3.2.1 Titre measurement of the source RABV

The RABV titre is measured by making 10-fold dilutions in EMEM10 from 10^{-1} to 10^{-8} . 0.1 mL of each dilution is added to wells in a chamber slide and incubated at the appropriate temperature and CO₂ level for 90 min. 0.2 mL of cell suspension containing 2.5×10^5 cells/mL is added to each well and the slide is incubated at the appropriate temperature and CO₂ level for 40 h. The slide is then fixed, stained and evaluated for RABV-infected cells; 20 fields in each well showing a good monolayer of cell growth are chosen to observe for RABV-infected cells. The fields are recorded as positive for infection if they show one or more fluorescing foci. The ED₅₀ titre of the RABV is interpolated using the Reed–Muench method as demonstrated in Fig. A19.1.

1.3.2.2 RABV seed production

The RABV should show infectivity at the 10^{-6} dilution to be used in production of RABV indicating at least 1×10^6 infectious units/0.1 mL (one fluorescing focus observed at the 10^{-6} dilution). For RABV production, multiplicity of infection (MOI) of 0.3 is described (1 infectious unit per every 3 cells). MOI of 0.1 and 0.01 have been successfully used and may vary per cell type used. If using 0.1 or 0.01 the amount of RABV added will be reduced accordingly.

A T150 cm² flask is trypsinized and resuspended to 30×10^6 cells/20 mL EMEM10. The cell suspension is centrifuged at $500 \times g$ for 10 min; 17.3 mL of the supernatant is aspirated and discarded and 10×10^6 infectious units of CVS-11 RABV are added and the tube vortex mixed. The cell–RABV suspension is incubated at the appropriate temperature and CO₂ level for 15–20 min and gently mixed once during the incubation to allow virus infection of the cells; 10 mL of EMEM10 is added to the tube, the suspension vortex mixed and then centrifuged at $500 \times g$ for 10 min. The supernatant is aspirated and discarded. The cell–RABV suspension is resuspended in 10 mL of EMEM10 and transferred to a T150 cm² flask filled with 20 mL EMEM10. Three 8-well (Lab-Tek) slides are prepared; label one

Determination of Rabies Virus 50% End Point Titer Calculated by the Reed-Muench Method Color coding is used for tracking steps

INSTRUCTIONS

Virus Dilution

Example RFFIT Lab Tek Slide

Fields Containing Infected Foci

Fields Containing Uninfected Foci

- 1 Determine the cumulative number of **infected** fields for each dilution as follows:
Beginning with the **highest** dilution, add the number of infected fields counted to the number counted in the next **highest** dilution. **Repeat for all dilutions**

Example of Cumulative infected fields

- 2 Determine the cumulative number of **uninfected** fields for each dilution as follows:
Beginning with the **lowest** dilution, add the number of uninfected fields to the number counted in the next **lowest** dilution. **Repeat for all dilutions**

Example of Cumulative uninfected fields

Following the instructions given for ED₅₀ of neutralizing antibody

- 3 Determine the ratio of infected fields for each dilution using the formula:

Example of Ratio of Infected Fields

- 4 Determine the percentage of infected fields for each dilution using the formula:

Example of Percentage of Infected Fields

- 5 Determine the **highest** dilution that contains less than 50% infected fields, then determine the **lowest** dilution that contains greater than 50% infected fields.

Example of % Infected Fields Next < 50%

Example of % Infected Fields Next > 50%

- 6 Determine the interpolative value using the formula:

Example of Interpolative Value

- 7 Determine Log₁₀ of the dilution factor. In this example 10-fold dilutions are used.

Example of Log₁₀ Dilution Factor

- 8 Determine the corrected interpolative value ("difference of logarithms") using the formula:

Example of Corrected Interpolative Value

- 9 Identify the highest dilution that contains < 50% infected fields.

Example of Dilution Next < 50% Infected Fields


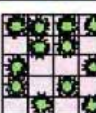
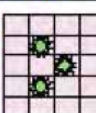

- 10 Determine the log reciprocal of the 50% endpoint dilution (log ED₅₀) using the formula:

Example of Log ED₅₀

- 11 Determine the 50% endpoint dilution (ED₅₀) by taking the inverse log of log ED₅₀.

Example of Log ED₅₀

SAMPLE PROBLEM

10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
			
20	12	3	0
0	8	17	20

← 15+20=35 - 3+12=15 - 0+3=3 - 0

0 - 0+8=8 - 8+17=25 - 25+20=45 →

Cumulative infected fields			
Cumulative uninfected fields			
Cumulative infected fields + Cumulative uninfected fields			
35/35=1	15/23=0.65	3/28=0.11	0/45=0

1x100=100% 0.65x100=65% 0.11x100=11% 0x100=0%

11%

65%

50% - % infected fields next < 50%	
% infected fields next > 50% - % infected fields next < 50%	
50 - 11	= 0.722
65 - 11	

Log₁₀ 10 = 1.0

0.722 x 1.0 = 0.722

-5

Log₁₀ dilution next < 50% infected fields - Corrected interpolative value

5 - 0.722 = 4.278

10^{4.278} = 18967 ED₅₀ = 1:18967

Fig. A19.1. Example of rabies virus 50% end-point titre calculation by Reed-Muench method¹

¹ Reproduced with permission from: Yager ML, Moore SM. The rapid fluorescent focus inhibition test. In: Rupprecht C, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention (Volume 2). Elsevier; 2015:199-215.

20 hours, one 40 hours and one 64 hours. The 20-hour slide receives 0.4 mL of cell/RABV suspension in each well. The 40 and 64 hours slides receive 0.2 mL of cell/RABV suspension and 0.2 mL of EMEM10 in each well. The flask and slides are incubated at the appropriate temperature and CO₂ level. One slide is acetone fixed, stained and evaluated after 20, 40 and 64 h; 24 h after a slide shows 100% RABV infectivity, the supernatant is removed from the flask, centrifuged at 4000 x *g* for 10 min to remove cell debris, and aliquoted in a volume useful for future stock virus production (e.g. 0.5 mL) for storage at –80 °C. Measure the titre of the prepared “seed” RABV as described above.

1.3.2.3 Production of the stock RABV

Only “seed” RABV should be used to prepare “stock” RABV for use in the RFFIT procedure to avoid changes in the virus proteins, especially the antigenic sites on the glycoprotein. The stock RABV is prepared as described above for the seed production and is aliquoted for storage at –80 °C in volumes appropriate to testing needs and to avoid multiple freeze–thaw cycles which will progressively reduce the RABV titre.

1.3.2.4 RFFIT challenge RABV dilution

The stock RABV titre is measured as described above. The dilution, which contains 10 infected fields and 10 uninfected fields, is the ED₅₀ dilution of the RABV and contains 1 FFID₅₀/0.1 mL. After determining the ED₅₀ dilution of the RABV, the challenge dose used for the RFFIT can be determined. The target challenge dose of virus is 50 FFID₅₀/0.1 mL with an acceptable range of 30–100 FFID₅₀/0.1 mL. The working dilution corresponding to 50 FFID₅₀ can be calculated as in the following example:

RABV ED₅₀ dilution is 10^{-5.6} dilution = 1 FFID₅₀/0.1 mL

50 FFID₅₀/0.1 mL: 5.6 – log 50 = 5.6 – 1.7 = 3.9 (dilution of 10^{-3.9})

1.3.3 SRIG preparation

A SRIG, typically provided as a lyophilized product, should be rehydrated as directed on the label to obtain a working stock containing 2 IU/mL. At least two international SRIGs are available. The WHO 1st international SRIG can be obtained from FDA/CBER (Lot R-3) and the WHO 2nd international SRIG can be obtained from NIBSC (<http://www.nibsc.org/>). The prepared SRIG should be aliquoted and stored frozen at –80 °C for individual use.

1.3.4 FITC-labelled anti-rabies conjugate

The FITC conjugate is prepared per the manufacturer's instructions. Several different dilutions can be made to determine the optimal working dilution providing the bright apple-green staining of intracellular virus with the least nonspecific background fluorescence.

References

1. Chosewood LC, Wilson DE, editors. Biosafety in microbiological and biomedical laboratories, 5th edition. Atlanta (GA): Centers for Disease Control and Prevention; 2009.
2. Zalan E, Wilson C, Pukitis D. A microtest for the quantitation of rabies virus neutralizing antibodies. *J Biol Stand.* 1979; 7:213–20. doi:10.1016/S0092-1157(79)80024-4.

Annex 2

A2. RFFIT modified for 96-well microtitre plate platform principle

RFFIT performed on a 96-well microtitre plate platform rather than in Lab-Tek chamber slides follows the sample principle as the classical RFFIT procedure. A cell line that is a subculture of BHK cells called BSR has been used in this method. Establishment of the cell line and growth and qualification of CVS-11 can be performed as for classical RFFIT. Each set of samples, including internal standards, described below is tested in two independent sets to provide two results that are averaged for the final results (see calculation of the titres, step 2.5.9, below). The steps that vary from the RFFIT procedure in this chapter are listed below.

2.2 Reagents (not previously mentioned for classical RFFIT)

- 2.2.1 Culture medium DMEM10: Dulbecco's MEM (DMEM), supplemented with high glucose (4500 mg/L), L-alanyl-L-glutamine (GlutaMAX) (862 mg/L), pyruvate (110 mg/L) and 10% of inactivated fetal bovine serum (FBS).
- 2.2.2 Glycerin 80% (in PBS)
- 2.2.3 Evans Blue (source)

2.3 Equipment (not previously mentioned for classical RFFIT)

- 2.3.1 96-well plates with flat bottom (clear colour, adapted to adherent cell culture)

2.4 Biological material

- 2.4.1 BSR cells (1) (available from some WHO collaborating centres on rabies; see [Appendix 2](#))
- 2.4.2 RABV CVS-11

2.4.2.1 Titre measurement of challenge RABV

For each stock suspension of CVS-11 RABV used for RFFIT, the percentage of FFID is determined. This titration is performed in parallel on two lines of a 96-well plate. Several remaining wells are reserved for cell control wells which do not receive RABV inoculation. Successive 2-fold dilutions of the RABV in DMEM10 are prepared in duplicate in a separate 96-well plate or in tubes. For this purpose, 70 µL of the viral suspension is diluted in 70 µL of DMEM10. Distribute 100 µL of DMEM10 into the final 96-well plate. Then transfer 50 µL of each of the RABV dilutions previously carried out. Incubate the plate for 1 h at 37 °C in humid atmosphere under 5% CO₂. Distribute 50 µL of cell suspension to each well (cells diluted in DMEM10 to a concentration of 1 x 10⁶ cells/mL to result in about 50 000 cells per well), including the cell control

wells. Incubate for 24 h at 37 °C in humid atmosphere under 5% CO₂. Discard the supernatant from the plate into a virucidal solution and gently rinse three times with PBS, discarding the liquid contents into a virucidal solution each time. Fix the wells with cold acetone diluted to 80%. Discard the acetone and let the plate air dry. Add 50 µL of FITC anti-rabies conjugate (with 1:2000 Evans Blue added) to each well. Incubate the plate for 30 min at 37 °C in a humid incubator. Rinse the plate three times with PBS. Drain the plate and add a drop of 80% glycerin to each well. The 96-well plate is evaluated under a fluorescence microscope (typically with 160–200x) and should be equipped with a focal lens long enough to read the plates in an inverted position (this step can be simplified using an inverted fluorescence microscope). Each well is observed for the presence of fluorescing foci. The percentage of infected cells is estimated by the reader and a percentage of infected cells (from 0% to 100%) is attributed to each well.

2.4.2.2 Determination of challenge dose

The RABV titration is repeated two times independently and the mean percentage is used for calibration of the RABV titre. A dilution giving a percentage of infection between 80% and 95% of the cells is the working dilution and designed as the challenge dose or dilution giving 80 FFID.

2.5 Method – Modified RFFIT on 96-well microtitre plates

2.5.1 In the test plate (see example in Fig.A19.1), add 100 µL of DMEM10 to each well.

2.5.2 Sample preparation

Sera are heat-inactivated at 56 °C for 30 min before testing.

2.5.3 Sample dilution

Using DMEM10, prepare an initial 1:3 dilution of the sera in tubes or in a 96-well plate. Then, distribute to the first well 50 µL of the 1:3 dilution prepared previously. Perform directly in the test plate successive 3-fold dilutions of the sera by transferring 50 µL of the previous well. Discard 50 µL of the final dilution.

2.5.4 Control systems

See Fig. A19.2 for an example of a titration plate layout.

2.5.4.1 Reference serum

2.5.4.2 SRIG diluted in the same manner as the test samples.

2.5.4.3 Internal standards

2.5.4.3.1 High positive serum (IS high pos) diluted in the same manner as the test samples.

2.5.4.3.2 Low positive serum (IS pos) diluted in the same manner as the test samples.

2.5.4.3.3 Negative serum (IS neg) diluted in the same manner as the test samples.

By courtesy of Susan Moore, Kansas State University, Manhattan, USA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Serum 1 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	Serum 6 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)
B	Serum 2 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	Serum 7 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)
C	Serum 3 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	Serum 8 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)
D	Serum 4 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	Serum 9 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)
E	Serum 5 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	Serum 10 (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)
F	SRIG (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	CVS (1:2)	(1:4)	(1:8)	(1:16)	(1:32)	(1:64)
G	IS (pos) (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	IS (neg) (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	Cell control
H	IS (high pos) (1:9)	(1:27)	(1:81)	(1:243)	(1:729)	(1:2187)	(1:6561)	(1:10883)	(1:59040)	(1:177147)	(1:531441)	Cell control

Fig. A19.2. Plate layout diagram for the modified RFFIT on 96-well microtitre plates (dilutions are indicated in parentheses)

CVS, challenge virus standard strain; IS (high pos): high RVNA positive serum; IS (neg): negative RVNA serum; IS (pos): low positive RVNA serum; RVNA, rabies virus neutralizing antibodies; SRIG: standard rabies immunoglobulin

2.5.4.4 Cell control

In the wells used for the cell control, add an additional 50 µL DMEM10 (final volume of DMEM10 is 150 µL as these wells will not receive an inoculation of RABV).

2.5.4.5 RABV back titration

The back titration of the challenge RABV is prepared separately in tubes or in a plate. Two-fold dilutions of the RABV are prepared and then 50 µL of each of these dilutions are transferred into the corresponding RABV control wells (in green in Fig. 1). Typical dilutions used are 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64.

2.5.5 Challenge RABV dilution preparation and inoculation

2.5.5.1 Add 50 µL of challenge RABV suspension (working dilution previously determined to give 80–95% infection) to each well except the cell control wells and the RABV back titration wells. Incubate for 1 h at 37 °C in a humid atmosphere under 5% CO₂ to allow neutralization.

2.5.6 Cell suspension preparation and addition

Prepare the cells as per the RFFIT procedure and adjust the concentration to 1 x 10⁶ cells/mL. Distribute 50 µL of the cell suspension (50 000 cells) in each well. Incubate for 24 h at 37 °C in a humid atmosphere under 5% CO₂.

2.5.7 Acetone fixation and staining

Discard the supernatant from the plate into a virucidal solution and gently rinse three times with PBS, discarding the liquid contents into a virucidal solution each time. Fix the plate with 80% acetone for 30 min at 4 °C. Discard the acetone and let the plate air dry. Add 50 µL of anti-rabies FITC conjugate (with 1:2000 Evans Blue added) to each well and incubate the plate for 30 min at 37 °C within a humid incubator. Rinse the plate three times with PBS. Drain the plate and add a drop of 80% glycerin to each well.

2.5.8 Microscopic evaluation

2.5.8.1 Using a microscope equipped by a longer focal lens to read the plates in an inverted position (this step can be simplified using of an inverted fluorescence microscope), at final magnification 160–200x, each well is observed in total for the presence of fluorescing foci. The percentage of infected cells is estimated by the reader and a percentage of infected cells (from 0% to 100%) is attributed to each well.

2.5.9 Calculation of the titres

As per the classical RFFIT method, use the Reed–Muench method to calculate the 50% end-point titre using the percentage infected values. The IU/mL values are calculated as per the RFFIT method. The final result is an average of two of the IU/mL values obtained from independent sets. Note: additional tests (retesting) can be performed if one titre value is above 0.5 UI/ml and the second one is below the value and/or if the ratio between the two values is equal to or greater than 3. In those cases, the mean value is calculated when titre values meet these two conditions (ratio between the values is less than 3 and all below or above 0.5 UI/mL). In addition, for sera with titres above the upper limit of the assay, they should be tested again with a pre-dilution step (e.g. pre-dilution of 1:10 or 1:50).

Acceptance criteria for the internal standards are based on their titre values, which have to be comprised in specific intervals previously defined and validated by the laboratory. These acceptance criteria are similar to those already found in the description of the classical RFFIT method.

The titre values of tested serum samples are validated only after all acceptance criteria are satisfied.

2.6 Quality control and quality assurance

Acceptance criteria for the controls and standards

2.6.1 Check for the viability of the cells before fixing.

2.6.2 Check for the absence of infection in the cell control.

2.6.3 Check that the challenge dose of rabies RABV is infecting between 80% and 95% of the cells.

Chapter 20

The fluorescent antibody virus neutralization test

Introduction

Preventive vaccination against rabies is a powerful tool to prevent, control and eliminate the disease (1–4). The presence of rabies virus (RABV) virus neutralizing antibodies (VNAs) in serum is considered a reliable indicator of adequate vaccination (5–7) ensuring satisfactory protection against rabies (8, 9). The level of VNAs is expressed in International Units per millilitre (IU/mL). To be considered adequate, the minimal level of VNAs required by international authorities is equal to 0.5 IU/mL (10, 11). Rabies serum neutralization tests offer a consistent, reliable way to measure the level of rabies VNA contained in serum samples.

The fluorescent antibody virus neutralization (FAVN) test was adapted from the original rapid fluorescent focus inhibition test (RFFIT) and thoroughly evaluated against both recommended methods such as the RFFIT and the mouse neutralization test (MNT) (12). The results showed consistency among all three tests (12). This method, using 96-well microplates instead of 8-well (Lab-Tek) chambers, was mainly intended to provide a reliable reading. Indeed, the FAVN test uses a qualitative microscopic reading (all or nothing), which is less time consuming than tests that employ the counting of infected fields or foci. The FAVN test allows easy distinction of low positive titres in serum samples thanks to its high sensitivity. Currently, besides the RFFIT, the FAVN test is one of the only two reference serum neutralization tests prescribed by WHO and OIE to detect the level of VNAs (10, 11).

The principle of the FAVN test is the *in vitro* neutralization of a constant amount of RABV, using the challenge virus standard CVS strain adapted to cell culture, before inoculating RABV permissive cells (BHK-21 C13). Each dilution of the sera under test and of the challenge virus used for back titration is tested in quadruplicates. The serum titre is the reciprocal of dilution at which 100% of the virus is neutralized in 50% of the wells. This titre is expressed in IU/mL by comparing this dilution with the neutralizing dilution of a standard serum tested under the same experimental conditions (OIE serum of dog origin or WHO standard rabies immunoglobulin (SRIG) or internal standard previously calibrated against the OIE serum or the WHO standard).

Materials

Reagents

Cell culture medium

- DMEM (Dulbecco's modified Eagle's medium + glucose 4500 mg/ L + L-glutamine – pyruvate); for FAVN testing and virus production
- GMEM (Glasgow Minimum Essential Medium + L-glutamine – pyruvate); the maintenance medium for cell culture
- fetal calf serum (FCS): heat-inactivated at 56 °C for 30 min

Antibiotics

- plasmocin (25 mg/mL)
- mixture of penicillin (100 units), streptomycin (100 µg) and amphotericin B (0.25 µg)
- sterile PBS buffer, pH 7.2 without Ca²⁺ and Mg²⁺
- trypsin ethylene diaminetetraaceticacid (EDTA) 10x
- acetone 80%
- phosphate buffered saline (PBS)
- positive reference serum: OIE reference serum or WHO international standard or internal standard (previously calibrated against the OIE serum or the WHO standard)
- naive reference serum
- conjugate: fluorescein isothiocyanate (FITC) anti-rabies monoclonal globulin (Fujirebio Diagnostics Inc.)

Equipment

- humidified incubator at 36 °C ± 2 °C with 5% CO₂
- dry incubator at 37 °C
- biosafety cabinet
- 80 °C freezer or liquid nitrogen tank
- fluorescence microscope suitable for FITC fluorescence equipped with 10x eye-piece and 10x objective (the global magnification of the microscope ranges between 100 and 125 due to the extra magnification of some epi-fluorescence systems)

Biological materials

- cell line: BHK-21 C13 cells (ATCC number: CCL-10) maintained in GMEM supplemented with 10% of heat-inactivated FCS and antibiotics (v/v). Cells are routinely passaged twice a week
- virus strain: CVS-11 (ATCC number: VR 959)

Methods

Production of CVS-11 virus on cells

Growth of cells

The BHK-21 C13 cells are used to produce the CVS virus. They are trypsinized during the exponential phase of their kinetic growth. If the confluence of the monolayer is complete, the culture should be split. The cells in the cell suspension should not be clumped, and around 50×10^5 cells are needed for seeding a 75 cm² cell culture flask.

Cells are collected within a volume of 20–30 mL in cell culture medium containing 10% (v/v) heat-inactivated FCS.

Infection of cells

The multiplicity of infection (number of infective particles per cell) is adjusted between 0.1 and 0.5. The vessel containing the virus–cell suspension is incubated for 60 min at $35 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$. The contents of the vessel are gently stirred every 10–15 min.

Virus growth

The virus/cell suspension is centrifuged at $800\text{--}1000 \times g$ for 15 min and the cell pellet is re-suspended in the cell culture medium supplemented with 10% heat-inactivated FCS. Virus is harvested 2 days later.

Harvest and storage

The culture supernatant is clarified by centrifugation at $800\text{--}1000 \times g$ for 15 min at $4 \text{ }^\circ\text{C}$. If several flasks have been used, the different clarified supernatants are mixed and then aliquoted and frozen at $-80 \text{ }^\circ\text{C}$. The infective titre of the harvest is established at least 3 days after freezing.

Determination of virus titre by TCID₅₀ assay (50% tissue culture infective dose)

The day before titration, a cell suspension (BHK-21 C13 cells), containing 10^5 cells/mL is prepared in complete medium containing 10% heat-inactivated FCS, and is distributed, 200 μL per well, into 96-well microplates. The plates are then incubated for 24 h at $35 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$ with 5% CO₂.

The serial dilutions of the virus are performed in 5 mL tubes using incomplete medium as diluent. Twelve serial 10-fold dilutions from 10^{-1} to 10^{-12} are prepared (0.9 mL of diluent with 0.1 mL of the previous dilution).

For infecting the cell monolayer, the medium in the microplates is discarded and 50 μL of each virus dilution is distributed per well (six replicates per dilution).

The microplates are then incubated for 1 h at $35 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$ with 5% CO₂, and then 200 μL of cell culture medium, containing 5% FCS, is added.

The microplates are incubated for 3 days at $35\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ in 5% CO_2 .

After around 72 h, the cells are stained using the FITC conjugate, as detailed below for the FAVN test procedure.

Reading is qualitative. Every well that shows specific fluorescence is considered to be positive. The titre is calculated using either the neoprobit graphic method or the Spearman–Kärber method.

The CVS titration must also be performed by the FAVN test to establish the infective titre by the TCID₅₀ assay in the FAVN test conditions.

FAVN testing

One FAVN test is routinely made up of five plates (96-well microtitre plate): one control plate and four plates with sera to be tested. The sera to be tested are previously heat-inactivated at $56\text{ }^{\circ}\text{C}$ for 30 min. The sera are stored at $-20\text{ }^{\circ}\text{C}$ until use.

Distribution of the cell culture medium

The medium used in FAVN testing is the DMEM medium supplemented with 10% heat-inactivated FCS and antibiotics. It is distributed according to the following patterns:

For the control plate

- CVS back titration: 150 μL of medium per well (A1–A4 -> H1–H4: 32 wells)
- naive reference serum titration: 100 μL of medium per well (A5–A8 -> C5–C8: 12 wells)
- positive reference serum 0.5 IU/mL titration: 100 μL of medium per well (D5–D8 -> H5–H8: 20 wells)
- serum to be tested titration (see the template of the microplate): 100 μL of medium per well (D9–D12 -> H9–H12: 24 wells); for the final (last) dilution: 200 μL of medium per well (C9–C12: 4 wells)
- cell control: 150 μL of medium per well (A9–A12: 4 wells)
- virus control: 100 μL of medium per well (B9–B12: 4 wells)

For the plates with sera to be tested

Four sera are tested per plate according to the scheme.

For the sera to be tested (see the template of the microplate): 100 μL of medium per well (all wells except A6–H6 (8 wells) and A12–H12 (8 wells): 80 wells); for the final (last) dilution, 200 μL of medium per well (A6–H6: 8 wells; A12–H12: 8 wells).

Naive reference serum

	1	2	3	4	5	6	7	8	9	10	11	12	
A													Cell control
B													CVS control
C					X	X	X	X					4.23 (final dilution)
D													2.39
E													1.91
F													1.43
G													0.95
H	X	X	X	X	X	X	X	X	X	X	X	X	0.48

CVS back titration Positive reference serum Serum to be titrated
0.5 IU/mL

CVS, challenge virus standard; X: place where undiluted virus and sera will be deposited

Distribution and dilution of the reference and test sera

The reference and test sera are distributed in quadruplicate; 50 µL of serum are distributed per well in the four first wells (noted with X on the plate). All the dilutions are then performed directly in the 96-well microtitre plates. Each dilution is mixed well by pipetting at least eight times.

For the naive and the positive reference sera

	1	2	3	4	5	6	7	8	9	10	11	12	
A	X						X						
B	X						X						
C	X						X						
D	X						X						
E	X						X						
F	X						X						
G	X						X						
H	X						X						
	0.48	0.95	1.43	1.91	2.39	4.23 Final dilution	0.48	0.95	1.43	1.91	2.39	4.23 Final dilution	

X: place where undiluted virus and sera will be deposited

The serial dilution is made with a dilution step of three (50 µL transfer). For the last line of wells, the 50 µL are discarded. The final volume in each well is equal to 100 µL.

For the test sera

The serial dilution is made with a dilution step of three (50 µL transfer). For the final dilution, the following steps are undertaken:

- For the last but one line of 4 wells, 40 µL is discarded.
- 10 µL are transferred in the last line of wells which contain 200 µL.
- The dilution is then mixed well by repeated pipetting.
- 180 µL are discarded and 70 µL of medium (DMEM supplemented with 10% heat-inactivated FCS and antibiotics) is added to obtain a final volume of 100 µL.
- The final volume in each well is equal to 100 µL.

This dilution is very high (corresponding to a dilution factor of 24547) to make sure that the virus has been distributed. At the final dilution, most of the high titred sera will provide fluorescence; the “green line” on the microplate is a guarantee that the virus has been correctly distributed; the final dilution avoids obtaining complete microplates without any fluorescence.

Addition of challenge virus standard strain

The stock of CVS virus is stored at –80 °C and aliquoted in cryotubes. One vial containing the viral suspension is thawed rapidly under cold running water and stored in ice. One dilution from this tube is performed using the DMEM supplemented with 10% FCS and antibiotics as diluent. This dilution corresponds to the working dilution of the virus which allows to reach around 100 TCID₅₀ per 50 µL (TCID₅₀ = median tissue culture infective dose). The calibration and validation of the working dilution of CVS-11 virus allowing reaching 100 TCID₅₀/50 µL should be undertaken before using it to routinely carry out FAVN tests. The diluted virus should be used rapidly and kept on ice during its distribution to avoid the decrease of the titre during the test. The range allowed for the virus dose titre must be 30–300 TCID₅₀/50 µL.

For the plate with sera to be tested

Some 50 µL of diluted virus is added in each well. The final volume in each well is equal to 150 µL.

For the control plate

The virus should be distributed in the control plate at the end of the test to ensure that the titre of the virus remains still satisfactory throughout the test (that is, close to 100 TCID₅₀/50 µL as prescribed in the OIE manual (11).

Some 50 µL of diluted virus is added in each well of the control plate except for the wells for virus back titration (A1–A4 -> H1–H4: 32 wells) and cell control (A9–A12: 4 wells) [see the template]. The final volume in each well is equal to 150 µL.

For the virus back titration, 50 μL is added in the first four wells (H1–H4) and a serial dilution is performed with a dilution step of four (transfer of 50 μL from row to row). For the last line of wells, the 50 μL are discarded and the final volume in each well is equal to 150 μL .

Incubation of the plates

The microplates are incubated for 1 h at $36\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}/5\% \text{CO}_2$.

Distribution of the cell suspension

The cells are maintained in GMEM supplemented with 10% FCS and antibiotics. For the FAVN test, a subconfluent culture of BHK-21 cells aged 3 days should be trypsinized. The concentration of cells should be counted by using a cell counting chamber (for example, a haemocytometer [Neubauer chamber]). The final cell concentration must be calculated following the manufacturer's instructions, depending on the type of counting chamber used. The concentration of cells is expressed in 10^5 cells/mL.

According to the number of cells counted, the cells are re-suspended to obtain a final concentration of 4×10^5 cells/mL using DMEM supplemented with 10% FCS and antibiotics as diluent, and then 50 μL of the cell suspension is distributed to each well.

Incubation of the plates

The microplates are incubated for approximately 48 h at $36\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}/5\% \text{CO}_2$.

Fixation

After 48 h of incubation the medium is discarded. The microplates are rinsed first in PBS buffer, then in 80% acetone. The microplates are fixed with 80% acetone at room temperature for 30 min in a fume hood. The acetone is discarded, and the microplates are air dried at room temperature.

All the plates are checked for possible cytotoxicity (lack of monolayer, cell free patches in the monolayer, detachment of monolayers). The observations are written on the raw reading sheet.

Staining

Some 50 μL of the FITC conjugate (diluted appropriately in PBS) are distributed in each well of the plates. The microplates are gently rocked to ensure good distribution in the entire surface of the well. The microplates are incubated for 30 min at $37^{\circ} \pm 2\text{ }^{\circ}\text{C}$, then the fluorescent conjugate is discarded, and the microplates are rinsed twice with PBS buffer. The microplates are inverted briefly on absorbent paper to remove the residual PBS.

Reading

The wells are observed thoroughly. The reading is qualitative, and this is an “all or nothing” reading.

If there is one or several fluorescent cells, the well is considered as positive; if there is no fluorescent cell, the well is considered as negative. The control plate should be read first.

Calculation of the titres

The logD₅₀ titres of the CVS titration, the naive reference serum, the positive reference serum and the test sera are calculated according to the Spearman-Kärber method:

$$\log_{10} (\text{dilution } 50) = -\left(x_0 - \frac{d}{2} + d \sum \frac{r_i}{n_i}\right)$$

Where:

- log₁₀ (dilution 50) = logD₅₀ = logarithm of the dilution where 50% of the positive wells is found; that is, 50% of the fluorescence;
- x₀ = – (log₁₀ of the smallest dilution with all negative wells)
- d = log₁₀ of the dilution step;
- r_i = number of negative wells; and
- n_i = number of replicates.

For the sera:

logD₅₀ of the serum = [(number of negative wells/4) x log of dilution step – (log of dilution step/2) + log of the lowest dilution where there are four negative wells]

And for the CVS virus:

logD₅₀ of the virus = [(number of positive wells / 4) x log of dilution step – (log of dilution step / 2) + log of the lowest dilution where there are four positive wells]

Conversion of the titres

The conversion of the logD₅₀ titre of the serum in the IU/mL titre is performed by using the following formula:

$$\text{Serum titre (IU/mL)} = \frac{[(10^{(\text{serum logD}_{50} \text{ value})}) \times \text{theoretical titre of Positive Ref serum } 0.5 \text{ IU/mL}]}{(10^{(\text{theoretical logD}_{50} \text{ of Pos Ref serum})})}$$

For the theoretical logD₅₀ of positive reference serum, two kinds of values can be used to convert the logD₅₀ titre in IU/mL: either the “value of the day” or the mean logD₅₀ values of positive reference serum.

Validation of the test

The results of titration of CVS, naive serum and positive serum are reported on a control card for each of these three controls.

Every person who performs the FAVN test in the laboratory should have his or her own control card for CVS virus, and naive and positive reference sera. The control test results of the FAVN test are compared with the accumulated control test results from previous FAVN tests of the technician(s).

The test is validated only if the current values of the three controls are not statistically different from the mean \pm 2 standard deviation (SD) of all values obtained in the previous tests. If one of the controls is statistically different from the mean \pm 2 SD, the test is not validated, and the sera must be titrated once again.

Discussion

The FAVN test has been recommended by the OIE since 1998 (13) and by WHO since 2005 (10). European Commission Regulation EC No. 998/2003 (14) also requires measuring VNA as a proof of vaccination against rabies to allow the free movement of pets within the European Union and between certain developing and European countries. As such, the FAVN test is the most widely used by the approved rabies serology laboratories (http://ec.europa.eu/food/animal/liveanimals/pets/approval_en.htm) in the context of international trade. Indeed, 86% of these laboratories used this serum neutralization test to perform rabies serological controls on domestic carnivores.

The FAVN test is also used on wildlife samples to evaluate the impact of oral vaccination campaigns (15). However, field samples are often of poor quality (haemolysis, bacterial contamination) (16) and cells used in the FAVN test are sensitive to any cytotoxic products and contaminating agents present in samples, rendering it impossible to obtain reliable results (17, 18) and leading to inadequate monitoring of the effectiveness of oral vaccination campaigns. To overcome the cytotoxicity problem of field samples, some modified FAVN tests were developed (19, 20). The FAVN test is also widely used in seroprevalence studies for vaccinated dog and cat populations (21) in serological surveys in wild and domestic animals as well as in immunogenicity studies of animal or human vaccines (more than 226 citations).

Critical parameters

As described in the OIE manual (11), some steps of this procedure may be modified to comply with the health and safety requirements and individual working practices of the laboratory, but others must not be changed as they are standard operating procedures.

The following steps must not be changed for the virus titration:

- Inoculation of a 24-h monolayer.
- 10-fold dilutions prepared using 0.9 mL of diluent and 0.1 mL of virus suspension.

- Four to six 50 μ L replicates per dilution.
- Incubation for 72 h.
- Qualitative reading (i.e. the well is positive or negative).
- In every titration session, a vial of a control batch of virus is titrated and this titre is integrated in a control card to validate the titration process.
- Calculation according to neoprobit graphic or Spearman–Kärber methods.

The following steps must [not](#) be changed for the FAVN test:

- RABV: only the CVS-11 strain should be used.
- Cells culture: only BHK-21 cells (ATCC number – CCL 10) should be used.
- The FAVN test must be performed only in 96-well microplates.
- Control cards should be used for RABV, naive serum and positive standard serum.
- The back titration of the CVS virus, as well as naive serum and positive standard serum, must be present on the control plate.
- A minimum of four 3-fold dilutions of sera are required; the reading method is an “all or nothing” only.
- Four replicates of each serum should be diluted.
- For the conversion of log D_{50} in IU/mL, the laboratories should use only the log D_{50} value of the standard serum.

Precautions

Pre-exposure vaccination against rabies and regular serological controls are mandatory for all people working with RABV. All manipulations using RABV are performed in a biosafety cabinet.

Limitations

Although the FAVN test offers a valuable and reliable way of assessing the effectiveness of rabies vaccination by detecting VNA in serum samples, this method is time-consuming, expensive and requires highly trained technicians and the maintenance of cell culture as well as special laboratory facilities due to the need for stringent precautions when handling live RABV. In addition, since they are based on cell cultures, they are sensitive to any cytotoxic products and contaminating agents present in samples.

Future considerations

As technology progresses, new methods are being developed and validated to replace neutralization methods (22). For example, molecular methods have led to the development of a lentiviral vector system that expresses the viral glycoprotein (23). This technology offers a safe alternative to the handling of live RABV and requires only low serum volumes for analysis compared with neutralization tests. Another example is rapid test strips using colloidal gold particles, developed in 2010 for the detection of RABV antibodies (24). A magnetic protein micro-

bead-aided indirect fluoroimmunoassay has also been described (25) to determine the presence of canine virus-specific antibodies including RABV antibodies. Recently, a commercial rabies ELISA kit (BioPro Rabies ELISA Ab kit) for testing sera from domestic and wild carnivores has been assessed (26, 27). This test may become an alternative to serum neutralization tests for monitoring rabies oral vaccination campaigns. Moreover, a promising technique for field blood sampling has been developed to improve the monitoring of oral vaccination in Europe (28). This method, using the filter paper method to collect blood samples of dead animals, coupled with an ELISA test is an attractive and cost-effective alternative, easy to use for collecting blood in even extreme field conditions, for further monitoring RABV antibodies in wildlife after oral vaccination campaigns (28).

References

1. Rupprecht CE, Slate D. Rabies prevention and control: advances and challenges In: Dietzgen RG, Kuzmin, IV, editor. Rhabdoviruses: molecular taxonomy, evolution, genomics, ecology, host-vector interactions, cytopathology and control. Norfolk (UK): Caister Academic Press; 2012:215–52.
2. Singh MP, Goyal K, Majumdar M, Ratho RK. Prevalence of rabies antibodies in street and household dogs in Chandigarh, India. *Trop Anim Health Prod.* 2011; 43:111–4. doi:10.1007/s11250-010-9661-8.
3. Cliquet F, Aubert M. Elimination of terrestrial rabies in Western European countries. *Dev Biol (Basel).* 2004; 119:185–204.
4. Cliquet F, Robardet E, Must K, Laine M, Peik K, Picard-Meyer E, et al. Eliminating rabies in Estonia. *PLoS Negl Trop Dis.* 2012; 6:1–17. doi:10.1371/journal.pntd.0001535.
5. Clark KA, Wilson PJ. Postexposure rabies prophylaxis and preexposure rabies vaccination failure in domestic animals. *J Am Vet Med Assoc.* 1996; 208:1827–30. PMID:8675469.
6. Johnson N, Cunningham AF, Fooks AR. The immune response to rabies virus infection and vaccination. *Vaccine.* 2010; 28:3896–901. doi:10.1016/j.vaccine.2010.03.039.
7. Moore SM, Ricke TA, Davis RD, Briggs DJ. The influence of homologous vs. heterologous challenge virus strains on the serological test results of rabies virus neutralizing assays. *Biologicals.* 2005; 33:269–76. doi:10.1016/j.biologicals.2005.06.005.
8. Aubert MFA. Practical significance of rabies antibodies in cats and dogs. *Rev Sci Tech OIE.* 1992;11:735–60.
9. Brown LJ, Rosatte RC, Fehlner-Gardiner C, Knowles MK, Bachmann P, Davies JC, et al. Immunogenicity and efficacy of two rabies vaccines in wild-caught, captive raccoons. *J Wildl Dis.* 2011; 47:182–94.

10. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (http://apps.who.int/iris/bitstream/handle/10665/85346/9789240690943_eng.pdf, accessed 25 May 2018).
11. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees), 7th edition. ed. Paris: World Organisation for Animal Health; 2012.
12. Cliquet F, Aubert M, Sagne L. Development of fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *J Immunol Methods*. 1998; 212:79–87. doi:10.1016/S0022-1759(97)00212-3.
13. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds, and bees), 3rd edition. Paris: World Organisation for Animal Health; 1998.
14. Regulation (EC) No 998/2003 of the European Parliament and of the council of 26 May 2003 on the animal health requirements applicable to the non-commercial movements of pet animals and amending Council Directive 92/65/EEC. Brussels: European Commission; 2003.
15. De Benedictis P, Mancin M, Cattoli G, Capua I, Terregino C. Serological methods used for rabies post vaccination surveys: an analysis. *Vaccine*. 2012; 30:5611–5. doi:10.1016/j.vaccine.2012.06.089.
16. Cliquet F, Sagne L, Schereffer JL, Aubert MFA. ELISA test for rabies antibody titration in orally vaccinated foxes sampled in the fields. *Vaccine*. 2000; 18:3272–9. PMID:10869772.
17. Barton LD, Campbell JB. Measurement of rabies-specific antibodies in carnivores by an enzyme-linked immunosorbent assay. *J Wildl Dis*. 1988; 24:246–58. PMID:3286905.
18. Cliquet F, Muller T, Mutinelli F, Geronutti S, Brochier B, Selhorst T, et al. Standardisation and establishment of a rabies ELISA test in European laboratories for assessing the efficacy of oral fox vaccination campaigns. *Vaccine*. 2003; 21:2986–93. PMID:12798642.
19. Hostnik P, Grom J. An indirect immunofluorescent test for detection of rabies virus antibodies in foxes. *J Wildl Dis*. 1997; 33:143–5. PMID:9027703.
20. Bedekovic T, Lemo N, Lojkic I, Mihaljevic Z, Jungic A, Cvetnic Z, et al. Modification of the fluorescent antibody virus neutralisation test-Elimination of the cytotoxic effect for the detection of rabies virus neutralising antibodies. *J Virol Methods*. 2013; 189:204–8. doi:10.1016/j.jviromet.2013.01.022.
21. Shiraishi R, Nishimura M, Nakashima R, Enta C, Hirayama N. Neutralizing antibody response in dogs and cats inoculated with commercial inactivated rabies vaccines. *J Vet Med Sci*. 2013; 76:605–9. PMID:24389741.
22. Wasniewski M, Barrat J, Fooks AR, Franka R, Müller T, Sabeta C, Cliquet F. Production and calibration of the second batch of OIE anti-rabies positive reference serum. *Rev Sci Tech*. 2017; 36:779–88. doi:10.20506/rst.36.3.2713.

23. Wright E, Temperton NJ, Marston DA, McElhinney LM, Fooks AR, Weiss RA. Investigating antibody neutralization of lyssaviruses using lentiviral pseudo-types: a cross-species comparison. *J Gen Virol*. 2008; 89:2204–13. doi:10.1099/vir.0.2008/000349-0.
24. Wang H, Feng N, Yang S, Wang C, Wang T, Gao Y, et al. A rapid immunochromatographic test strip for detecting rabies virus antibody. *J Virol Methods*. 2010; 170:80–5. doi:10.1016/j.jviromet.2010.09.002.
25. Wang X, Ren L, Tu Q, Wang J, Zhang Y, Li M, et al. Magnetic protein microbead-aided indirect fluoroimmunoassay for the determination of canine virus specific antibodies. *Biosens Bioelectron*. 2011; 26:3353–60. PMID:21300534.
26. Wasniewski M, Cliquet F. Evaluation of ELISA for detection of rabies antibodies in domestic carnivores. *J Virol Methods*. 2012; 179:166–75. doi:10.1016/j.jviromet.2011.10.019.
27. Wasniewski M, Guiot AL, Schereffer JL, Tribout L, Mähar K, Cliquet F. Evaluation of an ELISA to detect rabies antibodies in orally vaccinated foxes and raccoon dogs sampled in the field. *J Virol Methods*. 2013; 187:264–70. PMID:23201293.
28. Wasniewski M, Barrat J, Combes B, Guiot AL, Cliquet F. Use of filter paper blood samples for rabies antibody detection in foxes and raccoon dogs. *J Virol Methods*. 2014; 204:11–6. doi:10.1016/j.jviromet.2014.04.005.

Chapter 21

An indirect fluorescent antibody test for the detection of rabies virus immunoglobulin G and immunoglobulin M antibodies

Introduction

Serological methods for the detection of rabies virus (RABV)-specific antibodies include antigen binding methods, antibody function tests, and antigen function assays (1–4). Today, routine techniques include antigen binding assays, such as the enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody (IFA) tests, and antigen function assays for detecting neutralizing antibodies, such as the rapid fluorescent focus inhibition (RFFIT) [Chapter 19] and fluorescent antibody virus neutralization (FAVN) [Chapter 20] tests. Rabies serology is a powerful tool in serodiagnosis and research. Serological assays determine the immune status following pre-exposure rabies vaccination and determinations for booster requirements, activities on surveillance and epidemiology of rabies in human and other animal populations, and to help diagnose rabies in patients suspected of the disease, as summarized with emphasis on references detailing experience with the IFA (5–28).

Diagnosis of rabies on postmortem fresh or fresh frozen central nervous system (CNS) tissue by the direct fluorescent antibody test (DFAT) is the gold standard method, but to diagnose rabies antemortem in suspect patients, several tests have been developed for the detection of RABV antigens, antibodies or nucleic acids in a variety of non-CNS sample types (29). Among these, one of the simplest and most rapid serological methods for the detection of antibodies directed against RABV in serum and CSF is the IFA test (5, 6, 30).

The rabies IFA test is a standard antigen binding assay that measures RABV-specific antibodies that bind to viral antigens within infected cells, as depicted in Fig. 21.1. Briefly, slides are made that contain fixed whole cells infected with RABV. Serum and CSF are tested independently for the presence of RABV-specific IgG and IgM antibodies by placing serial dilutions of each sample in wells on the slides. If RABV-specific antibodies are present in the sample, they will bind with the antigens. Immunoglobulins not bound are removed in a washing step. In the final step, binding of the target, or primary, antibody with RABV antigens can be visualized when the antigen–antibody complexes are reacted with a secondary antibody, such as a fluorescein isothiocyanate (FITC)-labelled anti-human globulin (IgM or IgG depending upon the test) and examined using immunofluorescence microscopy. Immunoglobulins induced by whole RABV, as described here, contain a mixture of antibodies with specificities for several component antigens. The antibodies detected by the rabies IFA test are primarily directed against the RABV ribonucleoprotein (RNP); however, binding assays can be modified to include purified viral proteins as target antigens (e.g. nucleoproteins) to more specifically assess the subclasses of immunoglobulins that bind (4, 31, 32).

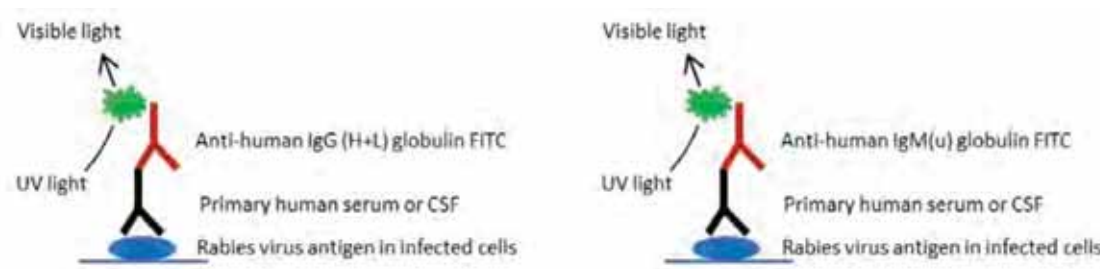


Fig. 21.1. Basic principle of the IFA test using human serum or CSF samples
CSF, cerebrospinal fluid; FITC, fluorescein isothiocyanate, UV, ultraviolet

The IFA test can detect both immunoglobulins IgG and IgM. The IgM antibody is the first immunoglobulin produced following virus infection and is transient in nature. The IgM titres decline to undetected levels after several months; thus, they are indicative of a more recent infection. IgG is the longer term response to the infection. It will appear later than IgM, but persist when IgM levels decline. Measuring a fourfold or greater rise in IgG antibody titre between paired acute and convalescent samples is also routinely used as indicative of a recent infection, whereas a standing IgG titre between acute and convalescent paired samples would suggest previous exposure or prior vaccination, depending upon type, such as suckling mouse brain (SMB) vaccine. If a patient tests negative with early samples and positive with later samples (i.e. seroconversion), results are consistent with exposure to RABV.

Methods

Materials

Equipment

- water bath at 56 °C
- pipetting devices: 1000 µL, 200 µL, 20 µL
- CO₂ water-jacketed incubator: 37 °C, 0.5% CO₂
- fluorescence microscope with FITC filter set (10x, 20x and 40x objectives)
- microcentrifuge, to accommodate 2 mL microcentrifuge tubes
- haemocytometer
- Class II, Type A Biosafety Cabinet
- inverted microscope
- freezer: -20 °C, explosion-proof
- humidified chamber or incubator: 37 °C

Supplies

- 4-well PTFE-coated microscope slides
- laboratory marking pen/pencil
- disposable personal protective equipment
- racks for tubes
- aerosol (filter) barrier tips
- sterile 2.0 mL microcentrifuge tubes
- stainless steel tray(s)
- plastic lids to cover slides for incubation
- slide coverslips
- centrifuge tubes (15 mL and 50 mL)
- cell culture flasks (125 cm² or 250 cm²)
- Coplin or similar jars
- serological pipettes, sterile (1 mL, 5 mL, 10 mL)
- 0.45µm cellulose acetate low protein binding filter

Reagents

- phosphate-buffered saline (PBS: pH 7.2–7.4)
- low glycerol mounting medium, 0.05 M Tris-buffered saline pH 9.0 with 20% glycerol
- affinity-purified antibody fluorescein-labelled goat anti-human IgG (H+L)
- affinity-purified antibody fluorescein labelled goat anti-human IgM (µ)
- IFA diluent (e.g. Bion or similar reagent) to reduce nonspecific fluorescence associated with some serum samples
- IgG removal reagent (e.g. Marsorb G or similar reagent) to remove IgG from a sample in IgM assays
- acetone, reagent grade

Biological materials

- murine neuroblastoma (MNA) cells (ATCC CCL 131) or baby hamster kidney (BHK-21) cells (ATCC CCL 10)
- RABV isolate (e.g. challenge virus standard, CVS-11)
- positive control human serum
- negative control human serum

Protocol

Specimen collection and handling

Routine clinical samples for the IFA test may include serum (i.e. whole blood) and CSF collected aseptically to preserve the integrity of the specimen. To collect serum, whole blood is collected in a tube approved for serum collection (e.g. red top or serum separator tube) and allowed to clot (30 min at room temperature).

To separate serum from the clot, the tube is centrifuged for clarification (e.g. at ~2000 r/min for 20 min). Remove the supernatant (i.e. serum) to a sterile polypropylene tube for storage. Serum and CSF samples should be refrigerated (2–8 °C) upon collection or frozen at –20 °C until testing. Samples should be shipped cooled or frozen on dry ice. To avoid potential reduction of immunoglobulin titres, samples should not be repeatedly frozen and thawed. This may especially affect accurate detection of IgM. Samples containing particles (artifacts) may be clarified by centrifugation or filtered using a 0.45 µm low protein binding filter. Human serum and CSF samples may contain potentially infectious agents; therefore, work should be performed by trained and vaccinated individuals working in a certified biosafety cabinet using at least Biosafety Level 2 practices, equipment and facilities (33).

Preparation of RABV antigen control slides

The substrate used for the IFA test is a monolayer of RABV-infected MNA cells (see reference 34 for isolation of RABV in MNA cells; for alternative procedures using BHK-21 C-13 cells, *see references 35–36*). Cultured flasks that are at or near confluence work best (e.g. normally a monolayer aged 3–4 days). A suspension of cells is adjusted to a concentration to 0.5×10^6 cells per mL. CVS-11, or an alternative fixed RABV strain (e.g. Flury LEP or ERA) is then added to the MNA cell suspension (35, 36). The amount of virus added should be predetermined to produce an infection rate of 20% to 30%. This allows uninfected cells to serve as negative control cells in the same field as infected positive cells, providing a contrast between specific staining cells and non-reactive cells when viewed under a microscope. One drop (~50–75 µL) of cell–virus suspension is added to each well of a 4-well PTFE-coated slide. Slides are incubated at 37 °C and 90% humidity, in 0.5% CO₂ for 20 h. After 20 h, slides are rinsed in PBS and fixed for a minimum of 30 min (to overnight) in cold acetone at –20 °C. After fixation, slides are air-dried and stored at –20 °C to –70 °C until use. For quality control, before freezing, representative slides should be stained using the DFAT protocol to ensure the proper ratio of infected cells has been achieved. Review the positive antigen slide using a fluorescence microscope to determine the percentage of cells fluorescing, indicating the infection rate, as well as the density of cells in each well of the slide. Negative cell control slides are prepared the same as above without the addition of virus. Before implementing a new lot of control slides, compare the new lot with the previous lot of antigen slides using known positive and negative serum or CSF samples. The staining patterns and intensity of the new lot should be comparable to the old lot.

Positive and negative control samples

For human serological testing, previously tested positive and negative human serum or CSF samples are generally used as the positive and negative controls for the IFA test. Standardized reagents, such as the United States SRIG (Laboratory of Standards and Testing, Food and Drug Administration, USA) have been developed under rigorous conditions to serve as quantitated positive control serum for use in antigen function assays such as the RFFIT and FAVN, as have other international standards. Similarly, human rabies immunoglobulin (HRIG) is a standardized quantitated rabies immunoglobulin indicated for the prophylaxis of previously unvaccinated individuals suspected of exposure to RABV. In the United States of America, both SRIG and HRIG are developed from pooled plasma

samples of individuals vaccinated with the human diploid cell vaccine (HDCV) or the purified chick embryo cell vaccine (PCEC) and are quantitated for potency by measuring the concentration of RABV-specific neutralizing antibodies. While it is expected that SRIG and HRIG contain the binding antibodies detected by IFA, these reagents are not specifically quantitated for binding antibody. It has been suggested that IgM in particular will peak at about 14 days after the first dose of vaccine is administered and will decline relatively quickly (9, 37). The IgM may not be well-represented in the pooled samples taken after completion of the 3-dose vaccination series; thus, use of these standardized reagents as positive controls for the IFA test would need to be carefully evaluated and validated before implementation.

IFA standard test procedure

1. Heat inactivate human serum and CSF at 56 °C for 45 min (animal serum/CSF for 30 min).

Note: Most users of heat inactivated serum do so because it has been included in protocols that originated years ago and have been carried forward. Heat inactivation was believed to destroy a component in serum called complement that may interfere with the detection of IgM and IgG in some assays (38, 39), although it has not been demonstrated to specifically affect the rabies IFA test. It was also believed to destroy adventitious agents such as viruses and mycoplasma, but it is important to note that heat inactivation may not completely destroy all pathogens and the sample should still be handled as potentially infectious material. When collection methods and filtration processes were improved along with the ability to gamma irradiate serum, these issues became much less relevant.

2. Following heat inactivation, prepare 2-fold dilutions of serum and CSF separately in IFA diluent or PBS. Serum dilution starts at 1:4 (i.e. 1:4, 1:8, 1:16). CSF starts at neat (undiluted) followed by 1:2, 1:4, 1:8, etc.
 - a. For detection of RABV IgM antibody in serum, addition of an IgG removal reagent to remove the IgG component prior to making dilutions can mitigate potential false reactions. Follow the manufacturer's instructions for use of the IgG removal reagent.
3. Air dry previously prepared 4-well slides of CVS-11 infected MNA cells for 5–10 min and label accordingly (Fig. 21.2). Label two CVS-11 infected MNA cell slides for each patient sample (i.e. serum and CSF) and the controls as follows: label one slide for each pair "IgG" and the other "IgM" and indicate the specimen ID. In addition, prepare one negative antibody control slide. Place slides on stainless steel tray with moistened paper towel.
4. Add 40 µL of test serum, CSF and control sera to representative wells. Cover slides with a plastic lid to produce a humidity chamber.
5. Incubate at 37 °C for 30 min.
6. Rinse each slide using a wash bottle containing PBS. Take care to avoid sloughing of the cell monolayer. Allow slides to soak once in a container of PBS for 5–10 min.

7. Blot off excess PBS. Slides can be inverted on to a dry paper towel, but caution must be taken to avoid disrupting the cell monolayer. Place slides back on tray with a moistened paper towel.
8. Follow manufacturer's instructions to reconstitute, store and prepare working dilutions of goat anti-human IgG and IgM conjugate (i.e. fluorescein-labelled antibody). Add 40 μ L of a working dilution of goat anti-human IgG or IgM conjugate to the appropriate wells of representative slides. Place slides on a tray with a moistened paper towel and cover them with a plastic lid.
9. Incubate in the dark at 37 °C for 30 min.
10. Rinse each slide and soak in PBS for 5 to 10 minutes as above.
11. Cover each dilution well with mounting medium such as 0.05 M TBS pH 9.0 with 20% glycerol (40), by applying drops of mounting medium to the coverslip. Place the stained slide onto the coverslip.
12. Slides should be read immediately or stored at -20 °C. Read the slides under a fluorescence microscope at a magnification of 200x to examine fluorescence and grade the slides; 400x magnification can be used if closer examination is needed.

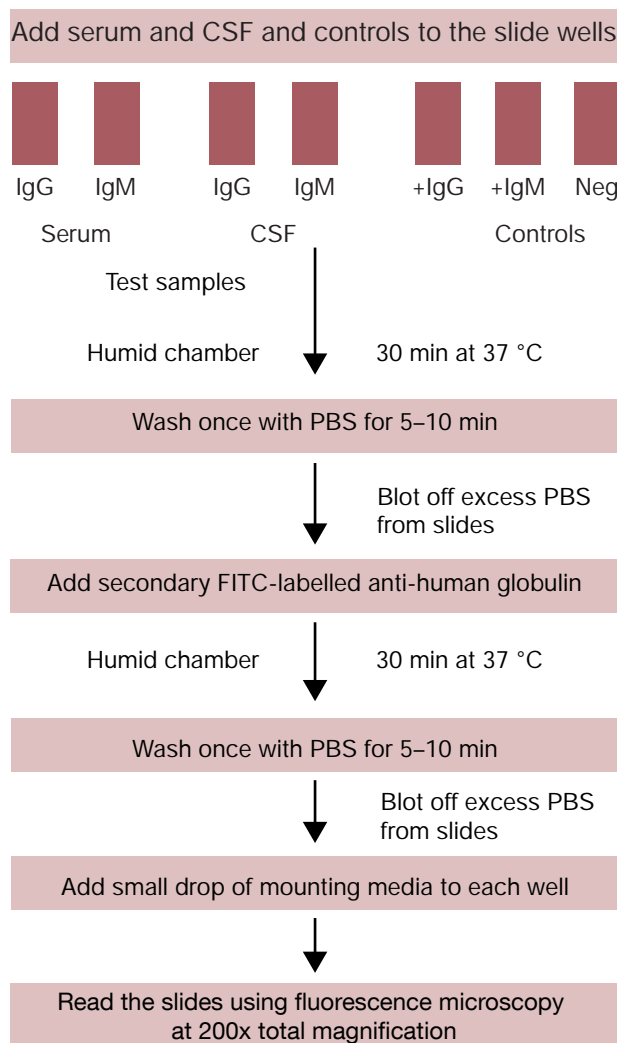


Fig. 21.2. Summary of the IFA test procedure

Pre-prepared slides of MNA cells (CVS-11 infected and normal cell controls). Control slides are prepared prior to use. Use 6 CVS-11 infected MNA cell slides; serum (2); CSF (2); IgG+ (1); IgM+ (1) and one normal MNA cell slide as negative control; serum + CSF IgG and IgM.

CSF, cerebrospinal fluid; CVS, challenge virus standard strain; MNA, murine neuroblastoma cell; PBS, phosphate buffered saline

Reading slides

1. Read all slides as follows:

a. Positive and negative control samples. Positive and negative control samples are included with every test and should perform as expected before reading test slides.

- i. Absence of fluorescent staining in the negative human control represents a negative reaction. Fluorescence observed in cells on this slide would suggest a nonspecific reaction.
- ii. The positive controls for both IgG and IgM should yield a 2+ to 4+ apple-green granular fluorescence in the cytoplasm/cell surface with one to several infected cells in any one field (200x total magnification). The remaining cells in the same field are uninfected and serve as a “within-field” negative control.
- iii. If positive and negative controls do not stain as expected (i.e. as described above), subsequent patient results are invalid and should not be reported. Additional controls may be tested and used according to above criteria.

b. Test samples

- i. Absence of characteristic apple-green fluorescent staining in the infected cells represents a negative reaction. This reaction should appear comparable with the reaction observed in the negative control.
- ii. In a positive patient, the characteristic apple-green fluorescent staining pattern should be observed, and the number of infected cells observed should approximate the number of infected cells seen in the positive control well.

2. Grade the intensity of test samples. Depending upon patient antibody concentration, the reactivity (i.e. intensity) of positive samples may vary from 1+ to 4+ apple-green fluorescence but will have a similar staining pattern and distribution as the positive control slide. For each patient sample well, record the intensity of the fluorescence within positive cells from 4+ to 1+ as follows:

4+ = brilliant apple-green fluorescing antigen within cells

3+ = a reduction in intensity but bright apple-green fluorescence

2+ = further reduction in intensity of fluorescence

1+ = weak or dull staining fluorescence

(-) = no fluorescence observed within cells

Interpretation

1. Antibody is considered to be present in the test sample if the staining pattern and morphology of the cells are similar to that of the positive control sample and exhibit an intensity of 1–2+ or greater. That sample can be resulted as “antibody detected”.

2. The result of each test can be quantified as a titre, which is recorded as the highest dilution of a patient’s sample (serum or CSF) in which RABV-specific immunoglobulins are detected with characteristic specific fluorescence resembling that of the positive control.

Note: To titre to end-point, additional dilutions of positive test samples may need to be performed (Fig. 21.3). Serial dilutions should be prepared in PBS (or alternative diluent), the same as above (typically eight 2-fold dilutions). The end-point is the last dilution that produces positive apple-green staining at 1–2+ intensity in the infected cells.

3. Nonspecific reactions: Staining alone cannot be considered positive if it is not typical. Occasional nonspecific staining may show dull apple-green fluorescence that appears diffuse across the well and is not cell-associated. Test results may only be reported if one is able to discern characteristic reactivity from the nonspecific fluorescence. If results are not clearly discernable, they should be reported as indeterminate. In cases where unusual or nonspecific staining is observed, the sample can be filtered using a 0.45 µm low protein binding filter and retested. If serological results are indeterminate, additional samples may be requested.

For patients suspected of RABV infection and demonstrating detection of or a rise in titre for both IgG and IgM in serum and CSF, a definitive diagnosis should be confirmed by other serological methods (e.g. RFFIT) as well as appropriate testing of other available samples (e.g. DFAT of fresh frozen skin biopsy; reverse transcriptase-polymerase chain reaction and sequencing of skin biopsy or saliva amplicons).

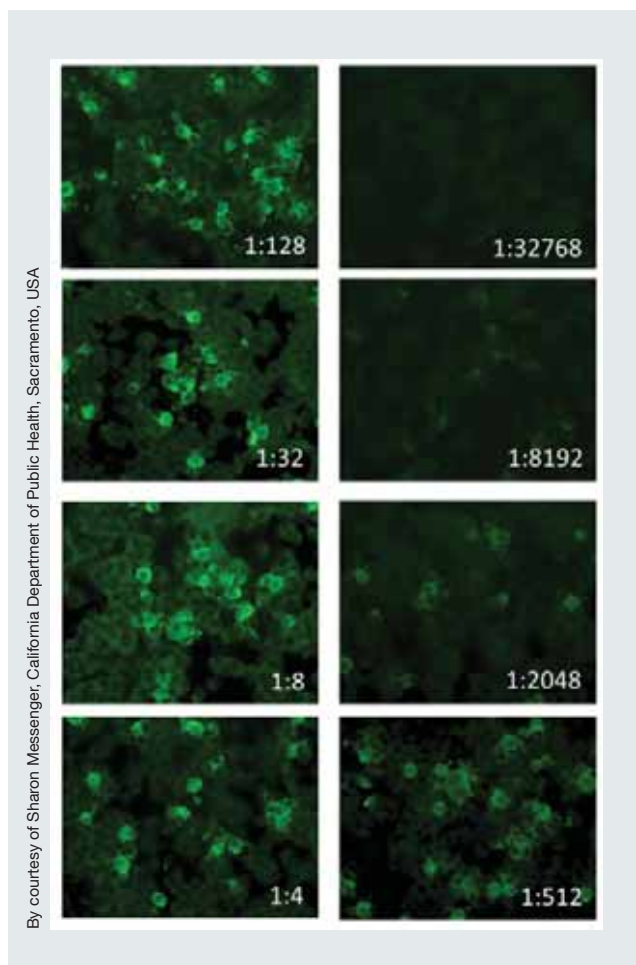


Fig. 21.3. End-point anti-RABV IgG antibody titre in serum from a positive rabid human sample (note gradual diminution in FITC-staining intensity with increasing dilution of sample).

Troubleshooting procedures for abnormal results

1. Positive control serum exhibits weak (i.e. 1+ –2+) or no fluorescence: repeat.
 - a. Possible mix-up when adding controls and test samples.
 - b. Positive control may be deteriorating or was improperly diluted: make new dilution of control.
 - c. Reagents may have been stored or were prepared improperly.
 - d. Slides were not read in a timely manner (e.g. left at room temperature for a long time or read the next day).
 - e. The fluorescence microscope may require maintenance (e.g. bulb may be old, the wrong light source was used, the scope was not aligned properly, etc.). Check microscope logs, determine number of bulb hours used, or service microscope if needed.
2. Negative control serum is positive: repeat.
 - a. Possible mix-up when adding control and test samples.
 - b. Possible artifacts or contamination in sample: make new dilution of control.
3. Too few antigen-staining cells on the cell spot to determine pattern of fluorescence: repeat with new slide.
 - a. Slides may have been washed too vigorously.
 - b. Slides should be at room temperature and dried before testing.
4. Excessive background on slides: repeat
 - a. Samples or reagents (e.g. conjugate) not diluted properly.
 - b. Slides may have dried out and well contents evaporated.

Discussion

There is no definitive period during which a rabid patient is expected to develop antibodies against RABV (2). It is not surprising for a virus that has evolved a strategy to evade the immune system that some rabies patients may succumb to the disease without developing any detectable antibodies. Each case is unique and the humoral immune response due to exposure to RABV is dependent upon not only the specific immune status of the host but also a complicated suite of factors of antigen presentation such as viral dose, route of entry and degree of, if any, viral replication in non-neural tissue versus the peripheral or CNS. The longer a rabies patient remains ill, the higher the probability of detecting antibodies. Thus, testing serial collections of serum and CSF is generally needed to document exposure to RABV.

Antibodies detected by the rabies IFA test, which are largely directed against the RABV RNP, have been shown to have some limited anti-viral activity (31–32). Neutralization tests, such as the RFFIT and FAVN, detect RABV neutralizing antibodies (RVNA) against the viral glycoprotein. The detection of binding antibodies may not always correlate with the detection of neutralizing antibodies (10, 41). Humans vaccinated with or exposed to RABV have been documented in which the IFA test detected antibodies earlier than neutralizing antibody assays and some cases in which the IFA was positive while the RFFIT was negative (5, 7, 8,

15, 25, 42–43). The opposite is rare. Because the IFA test is not a direct measure of neutralizing antibodies, the test cannot be used as a measure of immunity following vaccination (14).

Exposure history, compatible clinical signs and positive IFA reactions are indicators of RABV exposure. It is critical to obtain patient history, particularly evidence of pre-exposure rabies vaccination, when interpreting results of a serological test. Vaccination to RABV can cause positive reactions for both IgG and IgM in serum. No antibody, however, has been detected in the CSF of vaccinated persons (22). Thus, a suspected case of rabies in a patient (or evidence of exposure to RABV) can be confirmed by the IFA test if RABV-specific antibody is detected in the CSF of any patient or if RABV-specific antibody is detected in the serum of an unvaccinated individual.

Whenever possible, standardized reagents (e.g. cell line, challenge virus, reference standards, antisera) should be used for the IFA to compare performance over time and across laboratories, including routine proficiency tests. For testing of non-model organisms, however, special antiserum reagents (e.g. anti-species detector antibodies) may need to be developed for each species to be evaluated (3, 41).

End-point reactivity (intensity) may vary depending upon the type of microscope, the light source, age of bulb, filter assembly and filter thickness, etc. For this reason, 2+ staining intensity can be considered end-point. Use of controls and implementation of a comprehensive QA/QC programme which includes regular routine maintenance of critical equipment (i.e. the microscope) can minimize the effect of these microscope parameters on the interpretation of the result, but it is not unexpected that different laboratories testing the same specimen will obtain different end-point titres.

Because the interpretation of an IFA result is not seemingly accomplished by an objective measurement of an indicator (e.g. colour intensity of a chromogenic enzyme substrate) but rather by a trained microbiologist visually observing a pattern of diminishing fluorescence, it is sometimes discussed as an important limitation of the IFA. The perceived subjectivity, however, may also be viewed as a particular advantage of the method. The morphology and specific location of fluorescent staining can be evaluated directly to determine if it might represent a nonspecific reaction. Further, fluorescent patterns of different viral infections can be quite distinctive (e.g. accumulation of antigen within the nucleus, the cytoplasm or the cellular organelles represents microorganism-specific fluorescent staining) while dull or diffuse fluorescent staining may represent nonspecific fluorescence (44). Still, studies have shown that nonspecific staining can be morphologically similar to specific anti-RABV staining, and antibodies measured in the IFA test can cross-react with other encephalitic agents (36). Competency to identify specific and nonspecific staining as well as the microorganism-specific patterns of antigen distribution requires extensive training and experience. Additionally, as with any diagnostic test implemented in the laboratory, the performance specifications (i.e. accuracy, sensitivity, specificity, precision) of the IFA test must be thoroughly evaluated, validated and documented in each facility (4).

Clearly, serological tests for the confirmation of rabies using clinical samples are an indirect method of making a determination of exposure to viral antigens through the detection of antibodies developed following primary exposure in

naive patients or secondary response to patients previously exposed. The IFA test has proven value in the diagnostic arena, particularly in investigations of suspect rabies cases in which no one test has been proven to be superior as the most sensitive or earliest indicator of infection. Because of the recognition that the IFA test is not a direct measure of neutralizing antibodies, tests such as the RFFIT and FAVN have gained favour as the methods of choice to more directly measure the response to RABV exposure and vaccination. Thus, although IFA is not routinely performed in the developing world, despite its relative ease and rapidity, its utility continues to be demonstrated as part of the antemortem diagnosis of human rabies patients in the USA (45–47). Earlier studies directly comparing the performance of the IFA test to neutralizing antibody tests have shown that these tests largely correlate, but in cases in which discrepancies are observed, interpretation becomes less clear (5–7, 9–11). Additional studies that compare the performance characteristics of both tests are warranted, because such studies could provide guidance for conditions under which the more rapid and inexpensive IFA assay could be used as a surrogate for neutralizing assays (e.g. serosurveys of human or animal populations), and under which conditions this might not be prudent.

References

1. Thomas JB. The serum neutralization, indirect fluorescent antibody, and rapid fluorescent focus inhibition tests. In: Baer GM, editor. *The natural history of rabies*. New York (NY): Academic Press Inc; 1975:417–33.
2. Smith JS. Rabies serology. In: Baer GM, editor. *The natural history of rabies*. Boca Raton (FL): CRC Press; 1991:235–52.
3. Trimarchi CV, Smith JS. Diagnostic evaluation. In: Jackson AC, Wunner WH, editors. *Rabies*. London (UK): Elsevier Science; 2002:308–44.
4. Moore SM, Gordon CR, Hanlon CA. Measures of rabies immunity. In: Jackson A, editor. *Rabies: scientific basis of the disease and its management*. San Diego (CA): Academic Press Inc; 2013:461–95.
5. Thomas JB, Sikes RK, Ricker AS. Evaluation of indirect fluorescence antibody technique for detection of rabies antibody in human sera. *J Immunol*. 1963; 91:721–3. PMID:14106292.
6. Leffingwell L, Irons JV. Rabies antibodies in human serum titrated by the indirect FA method. *Publ Health Rep*. 1965; 80:999–1004. PMID:PMC1919712.
7. Larsh SE. Indirect fluorescent antibody and serum neutralization response in pre-exposure prophylaxis against rabies. *Ann Int Med*. 1965; 63:955–64. PMID:5320956.
8. Gispen R, Saathof B. Neutralizing and fluorescent antibody response in man after antirabies treatment with suckling rabbit brain vaccine. *Arch Ges Virusforsch*. 1965; 15:377–86. PMID:14292783.

9. Grandien M. Evaluation of tests for rabies antibody and analysis of serum responses after administration of three different types of rabies vaccines. *J Clin Micro*. 1977; 5:263–7. PMID:323275.
10. Lee T-K, Hutchinson HD, Zeigler DW. Comparison of rabies humoral antibody titers in rabbits and humans by indirect radioimmunoassay, rapid-fluorescent-focus-inhibition technique and indirect fluorescent-antibody assay. *J Clin Micro*. 1977; 5:320–5.
11. Zalan E, Wilson C, Pukitis D. A microtest for the quantitation of rabies virus neutralizing antibodies. *J Biol Standard*. 1979; 7:213–20. doi:10.1016/S0092-1157(79)80024-4.
12. Kasempimolporn S, Hemachudha T, Khawplod P, Manatsathit S. Human immune response to rabies nucleocapsid and glycoprotein antigens. *Clin Exp Immunol*. 1991; 84:195–9. PMID:2025949.
13. Madhusudhana SN, Shamsundar R, Saraswati S. Comparative evaluation of a simple indirect immuno fluorescence test and mouse neutralization test for assaying rabies antibodies. *Indian J Pathol Microbiol*. 2001; 44:309–12.
14. Human rabies prevention – United States, 2008. Recommendations of the Advisory Committee on Immunization Practices. *MMWR*. 2008; 57:1–36 (<https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5703a1.htm>).
15. Gilbert AT, Petersen BW, Recuenco S, Niezgoda M, Gomez J, Laguna-Torres VA, et al. Evidence of rabies virus exposure among humans in the Peruvian Amazon. *Am J Trop Med Hyg*. 2012; 87:206–15. doi:10.4269/ajtmh.2012.11-0689.
16. Black D, Wiktor TJ. Survey of raccoon hunters for rabies antibody titers: pilot study. *J Fla Med Assoc*. 1986; 73:517–20. PMID:3746238.
17. Orr PH, Rubin MR, Aoki FY. Naturally acquired serum rabies neutralizing antibody in a Canadian Inuit population. *Arctic Med Res*. 1988; 47 Suppl 1:699–700. PMID:3272719.
18. Follmann EH, Ritter DG, Beller M. Survey of fox trappers in northern Alaska for rabies antibody. *Epidemiol Infect*. 1994; 113:137–41.
19. Lopez RA, Miranda PP, Tejada VE, Fishbein DB. Outbreak of human rabies in the Peruvian jungle. *Lancet*. 1992; 339:408–11. PMID:1346669.
20. Cleaveland S, Barrat J, Barrat MJ, Selve M, Kaare M, Esterhuysen J. A rabies serosurvey of domestic dogs in rural Tanzania: results of a rapid fluorescent focus inhibition test (RFFIT) and a liquid-phase blocking ELISA used in parallel. *Epidemiol Infect*. 1999; 123:157–64. doi:10.1017/S0950268899002563.
21. Hill Jr RE, Beran GW, Clark WR. Demonstration of rabies virus-specific antibody in the sera of free-ranging Iowa raccoons (*Procyon lotor*). *J Wildlife Dis*. 1992; 28:377–85. doi:10.7589/0090-3558-28.3.377.
22. Hattwick MAW, Weis TT, Stechschulte CJ, Baer GM, Gregg MB. Recovery from rabies: a case report. *Ann Int Med*. 1972; 76:931–42. PMID:5063659.

23. Emmons RW, Leonard LL, DeGenaro Jr F, Protas ES, Bazeley PL, Giammona ST, et al. A case of human rabies with prolonged survival. *Intervirology*. 1973; 1:60–72.
24. Baraff LJ, Hafkin B, Wehrle PF, Emmons RW, Gunn RA, Overturf GD, et al. Human rabies. *West J Med*. 1978; 128:159–64.
25. Presumptive abortive human rabies – Texas, 2009. *MMWR*. 2010; 59:185–90.
26. Favi M, de Mattos CA, Yung V, Chala E, Lopez LR, de Mattos CC. First case of human rabies in Chile caused by an insectivorous bat virus variant. *Emerg Inf Dis*. 2002; 8:79–81. doi:10.3201/eid0801.010108.
27. Cohen C, Sartorius B, Sabeta C, Zulu G, Paweska J, Mogoswane M, et al. Epidemiology and molecular virus characterization of reemerging rabies, South Africa. *Emerg Infect Dis*. 2007; 13:1879–86. doi:10.3201/eid1312.070836.
28. Madhusudhana SN, Sukumaran SM. Antemortem diagnosis and prevention of human rabies. *Ann Indian Acad Neurol*. 2008; 11:3–12.
29. Protocol for postmortem diagnosis of rabies in animals by direct fluorescent antibody testing. Atlanta (GA): United States Centers for Disease Control and Prevention (<http://www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf>, accessed 25 May 2018).
30. Goldwasser RA, Kissling RE. Fluorescent antibody staining of street and fixed rabies virus antigens. *Proc Soc Exp Biol Med*. 1958; 98:219–23. doi:10.3181/00379727-98-23996.
31. Schneider LG, Dietzschold B, Dierks RE, Matthaeus W, Enzmann P-J, Strohmaier K. Rabies group-specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. *J Virol*. 1973; 11:748–55.
32. Wiktor TJ, György E, Schlumberger D, Sokol F, Koprowski H. Antigenic properties of rabies virus components. *J Immunol*. 1973; 110:269–76. PMID:4568184.
33. Biosafety in microbiological and biomedical laboratories (BMBL), 5th edition. United States Centers for Disease Control and Prevention and National Institutes of Health; 2007 (<http://www.cdc.gov/biosafety/publications/bmb15/>, accessed 25 May 2018).
34. Smith J. Rabies virus. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Tenover FC, editors. *Manual of clinical microbiology*, 6th edition. Washington (DC): American Society for Microbiology; 1995:997–1003.
35. Johnson HN, Emmons RW. Rabies virus. In: Lennette EH, Balows A, Hausler Jr WJ, Tenover FC, editors. *Manual of clinical microbiology*, 3rd edition. Washington (DC): American Society for Microbiology; 1980:875–83.
36. Rudd RJ, Appler KA, Wong SJ. Presence of cross-reactions with other viral encephalitides in the indirect fluorescent antibody test for diagnosis of rabies. *J Clin Microbiol*. 2013; 51:4079–82. doi:10.1128/JCM.01818-13.
37. Rubin R, Dierks RE, Cough P, Gregg M, Gerlach H, Sikes R. Immunoglobulin response to rabies vaccine in man. *Lancet*. 1971; 2:625–8.

38. Namekar M, Kumar M, O'Connell M, Nerurkar VR. Effect of serum heat-inactivation and dilution on detection of anti-WNV antibodies in mice by West Nile virus E-protein microsphere immunoassay. *PLoS One*. 2012; 7:e45851. doi:10.1371/journal.pone.0045851.
39. Manalo DL, Yamada K, Watanabe I, Miranda MEG, Lapiz SMD, Tapdasan E, et al. A comparative study of the RAPINA and the virus-neutralizing test (RFFIT) for the estimation of antirabies-neutralizing antibody levels in dog samples. *Zoonoses Public Health* 2017; 64:355–62. doi:10.1111/zph.12313.
40. Rudd RJ, Smith JS, Yager PA, Orciari LA, Trimarchi CV. A need for standardized rabies-virus diagnostic procedures: effect of cover-glass mountant on the reliability of antigen detection by the fluorescent antibody test. *Virus Res*. 2005; 111:83–8. doi:10.1016/j.viruses.2005.03.014.
41. Winkler WG. The rhabdoviruses. In: Kurstak E, Kurstak C, editors. *Comparative diagnosis of viral diseases*. New York (NY): Academic Press; 1981:529–50.
42. Bhatt DR, Hattwick MAW, Gerdson R, Emmons RW, Johnson HN. Human rabies: diagnosis, complications, and management. *Am J Dis Child*. 1974; 127:862–9.
43. Recovery of a patient from clinical rabies – California, 2011. *MMWR*. 2012; 61:61–5.
44. Rosebrock JA. Labeled-antibody techniques: fluorescent, radioisotopic, immunochemical. In: Balows A, Hausler WJ, Herrmann KL, Isenberg HD, Shadomy HJ, editors. *Manual of clinical microbiology*. Washington (DC): American Society for Microbiology; 1991:79–86.
45. Rabies death attributed to exposure in Central America with symptom onset in a U.S. detention facility – Texas, 2013. *MMWR*. 2014; 63:446–9.
46. Human rabies – Missouri, 2014. *MMWR*. 2016; 65:253–6.
47. Human rabies – Wyoming and Utah, 2015. *MMWR*. 2016; 65:529–33.

Chapter 22

The mouse neutralization test

Introduction

Adaptive immunity to rabies virus (RABV) is induced because of vaccination or infection. Vaccine-induced immunity is characterized by a slow onset and a durable response coupled with immunological memory. The neurotropic nature of RABV drives its entry into a motor neuron at the neuromuscular junction and transportation in a retrograde direction through the axon of the infected neuron (1). The immune responses triggered during RABV infection are peculiar because the nervous system is an immunologically privileged site (2). In addition, pathogenic variants of RABV may trigger peripheral immunosuppression (3). Therefore, seroconversion, if it is detected, occurs rather late in the course of the disease (4, 5). In humans, the presence of RABV-specific antibodies in the serum and CSF during the period of clinical disease can be a useful diagnostic indicator (4, 6, 7).

Typically, RABV-specific antibodies are demonstrated based on their ability to either bind to an antigenic target (i.e. via a binding assay) or neutralize the pathogen (i.e. a neutralization assay). A binding assay (e.g. ELISA) detects immunoglobulins that include both neutralizing and non-neutralizing antibodies. Conversely, neutralization assays (e.g. RFFIT, FAVN), which require a live indicator system such as a cell line or animal, detect only neutralizing antibodies, otherwise known as functional antibodies. Historically, the presence of virus neutralizing antibodies (VNAs) as a measure of rabies immunity has been demonstrated by a mouse neutralization test (MNT), which involves the use of a laboratory animal model as a sensitive indicator of the presence of unneutralized virus. As such, RABV does not typically produce cytopathic effect in cell culture or gross pathognomonic lesions in organs (8,9). Therefore, signs such as hind limb paralysis in mice and in affected animals the presence of RABV inclusions in the cytoplasm of infected neurons, as demonstrated by the DFAT, support evidence for proliferation in the CNS (10, 11).

The MNT was reported more than seven decades ago by Webster and Dawson (12). It is sensitive, specific and offers the benefit of using RABV (both fixed and street viruses) without a prior adaptation step. However, certain limitations, such as technical complexity, a long turnaround time, high cost, ethical issues, a need for animal containment facility and trained personnel, make the MNT less attractive today. A protocol on the MNT is only being included in this manual as an historical aside for those few institutions that still employ this test. However, it should be discontinued because alternative protocols, such as RFFIT and ELISA, exist. Similar concerns and technical issues as expressed in [Chapter 3](#) on biosafety and [Chapter 12](#) on RABV isolation in mice are also relevant here.

Method

Precautions

Besides technical competence in the use of animals, biosafety concerns are also an issue. For example, vaccinate all personnel involved in this test. Wear personal protective equipment, such as a gown, gloves and face shield, before entering the containment facility. Properly perform the clearance of trapped air from the syringe using a glass tube with cotton wool to avoid accidental exposure through aerosols. Carefully dissect the animal brain by taking care not to allow accidental exposure to RABV due to sharps injuries. Thoroughly homogenize the brain without generating a hazardous aerosol. Properly collect carcasses, bedding material and sharps (e.g. needles, glass slides, coverslips) contaminated with RABV in proper containers and decontaminate by incineration or autoclaving as appropriate before disposal.

Amplification of RABV for challenge

1. Rapidly thaw a vial of CVS 24, stored frozen at -80°C or -196°C as a 20% [w/v] mouse brain homogenate in cold running tap water and perform two serial 10-fold dilutions (1/10 and 1/100) using an ice-cold diluent, such as sterile PBS with 2% equine serum. Rapid thawing in cold running tap water and maintenance of the cold chain at all stages as appropriate helps to minimize thermal inactivation of RABV. Precise pipetting and dispensing is necessary to obtain accurate and reliable results.
2. Allot three groups of weanling Swiss albino mice (group size = 10) and administer 30 μL of inoculum, i.e. 10^{-1} dilution, 10^{-2} dilution and diluent alone, into the respective group of mice intracerebrally. Alternatively, BALB/c mice can also be used instead of Swiss albino mice. The particular strain of mice, body weight and age largely determine their susceptibility for RABV infection. Weaned Swiss albino mice of both genders with 12–14 g of body weight can be randomized to typically obtain unbiased experimental outcomes. The composition and quality of ingredients used for the preparation of the diluent ensures the stability of virus and avoids the nonspecific death of mice after IC inoculation. It is meaningful to screen every batch of horse serum for the presence of VNA, if any, to prevent a drastic drop in infectivity of challenge virus. The safety of diluent for mice can be verified by keeping a diluent control group in all the experiments.
3. Observe the inoculated mice daily for signs of rabies over a period of 14 days. Duration of observation is a function of incubation period of RABV, which can differ for fixed and street RABV depending upon their neurovirulence.
4. Consider mortality of mice occurring before day 4 post-inoculation as nonspecific and after day 4 post-inoculation as specific. Nonspecific death may be due to trauma caused by needle, faulty techniques and contaminated or improper diluent, etc.
5. Euthanize mice that show signs, such as paralysis of the hind limbs, or approach a moribund state.
6. Dissect brains aseptically from euthanized mice that received the highest

dilution of RABV, collect them in sterile microcentrifuge tubes and determine their weight by using an electronic weighing balance.

7. Prepare brain tissue homogenates (20% w/v) by trituration using a pestle and mortar [alternatively a glass homogenizer may also be used] and clarify by centrifugation at $200 \times g$ for 10 min at 10 °C, essentially following a standard protocol (11). In addition, sterile sand when used as an abrasive will aid in thorough homogenization. Trituration usually generates heat and hence maintenance of the cold chain is essential to minimize thermal inactivation of virus. Stability of RABV in the brain tissue homogenate can be preserved by adding heat inactivated-horse serum (2% v/v) to the diluent. Microbial contamination of brain tissue homogenate may be mitigated by adding antibiotic–antimycotic (2x) to the diluent.
8. Aliquot the clarified brain tissue homogenate in sterile cryovials and store them at either –80 °C or –196 °C. Storage of RABV-infected brain tissue homogenate at –20 °C is not advisable because in general viruses lose infectivity with time.
9. The median mouse intracerebral lethal dose (MICLD₅₀) for the amplified CVS 24 stock virus should be determined by titration in mice.

Titration of RABV

1. Thaw a vial of CVS 24 amplified by mouse brain passage quickly in cold running tap water.
2. Perform eight serial 10-fold dilutions of challenge virus using ice-cold diluent. Use calibrated pipettes and dedicated sterile tips for each aspiration and take care to dispense the content without touching the diluent.
3. Allot nine groups of weanling Swiss albino mice (group size = 10) and inoculate 30 µL IC of inoculum that includes various dilutions of RABV and diluent alone. Including a diluent control helps to appreciate whether nonspecific death is due to one or more components of the diluent. Using a 0.5 mL disposable syringe with a 26–29 gauge needle may aid in precise dispensing of 30 µL of inoculum without causing excess trauma.
4. Observe the inoculated mice daily as mentioned above, and record signs such as hind limb paralysis or acute deaths. Euthanasia of mice upon the first definitive signs is preferred.
5. Determine the titre of RABV by calculating the 50% end/point by the Reed–Muench method (13). Express the value as MICLD₅₀ /30 µL.

Neutralization of RABV by antibodies

1. Keep whole blood samples at room temperature to allow for better clotting and serum separation, in addition to prevention of haemolysis. Storage of clotted blood samples at 2–8 °C at least for a few hours results in better separation of sera due to contraction of clots. Separate sera from clotted blood samples by centrifugation at $200 \times g$ for 10 min at 10 °C and inactivate the complement by incubating at 56 °C for 30 min. Inactivation of complement is essential because it can cause cell lysis when activated by the classical pathway.

2. Perform five serial 5-fold dilutions of sera samples starting from 1:2.5 using ice-cold diluent and keeping samples chilled on wet ice until used.
3. Thaw an aliquot of challenge virus of known titre rapidly in cold running tap water and perform serial dilutions of challenge virus to achieve 100 MICLD₅₀ per 30 µL as described below:
 - For example: Consider titre of the CVS 24 stock as 10^{5.5} MICLD₅₀ per 30 µL.
 - Diluting the CVS 24 stock 10^{-5.5} times will result in 1 MICLD₅₀ per 30 µL.
 - Similarly, 100 (log 10²) MICLD₅₀ per 30 µL can be achieved as follows:
 - $\log 10^{5.5} / \log 10^2 = 5.5 - 2 = 3.5$. Antilog of 3.5 = 3162, which means diluting the CVS 24 stock 3162 times, and will result in 100 MICLD₅₀ per 30 µL. Perform three serial 10-fold dilutions such as 10⁻¹, 10⁻² and 10⁻³ followed by mixing 1 part of 10⁻³ diluted virus with 2.162 parts of the diluent to prepare enough volume of working virus stock. Precision in dilution and calculation are critical for the preparation of challenge virus of the right dose. The percentage mortality caused by back titrated virus per se will indicate accuracy of dose of the challenge virus.
4. Mix equal volumes of diluted sera (starting dilution, 1:2.5) and challenge virus (100 MICLD₅₀/30 µL) and incubate the mixture at 37 °C for 90 min with intermittent shaking for neutralization to occur. Include positive serum of a known titre as a reference. The final dose of challenge virus will be 50 MICLD₅₀ in all the dilutions and the dilution of serum will be 1:5.
5. Back titrate the challenge virus of 100 MICLD₅₀ per 30 µL by serial dilution to make 50 MICLD₅₀, 10 MICLD₅₀, 1 MICLD₅₀ and 0.1 MICLD₅₀. Incubate back titrated challenge virus along with serum–virus mixtures at 37 °C for 90 min with intermittent shaking during the neutralization step to maintain uniformity of treatment.
6. Keep the serum–virus mixtures and diluted virus stocks cold immediately on wet ice until used for inoculation.
7. Allot 15 groups of weanling Swiss albino mice (group size = 10) as required and inoculate 30 µL of serum–virus mixtures, diluted virus stocks and diluent alone into the respective group of mice through the IC route. Bringing the inocula to room temperature before IC inoculation may prevent cold-induced shock and eventual death of mice. Use a dedicated syringe and needle for inoculating each dilution of virus per individual animal.
8. Monitor the inoculated mice and record signs, such as hind limb paralysis, as mentioned above. Nonspecific deaths may be due to trauma caused by needles, faulty technique, etc.
9. Determine the percentage of mice which survived or became ill/died in various groups at the end of the observation period. Signs or death of mice after 4 days of inoculation is usually considered as RABV-specific, which may be confirmed by DFAT as per the standard protocol (10).

Interpretation of results

- 100% survival of mice in the diluent control group indicates the absence of unsafe components in the diluent.
- 80–100% survival of mice in the test and control group indicates the presence of neutralizing antibodies in test and reference sera.
- 100% death of mice indicates absence of neutralizing antibodies in test sera.
- Approximately 50% death of mice that received back titrated virus, especially 1 MICLD₅₀ per 30 µL, indicates accuracy of amount of virus inoculated.

Besides animal welfare concerns, considering the above animal protocol by comparison to binding or in vitro neutralization tests, which can be performed the same day or obtain results within 24–48 h with large numbers of test sera, it should be obvious that time and economic factors justify why the MNT for detection of RABV antibodies should be retired in all laboratory facilities.

References

1. Kelly RM, Strick, PL. Rabies as a transneuronal tracer of circuits in the central nervous system. *J Neurosci Methods*. 2000; 103:63–71. PMID:11074096.
2. Barker CF, Billingham RE. Immunologically privileged sites. *Adv Immunol*. 1977; 25:1–54. doi:10.1016/S0065-2776(08)60930.
3. Lafon M. Immunology. In: Rabies. Jackson AC, Wunner WH, editors. San Diego (CA): Academic Press; 2002:351–69.
4. Schuller E, Helary M, Allinquant B, Gibert C, Vachon F, Atanasiu P. IgM and IgG responses in rabies encephalitis. *Ann Microbiol*. 1979; 130A:365–72. PMID:484994.
5. OIE Terrestrial Manual. Paris: World Organisation for Animal Health; 2008; Chapter 2.1.13:304–23 (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.17_RABIES.pdf).
6. Crepin P, Audry L, Rotivel Y, Gacoin A, Caroff C, Bourhy H. Intravital diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *J Clin Microbiol*. 1998; 36:1117–121. PMID:9542950.
7. McColl KA, Gould AR, Selleck PW, Hooper PT, Westbury HA, Smith JS. Polymerase chain reaction and other laboratory techniques in the diagnosis of long incubation rabies in Australia. *Aust Vet J*. 1993; 70:84–89. PMID:8476363.
8. Crick J, King A. Culture of rabies virus in vitro. In: Campbell JB, Charlton KM, editors. Rabies. Boston (MA): Kluwer Academic Publishers; 1988; 7:47–66.
9. Anonymous. Rabies. In: OIE Terrestrial Manual. Paris: World Organisation for Animal Health; 2013; Chapter 2.1.13:1–26 (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.17_RABIES.pdf).
10. Dean DJ, Abelseth MK, Atanasiu P. The fluorescent antibody test. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies, 4th edition. Geneva: World Health Organization; 1996:88–95.
11. Koprowski H. The mouse inoculation test. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies, 4th edition. Geneva: World Health Organization; 1996:80–87.
12. Webster LT, Dawson JR Jr. Early diagnosis of rabies by mouse inoculation. Measurement of humoral immunity to rabies by mouse protection test. *Proc Soc Exp Biol Med*. 1935; 32:570–73.
13. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg*. 1938; 27:493–97.

Chapter 23

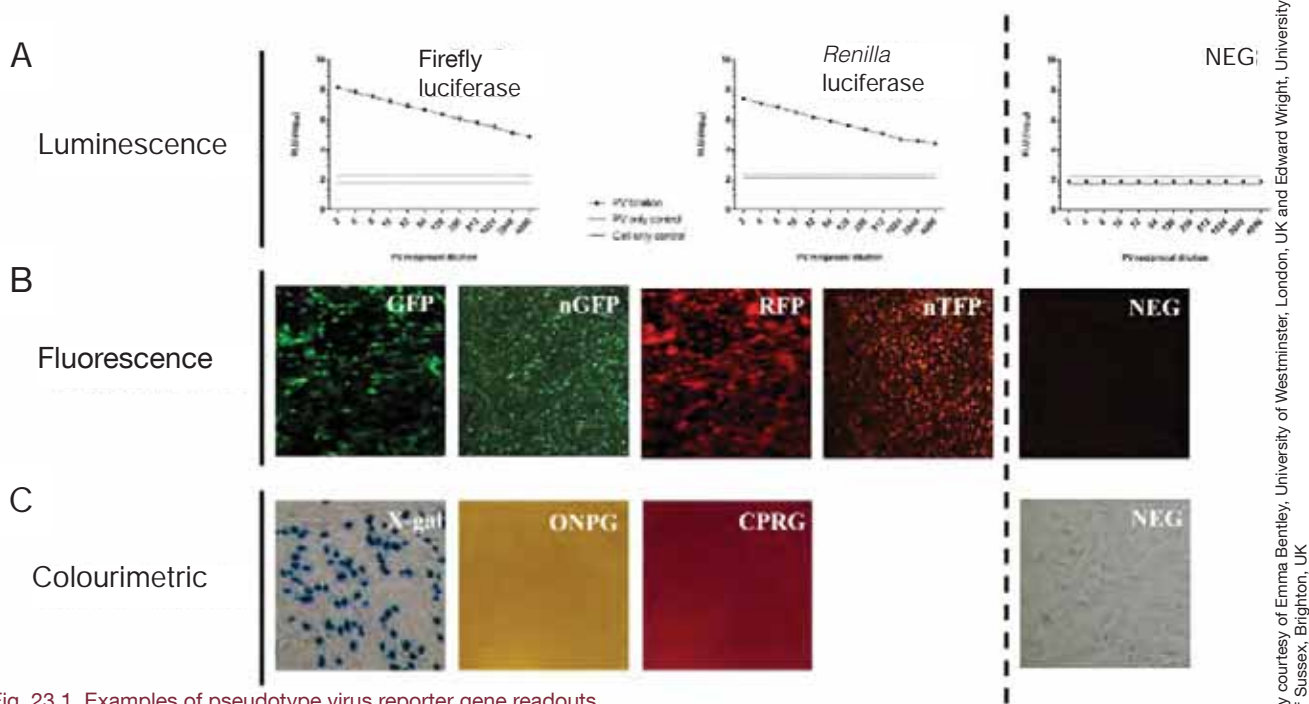
Demonstration of lyssavirus antibodies by pseudotype virus micro-neutralization assays

Introduction

Pseudotyped viruses (PTV) are defined as viral particles which consist of a core (matrix, capsid, nucleocapsid) originating from one virus, which is surrounded by a lipid envelope comprising a viral envelope protein (VEP) protruding from the outer surface. In most cases the genome has been engineered to incorporate a reporter gene while the genetic elements required for replication have been removed. This offers a safe system from which to study properties related to VEP, with PTV able to act as surrogates to highly pathogenic viruses, such as rabies virus (RABV), for serological and virus entry studies. Upon PTV transduction of susceptible cells, detection of reporter gene expression can be used to infer VEP interaction with antibodies and cell receptors, which can be easily and quickly quantified to determine the neutralizing potency of serological samples.

Retroviruses have widely been used as cores for PTV, and current production protocols are based on several decades of retroviral vector development (1). This development is closely allied to the gene therapy field, where retroviral vectors are favoured due to the ability to stably integrate transfer genes into the cell genome without transferring viral genes (2, 3). Early retroviral vectors were based on gammaretroviruses, often murine leukaemia virus (MLV). However, their utility was restricted by only being able to transduce dividing cells. Consequently, the system was soon expanded to include lentiviruses, most commonly human immunodeficiency virus (HIV), which can infect cells in the absence of division.

The use of PTV for serological evaluation is widely reported and increasing, with neutralization assays to detect neutralizing antibodies (nAbs) targeted towards the VEP having been developed for many viruses, providing both sensitive and specific results which correlate with live virus assays. For RABV and other lyssaviruses, pseudotypes have previously been reported for different species, acting as surrogates for live virus to evaluate levels of cross-neutralization afforded by RABV vaccines against European bat lyssavirus (EBLV) -1 and -2 (4). The assay was further applied in a larger serosurveillance study in Africa, a developing country where rabies is endemic, detecting rabies (CVS-11 isolate) antibodies within field serum samples from vaccinated dogs. Based upon unpublished data, further to this, pseudotypes have been generated to include at least one isolate from each lyssavirus species (5). In each case, the PTVNA was shown to be able to distinguish between lyssavirus species and the results were shown to correlate with, or in some cases were more sensitive than, the validated live virus FAVN assay. Additionally, it was demonstrated how serological studies via a PTVNA only require a small volume of serum for each assay, which is 5–10 fold lower than assays with live virus. They can also be generated to encode one of several reporter genes (Fig. 23.1) (6). Both of these factors enhance their suitability to be used in a wide range of laboratory environments and minimizes costs.



By courtesy of Emma Bentley, University of Westminster, London, UK and Edward Wright, University of Sussex, Brighton, UK

Fig. 23.1. Examples of pseudotype virus reporter gene readouts

Infection of target cells with PTV carrying: (A) luminescence reporter genes such as firefly and *Renilla* luciferase are quantified in relative light units; (B) fluorescent report genes such as GFP, RFP and nuclear localized GFP or RFP can be quantified as infectious units; and (C) the colourimetric report gene *lacZ* can be detected using several different substrates: 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), O-nitrophenyl- β -D-galactopyranoside (ONPG) or chlorophenol red- β -D-galactopyranoside (CPRG). For each group of genes, a negative (NEG) result is also shown.

Methods

Generation of PTV stock

Day 1: Seed human embryonic kidney (HEK) 293T/17 cells into a 6-well culture plate ($\sim 2 \times 10^5$ cells/well) or 10-cm culture dish ($\sim 2 \times 10^6$ cells) to be 60–80% confluent at the point of transfection.

Day 2: Prior to transfection the cell culture medium (DMEM) needs to be changed, replacing with 1.5 mL or 5 mL media for cells in a 6-well culture plate or 10-cm culture dish respectively. Following this, in separate 1.5 mL Eppendorf tubes, prepare the plasmid DNA and transfection reagent. Either polyethylenimine or FuGENE® 6 transfection reagent (Promega) are commonly used for this. Separately prepare the plasmid DNA (Table 23.1) and transfection reagent (Table 23.2) and incubate at room temperature for 5 min. It is important that the transfection reagent is added directly into serum-free medium, preferably OptiMEM. Next, add the transfection reagent to the plasmid DNA and incubate for a further 15–20 min, frequently mixing. The transfection mix is then added dropwise to the cells while swirling the culture plate/dish to evenly distribute. Incubate overnight in 37 °C, 5% CO₂ incubator.

Day 3: After 12–16 h incubation, aspirate off the cell culture medium and replace with the equivalent volume.

Day 4: Harvest the cell culture medium containing the PTV, pass through a 0.45 µm cellulose-based filter and store at 4 °C. Replace with the equivalent volume of media and continue incubation.

Day 5: Harvest cell culture medium containing the PTV as before. Combine the two PTV harvests. Ideally, store stocks at –80 °C where they remain stable for many years, otherwise storage at –20 °C, 4 °C and room temperature can be undertaken for months, weeks or days respectively.

Table 23.1. Plasmid DNA mix for transfection

Culture dish	Plasmid DNA			Transfection reagent	
	Gag-pol	Reporter	Envelope	PEI	Fugene-6
6-well	0.6 µg	0.9 µg	0.6 µg	OptiMEM 100 µL	dH ₂ O Up to 10 µL
10-cm	1 µg	1.5 µg	1 µg	200 µL	Up to 15 µL

DNA, deoxyribonucleic acid; PEI, polyethyleneimine

Table 23.2. Transfection reagent

Culture dish	PEI		Fugene-6	
	1 mg/mL PEI	OptiMEM	Reagent	OptiMEM
6-well	22 µL	100 µL	9 µL	100 µL
10-cm	35 µL	200 µL	18 µL	200 µL

DNA, deoxyribonucleic acid; PEI, polyethyleneimine

Titration of PTV stock

The titre of PTV can be assessed by titrating onto a susceptible target cell line (typically BHK-21 cells; ATCC CCL-10) in a 96-well culture plate. In duplicate, prepare a 2-fold serial dilution of PTV within cell culture medium from a 1:2 starting dilution, within a 100 µL final volume. Next, add 100 µL target cells (2×10^5 cells/mL) to each well. It is recommended that controls of target cells alone and PTV alone are included to provide the background level of reporter gene activity. Incubate the assay at 37 °C, 5% CO₂ for 48 h. Titres are determined as infectious units per mL (IFU/mL) or, when using a luminescent reporter, relative light units per mL (RLU/mL). Titrations of PTV with a luminescent reporter gene should be prepared using opaque (preferably white) 96-well culture plates; all other titrations are prepared using clear plates.

To standardize input into downstream assays, the 50% tissue culture infective dose per mL (TCID₅₀/mL) of PTV stocks should be determined. Prepare four replicates of a 5-fold serial dilution of PTV from a 1:5 starting dilution, within a total volume of 100 µL in a 96-well culture plate. Incubate at 37 °C, 5% CO₂ for 60 min. Next, add 100 µL target cells (2×10^5 cells/mL) to each well along with setting up controls of target cells alone. Incubate at 37 °C, 5% CO₂ for 48 h.

After reading plates according to the PTV reporter gene, calculate the TCID₅₀/mL using the Reed–Muench end-point method (7). Firstly, count the cumulative number of wells positive or negative for PTV transduction at each dilution and calculate the percentage negative for each. Set the negative cut-off to 2.5 times the average of the target-cell-alone control wells. The proportionate distance between the dilutions either side of the 50% point is then calculated and applied to the Reed–Muench formula:

$$\text{Proportionate distance} = \frac{(\% \text{ positive above } 50\%) - 50\%}{(\% \text{ positive above } 50\%) - (\% \text{ positive below } 50\%)}$$

$$\log_{10} \text{TCID}_{50} = \log_{10} \text{dilution above } 50\% - (\text{proportionate distance} \times \log_{10} \text{dilution factor})$$

Setting up a PTVNA

Set up a PTVNA in a 96-well culture plate by titrating sera samples in duplicate or triplicate, as required, using cell culture medium to a final volume of 50 µL. Next, prepare PTV by diluting in cell culture medium to add 50 µL containing 50–100 TCID₅₀ per well. As with PTV titration, it is important to include controls of target cells alone and PTV alone in the set up. Additionally, a control representing 100% infection levels that comprised target cells and PTV together should be used. After the PTV has been added, briefly centrifuge the culture plate at 500 r/min for 10 sec before incubating at 37 °C, 5% CO₂ for 60 min. Following incubation, add 100 µL target cells to each well (2 x 10⁵ cells/mL) and incubate the plate for a further 48 h.

Reporter gene assay

Luminescence

When using PTV incorporating a firefly luciferase reporter gene, assays can be read using the Bright-Glo assay system (Promega), prepared following the manufacturer's instructions. After incubation, remove cell culture medium from the 96-well culture plate and add 50 µL of 50% v/v assay reagent in DMEM to each well, incubating on a shaking platform for 3 min. Read the plate on a microplate luminometer, such as the GloMax® Multi+ (Promega). Each well of the 96-well culture plate will be assigned a value in RLU.

Fluorescence

When using PTV incorporating a fluorescent reporter gene, such as GFP, cells must be fixed in the 96-well culture plate by removing cell culture media, adding 0.5% glutaraldehyde (diluted in 1x PBS), and incubating for 10 min at 4 °C. After discarding the fixative, incubate cells with 1x PBS for 5 min at 4 °C. Cells are then washed once with 1x PBS with 100 µL added and left in each well. Transduced cells, emitting fluorescence, can be viewed using a fluorescence microscope or flow cytometer with the appropriate filters/lasers. Infectious units can be determined by counting the number of transduced cells within each well.

Colourimetric

When using PTV incorporating a lacZ reporter gene, cells are fixed as described above for the fluorescent reporters. Following incubation of the cells with 1x PBS for 5 min at 4 °C, cells are washed twice with 1x PBS before being stained by adding 50 µL X-gal (1 mg/mL) diluted in X-gal buffer for 30–60 min at 37 °C. Plates should be wrapped in foil to protect the cells from light. After staining, cells are washed once with 1x PBS with 100 µL added and left on each well. Transduced cells, which present with blue stained nuclei, can then be viewed using a light microscope. Infectious units can be determined by counting the number of transduced cells within each well.

Interpretation of results

Neutralization titres of samples are recorded as the inhibitory concentration at which 100% of PTV input is neutralized (IC₁₀₀). For luminescence reporters, this is when the RLU values reach background levels (similar to target cells alone or PTV alone controls); for fluorescence and colourimetric reporters, this is where no fluorescent/stained cells or colour change is observed. For whichever reporter was used, the neutralization titres can be recorded as the concentration or reciprocal dilution of the sample at which the IC₁₀₀ end-point is achieved. Where IC₁₀₀ end-point titres vary by more than one doubling dilution, the assay should be repeated, and the geometric mean recorded.

It is becoming increasingly common for pseudotype neutralization assays to be multiplexed, where separate PTV preparation bearing different VEP on their surface carry different reporter genes within their genomes (8–10). The use of different reporter genes enables the neutralization potency of each PTV to be accurately determined in the same well using the sample aliquot of sample. However, it is important to note that selection of reporter genes is critical and normally comprises a combination of luminescent, fluorescent and colourimetric genes. The use of secreted reporter proteins is highly discouraged for mono- or multiplex PTV assays as the reporter will be expressed into the cell culture medium during PTV production and therefore be present within stocks of harvested PTV. This will lead to the false-positive detection of reporter activity in downstream assays.

References

1. Temperton NJ, Wright E, Scott SD. Retroviral pseudotypes – From scientific tools to clinical utility. In: eLS. Chichester (UK): John Wiley & Sons, Ltd; 2015:1–11.
2. Sakuma T, Barry MA, Ikeda Y. Lentiviral vectors: basic to translational. *Biochem J.* 2012; 443:603–18. doi:10.1042/BJ20120146.
3. Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol.* 1998; 9:457–63. PMID:9821272.
4. Wright E, Temperton NJ, Marston DA, McElhinney LM, Fooks AR, Weiss RA. Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. *J Gen Virol.* 2008; 89:2204–13. doi:10.1099/vir.0.2008/000349-0.
5. De Benedictis P, Minola A, Rota Nodari E, Aiello R, Zecchin B, Salomoni A, et al. Development of broad-spectrum human monoclonal antibodies for rabies post-exposure prophylaxis. *EMBO Mol Med.* 2016; 8:407–21. doi:10.15252/emmm.201505986.
6. Wright E, McNabb S, Goddard T, Horton DL, Lembo T, Nel LH, et al. A robust lentiviral pseudotype neutralisation assay for in-field serosurveillance of rabies and lyssaviruses in Africa. *Vaccine.* 2009; 27:7178–86. doi:10.1016/j.vaccine.2009.09.024.
7. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol.* 1938; 27:493–7.
8. Wright E, Hayman DT, Vaughan A, Temperton NJ, Wood JL, Cunningham AA, et al. Virus neutralising activity of African fruit bat (*Eidolon helvum*) sera against emerging lyssaviruses. *Virology.* 2010; 408:183–9. doi:10.1016/j.virol.2010.09.014.
9. Zhou Y, Agudelo J, Lu K, Goetz DH, Hansell E, Chen YT, et al. Inhibitors of SARS-CoV entry--identification using an internally-controlled dual envelope pseudovirion assay. *Antiviral Res.* 2011; 92:187–94. doi:10.1016/j.antiviral.2011.07.016.
10. Molesti E, Wright E, Terregino C, Rahman R, Cattoli G, Temperton NJ. Multiplex evaluation of influenza neutralizing antibodies with potential applicability to in-field serological studies. *J Immunol Res.* 2014; 2014:457932. doi:10.1155/2014/457932.

Annex

- DMEM – high glucose (4500 mg/L glucose), L-glutamine, sodium pyruvate and sodium bicarbonate. Liquid, sterile-filtered and suitable for cell culture. Supplemented with 10% fetal bovine serum, 100 units penicillin/mL and 100 µg streptomycin/mL.
- OptiMEM – reduced serum medium, GlutaMAX liquid supplement and contains phenol red.
- Transfection reagents:
 - FuGENE 6 transfection reagent (Promega #E2691).
 - PEI transfection reagent (branched; Sigma #408727) is made up at 1 mg/mL in distilled water dH₂O, pH adjusted to 7 with dilute HCl [hydrochloric acid], sterilized using a 0.22 µm filter and stored at –20 °C until needed. It can then be kept at (+4 °C) for 1 month.
- Bright-Glo assay system (Promega #E2610).
- XGAL substrate buffer – 10 mmol deoxycholic acid, 5 mmol potassium ferrocyanide, 4.3 mmol magnesium chloride and 0.02% (v/v) NP40. X-gal is dissolved in dimethylformamide.
- β-galactosidase substrates:
 - X-gal (Sigma #B-4252)
 - ONPG (Sigma #N1127)
 - CPRG (Sigma #59767)

Chapter 24

A simplified fluorescence inhibition microtest for the determination of rabies virus neutralizing antibodies

Introduction

WHO recommends that anyone who is at risk for exposure to rabies virus (RABV) because of their residence or occupation, including laboratory workers dealing with RABV and other lyssaviruses, veterinarians and animal handlers, should receive pre-exposure vaccination (1). Such persons working with live virus in diagnostic laboratories, research laboratories and vaccine production laboratories, etc. who are at continuous risk of exposure to rabies should have the evaluation of virus-neutralizing antibodies (VNA) performed periodically every 6 months; for other professions (veterinarians, animal handlers, wildlife officers etc.) working in areas endemic for rabies it should be every 2 years. All persons should have a booster dose when the VNA titre against RABV falls below 0.5 IU/mL (1, 2).

Various virus neutralization tests are most commonly used for the detection of VNA. In 1935, Webster and Dawson developed the mouse neutralization test (MNT) for detection of VNA with the inoculation of residual RABV in mice (3). With the adaptation of the challenge virus standard (CVS) in cell culture, an *in vitro* technique was developed as a replacement of MNT, the rapid fluorescent focus inhibition test (RFFIT) performed on 8-well tissue-culture chamber slides. The use of 8-well slides limits the number of serum samples tested and complicates automation (4). Zalan and colleagues (5) developed a microtest for the quantification of rabies VNA based on RFFIT using 96-well microplates, the fluorescence inhibition microtest (FIMT). The FIMT was considered more rapid and economical than the RFFIT.

Based on the RFFIT and the FIMT, during 1992 a simplified fluorescence inhibition microtest (SFIMT) was standardized and implemented at the laboratory for rabies diagnosis of the Pasteur Institute in São Paulo, Brazil for titration of rabies VNA (6). The SFIMT is a cell culture virus neutralizing test performed in 96-well microplates with the advantage of an easy and rapid reading. During 2008, the SFIMT procedure was published in the Manual for laboratory diagnosis of rabies, with the collaboration of the Pasteur Institute in Brazil, the national reference laboratory for rabies diagnosis to the Ministry of Health from Brazil (7). Since 1995, the SFIMT has been used as a RABV serology test in Brazil for pre-exposure evaluation. Today, more than 22 000 sera samples are analysed each year by three Brazilian laboratories using this technique. Moreover, the surveillance of rabies in wildlife is an important tool for epidemiological assessments that may underpin the development of disease prevention and control strategies. Several studies using SFIMT have detected low titres of rabies VNA in wild carnivores and others wildlife in Brazil (8–11).

Methods

All aspects of the Biosafety Guidelines for the classification of RABV risk should be rigorously observed, with respect to the infrastructure of laboratories and safety requisites for the professionals involved.

Propagation of RABV in BHK-21 cells

1. Utilize samples of the strains Pasteur virus (PV) or challenge virus standard-11 (CVS-11) of RABV-infected cultured cells in a monolayer.
2. Initially, a BHK-21 cell monolayer should be established in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS).
3. After growing BHK-21 cells monolayers for 72 h at 37 °C, trypsinize cultures and resuspend cells in MEM + 10% FBS to obtain a final concentration of 5×10^5 cells/mL.
4. Dilute the working virus depending on the titre obtained previously, utilizing the dose that shows the maximal dilution at which there is approximately 100% infection of cells (cell culture infective dose: CCID100) (12).
5. The choice of the capacity and quantity of flasks for the propagation of virus should be in accordance with the volume of virus needed routine work. For example, if utilizing 225 cm² cell culture flasks, add to each flask 9 mL of cell suspension (5×10^5 cells/mL) and 9 mL of virus suspension diluted at the CCID100, and complete the volume with 72 mL of MEM containing 10% FBS. The flasks should be kept at a temperature of 34–37 °C for 72 h.
6. After 72 h, collect the viral suspension in 50 mL sterile conical centrifuge tubes and centrifuge at 3000 r/min ($900 \times g$) for 10 min at 4 °C to remove cells debris.
7. Collect the supernatant in aliquots of 40 mL and 1.0 mL and store at –80 °C before titration to obtain the working dilution.

Virus titration to determine the virus work titre/dilution (CCID100)

1. In the 96-well microplate do a 2-fold serial dilution of the virus in 12 wells of the microplate starting with a dilution of 1:2 (Log dilution 0.3), in triplicate.
2. Add 50 µL of the MEM with 10% of FBS in the 12 wells.
3. At the first well, with a micropipette add 50 µL of the one aliquot of the virus pre-thawed, mix it and transfer 50 µL of the mixture to the second well and do successively until the last well. Discard the final 50 µL.
4. Add 100 µL of MEM 10% FBS to all the 12 wells.
5. Prepare a suspension of BHK-21 cells with a concentration of 10^6 cells/mL.
6. Add 50 µL/well of the BHK-21 cells suspension (5×10^4 cells/well).
7. Incubate the microplate for 24 h at 37 °C with 5% of CO₂.
8. After 24 h, discard the MEM from the microplates by aspiration and put the microplate on a cold pack or in an ice bath.
9. Add to each well 150 µL of an ice-cold solution of acetone 80%. Fix the cells in the microplates in ice-cold acetone for 15 min.

10. Discard the acetone and let the microplates dry to evaporate all remaining acetone.
11. Add to each well 40 μL of FITC anti-rabies conjugate (13, 14) diluted in PBS 0.01 mol pH 7.3 with 0.1% Evans Blue and incubate for 1 h at 37 °C.
12. After 1 h, discard the microplate solution, then rinse the microplate three times in PBS and three times in clinical laboratory reagent water (CLRW).
13. Let the microplate dry and add 50 μL of 10% glycerin in PBS in each well on the microplate.
14. Observe the infection with an inverted fluorescence microscope with 100x magnification.
15. The virus titre is established as the highest dilution that shows 100% infection on BHK-21 cells, CCID100 (12).

SFIMT procedure

The SFIMT is performed in 96-well cell culture microplates. Serum samples to be tested must be heat inactivated at 56 °C for 30 min.

1. The standard serum is a human anti-rabies immunoglobulin diluted to contain 0.5 IU/mL and calibrated with the second international reference serum of 30 IU/mL.
2. The positive control serum is a mixture of several sera from immunized persons, titrated several times with 1.00 IU/mL. The negative control serum is a mixture of sera from individuals not vaccinated (< 0.08 IU/mL).
3. Serum dilution
 - 3.1. Prepare six dilutions of sera (standard, samples test and controls) (Fig. 24.1) on serial dilution 2-fold in 100 μL of MEM/10% FBS with 1% antibiotics (penicillin–streptomycin solution), starting at 1:10 (log dilution 1.0), as follows:
 - 3.2. In the first well, add 20 μL of the serum and 180 μL of MEM/10% FBS;
 - 3.3. From the second to the sixth well, add 100 μL of MEM/10% FBS;
 - 3.4. Mix the contents of the first well and transfer 100 μL of the mixture to the second well successively until the last well and discard the final 100 μL .

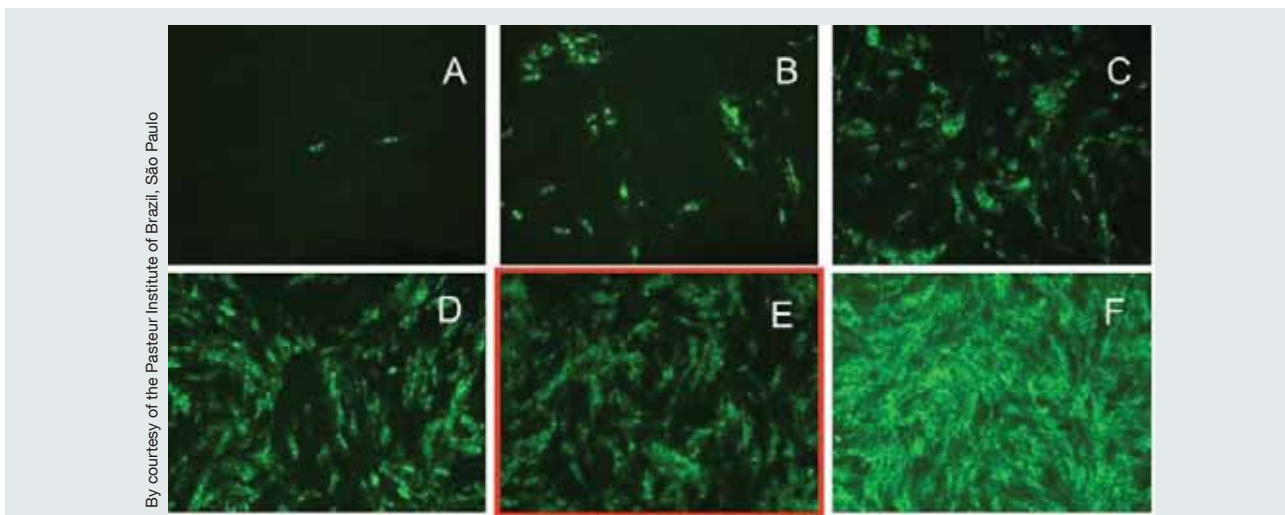
By courtesy of the Pasteur Institute of Brazil, São Paulo

	Log dilution	1.00	1.30	1.60	1.90	2.20	2.50	Log dilution		1.00	1.30	1.60	1.90	2.20	2.50
		1	2	3	4	5	6			7	8	9	10	11	12
Standard	A							Sample 8	A						
Sample 1	B							Sample 9	B						
Sample 2	C							Sample 10	C						
Sample 3	D							Sample 11	D						
Sample 4	E							Sample 12	E						
Sample 5	F							Positive	F						
Sample 6	G							Negative	G						
Sample 7	H							Virus titration	H	2X	1X	1:2	1:4	Cell	Cell

Fig. 24.1. SFIMT schematic of an assay showing positions, dilution of serum samples and controls

4. The virus is stored in 1 mL microtubes at -80°C . One tube is thawed rapidly under cold running water and placed in melting ice.
5. Add $50\ \mu\text{L}$ /well of the virus suspension previously diluted at CCID_{100} .
6. For the virus control in the assay, do a serial dilution of the working virus dilution on four wells of the microplate as follows:
 - 6.1. Add $50\ \mu\text{L}$ of MEM/10% FBS in the 4 wells;
 - 6.2. Add $100\ \mu\text{L}$ of the virus suspension in the first well;
 - 6.3. Add $50\ \mu\text{L}$ of the virus suspension in the second and third wells;
 - 6.4. Mix the contents of the third well, transfer $50\ \mu\text{L}$ to the fourth well mix and discard the last $50\ \mu\text{L}$;
 - 6.5. Add $50\ \mu\text{L}$ of MEM/10% FBS in the second well and $100\ \mu\text{L}$ of MEM/10% FBS in the third and fourth wells.
7. Incubate the microplate for 1 h at 37°C with 5% CO_2 .
8. Add $50\ \mu\text{L}$ /well of the BHK-21 cells suspension with 10^6 cell/mL (5×10^4 cells/well).
9. Incubate the microplate for 24 h at 37°C with 5% of CO_2 .
10. After 24 h of incubation, proceed as described previously in above fixation and staining steps 8–15 on pages 261–2.
11. The reading of the results is performed using an inverted fluorescence microscope with 100x magnification. The serum titre is the dilution with a 50% reduction of infection in cells, or 50% of reduction of fluorescence (Fig. 24.2).
12. The conversion of the dilution titre 50% of the serum in the IU/mL titre is performed by using the following formula:

$$\text{Serum titre (IU/mL)} = \frac{\text{serum standard titre (IU/mL)} \times 10^{(\text{serum log dilution } 50\%)}}{10^{(\text{standard log dilution } 50\%)}}$$



By courtesy of the Pasteur Institute of Brazil, São Paulo

Fig. 24.2 (A–F). Titration of virus neutralizing antibodies by SFIMT at log dilutions 1.0–2.5; log dilution 2.2 (E) shows 50% reduction of infection in BHK-21 cells infected with Pasteur virus

References

1. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012, http://apps.who.int/iris/bitstream/handle/10665/85346/9789240690943_eng.pdf; accessed 28 May 2018).
2. WHO Guide for rabies pre and post exposure prophylaxis in humans. Geneva: World Health Organization; 2014 (http://www.who.int/rabies/PEP_Prophylaxis_guideline_15_12_2014.pdf, accessed 28 May 2018).
3. Webster LT, Dawson JL. Early diagnosis of rabies by mouse inoculation. Measurement of humoral immunity to rabies by mouse protection test. *Proc Soc Exp Biol Med.* 1935; 32:570–3.
4. Smith J, Yager PA, Baer GM. A rapid reproducible test for determining rabies neutralizing antibody. *Bull World Health Organ.* 1973; 48:535–41. PMID:4544144.
5. Zalan E, Wilson C, Pukitis D. A microtest for quantification of rabies virus neutralizing antibody. *J Biol Stand.* 1979; 7:213–20.
6. Favoretto SR, Carrieri ML, Tino MS, Zanetti CR, Pereira OAC. Simplified fluorescent inhibition microtest for the titration of rabies neutralizing antibodies. *Rev Inst Med Trop Sao Paulo.* 1993; 35:171–5.
7. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. [Manual for laboratory diagnosis of rabies]. Brasília: Editora do Ministério da Saúde; 2008 (in Portuguese).
8. Megid J, Almeida MF, Albas A, Araujo Jr JP, Duarte JMB. Occurrence of rabies neutralizing antibodies in wild deer (*Blastocercus dichotomus*) from the western region São Paulo Estate, Brasil. *Int J Vet Med.* 2000 (<http://www.priory.com/vet/deer.htm>, accessed 21 March 2012).
9. Jorge RSP, Pereira MS, Morato RG, Scheffer KC, Carnieli Junior P, Ferreira F, et al. Detection of rabies virus antibodies in Brazilian free-ranging wild carnivores. *J Wildl Dis.* 2010; 46(4):1310–5. PMID:20966286.
10. Casagrande DKA, Favaro ABBBC, Carvalho C, Picolo MR, Hernandez JCB, Lot MS, et al. Rabies surveillance in bats in Northwestern State of São Paulo. *Rev Soc Bras Med Trop.* 2014; 47:709–15. doi:10.1590/0037-8682-0189-2014.
11. Onuma SSM, Chaves LB, Souza MCC, Lara H, May-Júnior JA, Taques IGG, et al. [Serological and molecular investigation of viral agents in free-living jaguars in the Pantanal of Mato Grosso, Brazil]. *Braz J Vet Res An Sci.* 2016; 53:270–9 (in Portuguese).
12. Batista AM, Cruz PS, Almeida E, Costa AEB, Scheffer KC, Chaves LB, et al. [Infection of BHK-21 cells cultured in stationary monolayers by PV and CVS virus strains]. *BEPA.* 2009; 6:4–11 (in Portuguese).
13. Atanasiu P, Tsiang H, Virat J. Obtenção D'IgG anti-núcleocapsides rabiques. [Purification and conjugation with peroxidase or fluorescein isothiocyanate]. *Ann Microbiol.* 1974; 125B:85–98 (in Portuguese).
14. Caporale GMM, Silva ACR, Peixoto ZMP, Chaves LB, Carrieri M, Vassão, RC. First production of fluorescent anti-ribonucleoproteins conjugate for diagnostic of rabies in Brazil. *J Clin Lab Anal.* 2009; 23:7–13.

Annex

Reagents

- clinical laboratory reagent water (CLRW)
- Eagle's Minimum Essential Medium
- fetal bovine serum (FBS) – heat-inactivated
- phosphate buffered saline (PBS) 0.01 M pH 7.3
- glycerin 85% PA
- trypsin ethylene diaminetetraacetic acid (EDTA)
- high-grade acetone 80% (diluted with CLRW), stored at 4 °C
- Evans Blue

Equipment

- biological safety cabinet (BSC)
- fluorescence microscope
- CO₂ incubator
- laboratory refrigerate centrifuge
- –80 °C ultra-freezer

Biological materials

- baby hamster kidney-21 cells (BHK-21 C13 [ATCC CCL-10])
- Pasteur virus (PV)
- challenge virus standard strain 11 (CVS-11 [ATCC VR 959])

Chapter 25

The immunoperoxidase inhibition assay

Introduction

The immunoperoxidase inhibition assay (IIA) is an alternative test for the detection of rabies virus (RABV) antibodies in sera. The IIA is a blocking assay based on the reaction between test sera and RABV antigens in infected cells. Positive test sera will inhibit linkage of an anti-RABV polyclonal serum (prepared in rabbits), which will then be revealed by the addition of an anti-rabbit IgG/peroxidase conjugate. The test is low in cost and does not require specific equipment, such as a fluorescence microscope, only basic cell culture facilities. The results are easily read in an ordinary light microscope and interpretation is straightforward, with reading based on an “all or nothing” principle. When compared to the “simplified fluorescence inhibition microtest” (1) with human sera, it revealed a strong positive correlation (2). As the IIA is based on a blocking reaction, it might also be potentially useful for detecting RABV antibodies in any species without specific conjugates. This assay may be advantageous over other similar methods designed to detect RABV-specific binding antibodies because it can be easily introduced into most laboratories, provided basic cell culture facilities are available.

Advantages of the IIA

The IIA is advantageous for the following reasons:

- does not utilize competing monoclonal antibodies;
- does not require preparation of large volumes of virus nor purification of viral proteins for antigen preparation;
- does not require expensive equipment for antigen preparation or ELISA plate readers;
- does not require a fluorescence microscope; readings can be performed with the aid of a regular light microscope with a 20x or 40x objective magnification;
- the polyclonal rabies anti-serum required can be prepared locally; and
- the test can be potentially useful for the detection of antibodies in different animal species.

Protocol

1.1 Cells

- For the IIA, cultivate the chicken embryo related (CER) cell lineage (3) in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin), splitting cells once weekly.

1.2. Viruses

- For the IIA, propagate RABV strain Pasteur virus (PV) in CER cells (prepared as in 1.1).
- For production of RABV antiserum in rabbits, use the challenge virus standard (CVS) after vaccination. Amplify CVS in mice aged 3–4 weeks following standard procedures (see [Chapter 8](#) on Virus isolation in animals: the mouse inoculation test) or prepare the virus in cell culture as described by Caporale and colleagues (4).

1.3. Preparation of polyclonal rabbit anti-rabies serum

- Vaccinate 3 rabbits intramuscularly with three weekly doses of a commercially available rabies vaccine.
- Twenty-one days after the last immunization, challenge the rabbits with 100 50% lethal doses (LD₅₀) of strain CVS.
- Collect serum samples from each animal 3 weeks after challenge.
- Titrate serum samples from each animal with a usual antibody detection assay and use as a secondary antibody in the IIA. The appropriate dilution of the polyclonal rabbit anti-rabies serum to be used in the IIA is determined by testing serial 2-fold dilutions of the serum in the test with the positive reference serum (diluted to 0.5 IU/mL) and the antibody-negative control serum as a source of “blocking” antibodies. The highest serum dilution capable of binding to antigen-containing cells with a clear positive reaction after staining and a minimal background staining should be selected for use in subsequent tests.
- Make polyclonal rabbit anti-rabies serum in dilution buffer ([as described in 2.1](#)).
- Perform the animal handling procedures according to institutional guidelines for animal welfare for experimentation on animals (http://www.oie.int/index.php?id=169&L=0&htmfile=titre_1.7.htm).

1.4. Preparation of plates

- Add CER cells in 96-well tissue culture plates (3–5 x 10⁵ cells per well in 100 µL volume).
- Incubate at 37 °C in a 5% CO₂ atmosphere overnight.
- Inoculate each well with 50 µL of a suspension containing 100 TCID₅₀ of strain Pasteur virus.
- Incubate at 37 °C in a 5% CO₂ atmosphere for 72 h.

- Remove the supernatant.
- Freeze the plates at $-20\text{ }^{\circ}\text{C}$ in sealed bags for at least 1 h.
- Remove plates from the freezer.
- Add 100 μL of fixation buffer to each well (as described in 2.2).
- Incubate for 20 min at room temperature.
- Wash the monolayers three times with 100 μL /well of wash buffer (as described in 2.3) and submit to the IIA.

1.5. Preparation of serum samples

- Inactivate the sera test at $56\text{ }^{\circ}\text{C}$ for 30 min.
- Dilute an international reference serum to a final concentration of 0.5 International Units of RABV neutralizing antibodies per mL (IU/mL) and include in all experiments as positive control serum.
- If possible (after Human Subjects Review and following all biosafety precautions for the use of human blood products), use serum collected from a person not previously vaccinated against rabies as a negative control, inactivate as described above and dilute 1:2 for use in all experiments.

Considerations

Make dilutions of test sera as well as positive and negative control sera in dilution buffer (as described in 2.1).

1.6. The IIA procedure

- Add test sera in duplicate and test in 5-fold dilutions (diluted as in 1.5) to plates (prepared as in 1.4).
- Add positive and negative control sera (diluted as in 1.5) to plates (prepared as in 1.4).
- Incubate for 1 h at $37\text{ }^{\circ}\text{C}$.
- Wash the plates as described in 1.4.
- Add 50 μL /well of an appropriate dilution (1:40) of polyclonal rabbit anti-rabies serum.
- Incubate for 1 h at $37\text{ }^{\circ}\text{C}$.
- Wash the monolayers three times with 100 μL /well of wash buffer (as described in 2.3);
- Add 50 μL /well of anti-rabbit IgG peroxidase conjugate (1:150).
- Incubate for 1 h at $37\text{ }^{\circ}\text{C}$.
- Wash the monolayers three times with 100 μL /well of wash buffer (as described in 2.3);
- Add 50 μL /well the substrate 3-Amino-9-ethylcarbazole (AEC, Sigma) prepared fresh each time, as recommended (5).
- Incubate the reaction at $37\text{ }^{\circ}\text{C}$ for 15–40 min depending on the visible intensity of the staining, as judged by comparing specifically stained wells (with negative control serum) and unstained wells (with positive reference serum).
- Remove the substrate.

- Add 100 µL/well of PBS.
- Read the plates on a standard inverted microscope with a 20x magnification objective.

Considerations

- Make all dilutions of test sera, polyclonal rabbit anti-rabies serum and the anti-rabbit IgG peroxidase conjugate and controls in dilution buffer (as described in 2.1).
- Use antibody-positive sera when it is capable of inhibiting 100% of the binding of the polyclonal rabbit anti-rabies serum, thus precluding binding of the conjugate, giving rise to unstained wells.
- Use antibody-negative sera when it is incapable of inhibiting the binding of the polyclonal rabbit anti-rabies serum, giving rise to stained wells.
- The peroxidase conjugate reacts with hydrogen peroxide in the presence of the substrate AEC, which precipitates on the antigen-containing cells, allowing visualization of the characteristic carmine red cytoplasmic staining.
- Calculate the antibody titre of the test sera on the basis of the last dilution capable of blocking 100% of the specific staining.
- Consider the test valid if the reference serum (diluted to 0.5 IU/mL) is able to block 100% of the AEC colour reaction by competing for antigen with the polyclonal rabies anti-serum. Likewise, polyclonal RABV anti-serum binding should not be inhibited by the negative control serum (diluted 1:2), shown by a coloured reaction in the wells.
- Express the IIA results in equivalent units per mL of serum (EU/mL) by calibration against the international reference serum. Antibody titres ≥ 0.5 EU/mL should be considered positive for antibodies, whereas antibody titres < 0.5 EU/mL should be considered negative (6).

Description of solutions

2.1 Dilution buffer

29.5 g NaCl; 1.55 g Na₂HPO₄; 0.23 g Na₂HPO₄, 0.5% Tween 80, H₂O q.s.p. 1 L.

2.2 Fixation buffer

4% paraformaldehyde in PBS (8.5 g NaCl, 1.55 g Na₂HPO₄, 0.23 g Na₂HPO₄, H₂O q.s.p. 1 L, pH 7.2).

2.3 Wash buffer

0.5% Tween 80 in PBS, pH 7.5.

References

1. Favoretto SR, Carrieri ML, Tino MS, Zanetti CR, Pereira OAC. Simplified fluorescent inhibition microtest for the titration of rabies neutralizing antibodies. *Rev Inst Med Trop Sao Paulo*. 1993; 35:171–5.
2. Batista HBCR, Lima FES, Maletich D, Silva ACR, Vicentini FKV, Roehle LR, et al. Immunoperoxidase inhibition assay (IIA) for rabies antibody detection. *J Virol Meth*. 2011; 174:65–8.
3. Cardoso TC, Queiroz da Silva LH, Albas A, Ferreira HL, Perri SHV. Rabies neutralizing antibody detection by indirect immunoperoxidase serum neutralization assay performed on chicken embryo related cell line. *Mem Inst Oswaldo Cruz*. 2004; 99:531–4. doi:S0074-02762004000500013.
4. Caporale GMM, Silva ACR, Peixoto ZMP, Chaves LB, Carrieri M, Vassão, RC. First production of fluorescent anti-ribonucleoproteins conjugate for diagnosis of rabies in Brazil. *J Clin Lab Anal*. 2009; 23:7–13.
5. Harlow E, Lane D. *Antibodies: a laboratory manual*, 2nd edition. New York (NY): Cold Spring Harbor Laboratory Press; 1988.
6. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012, <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 28 May 2018).

Annex. Reagents, equipment and biological materials required for the IIA

Reagents

- Eagle's Minimum Essential Medium (EMEM)
- fetal bovine serum (FBS)
- trypsin
- antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin)
- substrate 3-amino-9-ethylcarbazole (AEC)
- phosphate buffered saline (PBS)
- anti-rabbit IgG peroxidase conjugate

Equipment

- Biological Safety Cabinet
- standard inverted microscope
- CO₂ incubator
- freezer

Animals

- mice aged 3–4 weeks
- rabbits

Biological materials

- chicken embryo related (CER) cell lineage
- Pasteur virus (PV)
- challenge virus standard (CVS)
- rabies vaccine
- international reference serum
- negative control serum

Chapter 26

Demonstration of rabies virus antibodies by the counter immunoelectrophoresis test

Introduction

Titration of antibodies allows the immunity of individuals who receive anti-rabies pre- or post-exposure prophylaxis (PEP) to be quantified. A titre of 0.5 IU/mL in the serum neutralization test is considered the minimum adequate value indicative of an individual response after prophylaxis (1). Such tests are used to quantify virus neutralizing antibodies (VNA) against rabies virus (RABV) (1–3). The mouse neutralization test (MNT), the first to be developed (4, 5), was long considered as the reference test; however, it is costly due to animal use (6) and also raises ethical and animal welfare issues. The rapid fluorescent focus inhibition test (RFFIT), originally developed by Smith and colleagues (7) and Zalan and colleagues (8), was the test used to adapt the technique to cell culture. The RFFIT reveals the presence or absence of viral infection in cell culture after ~20 h, using the fluorescent antibody technique (9). The RFFIT (see also [Chapter 19](#)), together with the FAVN (fluorescent antibody virus neutralization) test (see also [Chapter 20](#)), are now considered the gold standard techniques (7,9) due to their good correlation with the MNT (see also [Chapter 22](#) and references 2, 10–12).

Counter immunoelectrophoresis (CIE) is another technique for the titration of RABV-specific antibodies. The CIE detects antibodies of the IgG class, viral anti-glycoprotein, the main immunoglobulin involved in response against the virus. For this reason, the CIE can determine the neutralizing potential of analysed sera (13, 14). The CIE is performed on agarose gel slides and is based on the reaction of the antibodies present in serial dilutions of the test serum against a constant concentration of viral antigen. After a first electrophoretic run, the hyperimmune indicator serum is located to allow for diffusion in the opposite direction. A precipitation line forms at any serum dilution where no antibodies are present. The technique is simple, low cost and provides results in a short period of time (6–8 h), and therefore is of utility for clinical practice (13, 15–18).

The comparison of RABV Ab titres detected by a modified CIE (MCIE) and MNT show good correlation between these techniques (13). Further, when compared with the standard MNT and RFFIT techniques, MCIE had good sensitivity and specificity for estimating antibodies (19, 20). In addition, the MCIE and MNT were not obviously different regarding the titre of detected antibodies (21). Several studies using this technique have been published (22, 23). Moreover, the MCIE was shown to be sensitive enough to detect the minimum acceptable level of Ab (0.5 IU/mL) (18).

Protocol

The CIE is performed according to the general procedure described by Diaz and Myers (13).

1. Centrifuge the sera or plasma to be tested (TS) at 1000 *g* for 10 min.
2. Transfer the supernatant into a sterile tube, inactivated at 56 °C for 30 min in a water bath.
3. Store at –20 °C or lower.
4. At the time of titration, thaw the sera and prepare a serial dilution $q = 2$ with PBS pH 7.2, starting at 1/1 (neat) to 1/16 with 50 μ L volumes per tube (final dilutions 1/2 to 1/32).
5. Add 50 μ L of the standardized Ag to pre-dilution with twice the working concentration (for example, working dilution = 1/10, pre-diluted to 1/20) in all TS tubes and 100 μ L in the negative control tube containing 100 μ L of negative serum (NS), so that the Ag concentration falls to the ideal (half) after mixing.
6. Incubate the serum–Ag mixture at 37 °C for 60 min.
7. Perform the electrophoretic run on 0.9% agarose gel slides, melted in a water bath or microwave oven at < 90 °C.
8. To produce the slides, pour the volume of 8.0 mL onto the 75 × 50 mm and 4.0 mL for the 75 × 25 mm control slides on a level surface.
9. Let the gel slides solidify and keep them refrigerated in a closed chamber for 15 min.
10. Make two sets of five wells on the gel using one cardboard template under each glass slide, one 6 mm series for the serum–Ag mixture and one 3 mm for the hyperimmune indicator serum (IS), at a distance of 8–10 mm from each other.
11. In the control slide, make two wells of 6 mm and two wells of 3 mm (Fig. 26.1).
12. Leave the cut gel plugs in the wells by immediately withdrawing the 6 mm wells and place the slides in the electrophoresis chamber filled with borate buffer pH 9.1.
13. Position the 6 mm wells on the anode side (positive) and the 3 mm wells on the cathode side (negative).
14. Distribute the serum–Ag mixture in the 6 mm wells containing TS and the negative control slide.
15. Carry out the first run with a constant current of 10 mA per slide for 45 min, keeping the connecting wicks moistened constantly.
16. After this period, distribute the IS in the 3 mm wells and proceed with another electrophoresis run for 120 min.
17. The electrophoretic run is performed using equipment such as an electrophoresis power supply, voltmeter, electrophoresis chamber, biosafety cabinet class IIA, refrigerated centrifuge that reaches 5000 *g*, and water bath at 37 °C and 56 °C (see Figs. 26.2–3). The wicks connecting the slides and buffer can be made with filter paper or viscose fabric (Fig. 26.4).

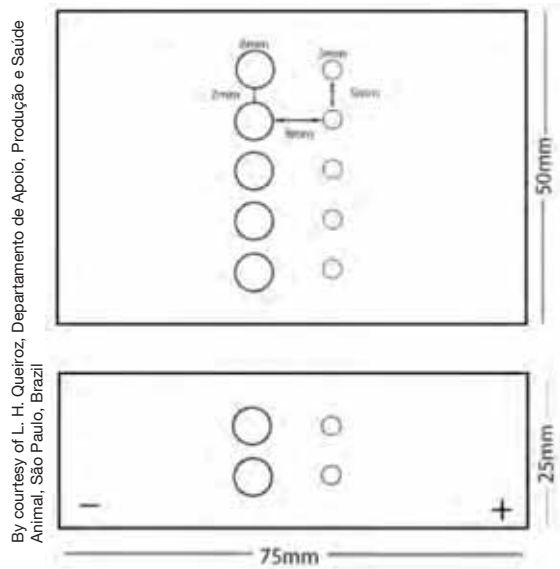


Fig. 26.1. Diagram (pattern) employed for the CIE agarose gel slides: the Ag-serum mixtures are placed in the 6 mm wells and the indicator serum in the 3 mm wells



Fig. 26.2. Main equipment used in the CIE test

By courtesy of W. C. Moreira, Instituto Municipal de Medicina Veterinária Jorge Vaitsman, Secretaria Municipal de Saúde, Rio de Janeiro, Brazil



Fig. 26.3. Power supply and electrophoresis chamber, with lateral channels and a central support for glass slides

By courtesy of W. C. Moreira, Instituto Municipal de Medicina Veterinária Jorge Vaitsman, Secretaria Municipal de Saúde, Rio de Janeiro, Brazil



Fig. 26.4. Electrophoresis chamber, with the glass slides and connecting wicks made of viscose fabric for cleaning use (such as Perfex)

By courtesy of W. C. Moreira, Instituto Municipal de Medicina Veterinária Jorge Vaitsman, Secretaria Municipal de Saúde, Rio de Janeiro, Brazil

Interpretation of test results

18. Read the slides with an indirect light source against a dark background (for example, a colony counter).
19. To consider the test as valid, verify if the control slide shows precipitation lines on the negative serum (NS) side and no line in the negative control well.
20. Evaluate the precipitation lines of the TS in relation to their position, sharpness, and shape, which should be comparable to those obtained in the control slide. The TS titre is expressed as the reciprocal of the end-point, which is the highest dilution that does not produce a precipitation line or relative potency, expressed in International Units using a reference standard (which can be diluted to 1.0 IU/mL).

$$RP = \frac{\text{reciprocal of end - point of ST}}{\text{reciprocal of end - point RS}} \times \text{IU mL of RS}$$

Where:

RP = relative potency

ST = serum test

RS = reference serum

IU = International Units

Antigen production

The Ag is produced from brains of 6–8 or more litters of suckling mice of the Swiss Webster line, aged up to 3 days, inoculated intracerebrally with a 20 μ L suspension of RABV CVS strain diluted to contain 100–1000 LD₅₀.

1. During the paralysis phase, euthanize and collect the brains, weigh them and produce a 40% (w/v) suspension in a glycine (0.01%) and sucrose (5%) solution.
2. Centrifuge the brain suspension at 5000 *g* for 30 min at 4 °C.
3. Collect the supernatant, keep an aliquot (stored at –70 °C) for titration in mice, and inactivate the remainder in a 56 °C water bath for 30 min.
4. Store at –20 °C or lower for at least 2 weeks prior to standardization.
5. Perform a viral inactivation test by inoculating intracerebrally a 10% suspension of the inactivated Ag in 10 suckling mice that are observed for 21 days.
6. Before standardizing the Ag, titrate the aliquot in weaned mice. If the titre is < 10⁶ LD₅₀/30 μ L, discard the lot, as it will have low activity in the CIE (15).

Antigen standardization

The Ag titre is determined by comparing the reference serum (RS) with the negative serum (NS).

1. Defrost the Ag and separate into aliquots of 0.5 or 1.0 mL.
2. Titrate by CIE, pre-diluting in PBS at pH 7.2 (seven to 10 different dilutions).
3. Prepare two tube sets, one containing 50 μ L aliquots of NS, identified with the respective dilution of the candidate Ag. In the other set, the RS, diluted to contain 1 IU/mL, is distributed in equal volume.
4. Homogenize the Ag, dilute (for example, q = 5 factor 1/2.5 to 1/25) and add 50 μ L of each dilution to the respective NS or RS tube series; this will double the final dilutions of the Ag (1/5 to 1/50).
5. Perform the other steps of the CIE: first the electrophoretic run, then distribute the indicator hyperimmune serum in the 3 mm wells and proceed to the second run. The titre of the Ag expressed in reciprocal dilution is determined by selecting the highest dilution that produces sharp, shiny, slim and curved precipitation lines in the NS (Fig. 26.5), and simultaneously does not form a precipitation band or line against the RS (24).
6. Repeat the titration at least three times on different days to determine the Ag titre.
7. Test known test samples with high, medium and low titres to confirm the results obtained before placing the new standardized Ag into use. The Ag titre can also be determined in conjunction with IS standardization by either block titration or so-called checkerboard titration procedure.

By courtesy of W. C. Moreira, Instituto Municipal de Medicina Veterinária Jorge Vaitzman, Secretária Municipal de Saúde, Rio de Janeiro, Brazil

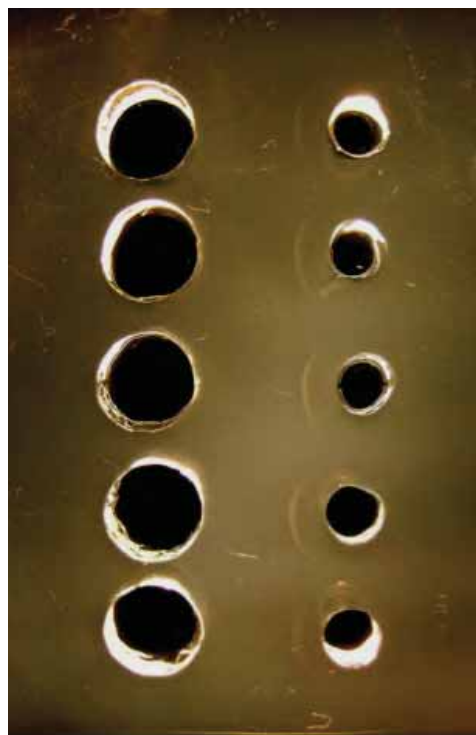


Fig. 26.5. Agarose gel layer showing the precipitation lines formed by the reaction between antigen and indicator serum

Production of hyperimmune indicator serum

Hyperimmune serum is used as an indicator to reveal the result of the CIE. It is prepared from a pool of sera of rabbits, hyperimmunized against RABV, with the sera standardized by CIE.

Vaccine production

A vaccine is produced by diluting the CVS strain to obtain 100–1000 LD₅₀ in 30 µL intracerebrally in mice.

1. Inoculate intercranially a litter of rabbits aged up to 3 days.
2. Euthanize the animals when neurological signs appear, then collect the brains and store them at –20 °C or lower.
3. Weigh to obtain mass and grind the brains, kept in a freezer or mixer, and dilute to 20% (w/v) in glycine (0.01%) and sucrose (5%) solution.
4. Centrifuge the brain suspension at 1500 *g* for 30 min, collect the supernatant and distribute in 1.0 mL volumes, keeping an aliquot for titration in mice and storing in a freezer at –20 °C or lower.
5. Titrate the separated aliquot in mice; the titre obtained should not be less than 10⁶ LD₅₀ in 30 µL i.c. Vaccine potency should be greater than 3.0 IU/dose by NIH test (see [Chapter 44](#) in Volume II of this manual [forthcoming]).

Vaccination and blood collection

1. At vaccination, prepare the vaccine by adding marcol–montanide mineral oil (10% marcol 88 and 90% montanide) in equal volume to the CVS virus suspension already inactivated in a water bath at 56 °C for 30 min. Alternatively, the vaccine can be inactivated by β-propiolactone at 1:1500 concentration (0.02 M sodium bicarbonate solution) in a water bath at 37 °C for 120 min, and then chilled overnight at 4 °C.
2. Inject intraperitoneally the 2 mL volume of the emulsion in three or more New Zealand rabbits weighing between 1.5 and 2.5 kg, once a week, for 4 weeks.
3. After the fourth dose of vaccine, two challenges must be performed in a one-week interval between each, inoculating intraperitoneally 1 mL of the non-inactivated vaccine.
4. Some 10 days after the last challenge dose, collect a blood sample through the marginal ear vein and titrate the serum by the CIE. All animals with VNA titres ≥ 1/128 will undergo total bleeding by cardiac puncture under anaesthesia, followed by euthanasia.

Obtaining hyperimmune serum

1. Defrost the pool of sera, transfer to sterile tubes and centrifuge at 2000 *g* for 30 min at 4 °C.
2. Collect the supernatant, filter on a 0.22 µm membrane and inactivate in a water bath at 56 °C for 30 min.
3. Place the aliquot in 50 mL vials and keep them frozen at –20 °C or lower.

Standardization of hyperimmune indicator serum

The IS used in the Ab titrations in the CIE is standardized against the Ag in the working dilution to determine the optimum serum dilution.

1. Thaw the IS, separate into aliquots of 0.5 or 1.0 mL and dilute the candidate serum in PBS at pH 7.2 (for example, 1/10 to 1/60).
2. Dispense 50 μ L NS aliquots into 10 tubes, add 50 μ L of half the standardized Ag working dilution into all tubes and incubate at 37 °C for 60 min.
3. Proceed to the first electrophoretic run.
4. For the second run, fill the 3 mm wells of the agarose gel with each of the IS dilutions to be tested and continue with the CIE procedure. The hyperimmune serum titre is assumed to be the reciprocal of the highest dilution that presents a clear, bright, thin and curved precipitation line.

The Ag and IS must be standardized prior to TS titration in order to determine the inter-assay precision of the new batch of reagents. A minimum of three determinations can be performed to determine precision on different days.

The acceptance criteria of the test include evaluating the assay, the samples and the reference serum.

Validation of the test

The validity of the test is determined by evaluating the control and RS slides. The control slide should have precipitation lines on the NS side and no line in the negative control well. For the samples, TS precipitation lines are evaluated for position, sharpness and shape, and should be comparable to those obtained on the control slide. A weak (unclear) line is interpreted as non-precipitation formation; if this occurs in two dilutions of the same slide, it indicates test invalidity due to sample. The same procedure should be applied when assessing the test validity due to RS. If any problem occurs on the control slide or RS, any test is considered invalid and should be repeated, but if the verified problem is in one of the TS, only the invalid sample should be repeated.

The performance of the test should be monitored through control graphs for Ag and reference serum, where statistical data are shown over time. Where deviations occur, evaluate the need to re-titre the standards and verify the condition of the media, buffers and solutions. This tool allows the behaviour of the assay to be evaluated, the presence of variables acting on the process to be identified, and decisions on preventive and/or corrective actions to be taken. A minimum of 20 data points for each quality parameter should be obtained and the mean, standard deviation (SD), mean and ± 2 and 3 times the SD for such data should be calculated (25, 26).

Discussion

The use of modern vaccines produced in cell culture (HDCV, PCEC and PVRV) is an alternative to using a vaccine produced in rabbits, with a titre exceeding 3.0 IU/dose. A commercial equine RIG or a commercial human RIG, as used in rabies PEP, whose minimum titres are generally 200 and 150 IU/mL, respectively, may also be used in place of laboratory animal sera.

References

1. WHO Expert Consultation on Rabies, third report. Geneva: World Health Organization; 2018 (WHO Technical Report Series, No. 1012, <http://apps.who.int/iris/bitstream/handle/10665/272364/9789241210218-eng.pdf>, accessed 28 May 2018).
2. Smith JS. Rabies serology. In: Baer GM, editor. The natural history of rabies, 2nd edition. Boston (MA): CRC Press; 1991:235–54.
3. Cliquet F, Aubert M, Sagne I. Development of fluorescent antibody virus neutralization test (FAVN test) for the quantitation of rabies-neutralising antibody. *J Immunol Methods*. 1998; 212:79–87. doi:10.1016/S0022-1759(97)00212-3.
4. Webster LT, Dawson JR. Early diagnosis of rabies by mouse inoculation. Measurement of humoral immunity to rabies by mouse protection test. *Proc Soc Exp Biol Med*. 1935; 32:570.
5. Atanasiu P. Quantitative assay and potency test of rabies serum and immunoglobulin. In: Meslin, FX, Kaplan, MM, Koprowski, H, editors. Laboratory techniques in rabies, 4th edition. Geneva: World Health Organization; 1996:314–8.
6. Rizzo LF. [Human anti-rabies immunoprophylaxis with latent mouse brain vaccine using reduced vaccination schedules]. *Rev Univ San Carlos*. 1983; 11:253–7 (in Spanish).
7. Smith JS, Yager PA, Baer GM. A rapid reproducible test for determining rabies neutralizing antibody. *Bull World Health Organ*. 1973; 48:535–41.
8. Zalan E, Wilson C, Pukitis D. A microtest for the quantitation of rabies virus neutralizing antibodies. *J Biol Stand*. 1979; 7:213–20.
9. Simani S, Amirkhani A, Farahtaj F, Hooshmand B, Nadim A, Sharifian J, et al. Comparison of three serological tests for titration of rabies antibodies in immunized individuals. *Arch Iranian Med*. 1999; 2:125–7.
10. Guillemin F, Tixier G, Soulebot JP, Chappuis G. [Comparison of two neutralizing anti-rabies antibody titration methods]. *J Biol Stand*. 1981; 9:147–56 (in French).
11. Guillemin F, Tixier G, Soulebot JP, Chappuis G. [Comparative results of rabies antibody assays by two methods using immunofluorescence]. *J Biol Stand*. 1981; 9:157–60 (in French).
12. Louie RE, Dobkin MB, Meyer P, Chin B, Roby RE, Hammar AH, et al. Measurement of rabies antibody: comparison of the mouse neutralization test with the rapid fluorescent focus inhibition test. *J Biol Stand*. 1975; 3:365–8.
13. Diaz AM, Myers DM. Determination of serum neutralization antibodies to rabies virus by a modified counterimmunoelectrophoresis test. *J Clin Microbiol*. 1980; 12:175–9. PMID:7229005.
14. Diaz AM, Myers DM. Comparison between a modified counterimmunoelectrophoresis test and the indirect fluorescent-antibody test for detection of antibodies to rabies virus in human sera. *J Clin Microbiol*. 1981; 14:446–8.
15. Diaz AM. [The counterimmunoelectrophoresis technique for the diagnosis of rabies. Technical Monograph Series, no. 13]. Organización Panamericana de la Salud; 1985 (in Spanish).

16. Diaz AM, Arispe E, Brunel C, Cavandoli C, Dellepiane N, Miranda A. [The counterimmunoelectrophoresis technique for the determination of anti-rabies antibodies]. *Bol Ofic Sanit Panamer*. 1986; 101:255–61 (in Spanish).
17. Diaz AM, Albas A, Valentini EJJ, Perdomo G. [Evaluation of the quality of the reagents used in the counterimmunoelectrophoresis technique for the determination of anti-rabies antibodies]. *Rev Inst Med Trop São Paulo*. 1991; 33:44–9 (in Spanish).
18. Chauhan A, Sood DK, Saha SM, Kapoor M, Aggarwal RK, Saxena SN. The quantitation of rabies specific antibodies. I. Modified counter immunoelectrophoresis. A rapid and sensitive method. *Biologicals*. 1991; 19:93–5. doi:10.1016/1045-1056(91)90005-5.
19. Silva LHQ, Bissoto CE, Carvalho C, Cardoso TC, Pinheiro DM, Perri SHV. Comparison between the counterimmunoelectrophoresis test and mouse neutralization test for the detection of antibodies against rabies virus in dog sera. *Mem Inst Oswaldo Cruz*. 2002; 97:259–61. doi:10.1590/S0074-02762002000200020.
20. Diaz AM. Rabies neutralizing antibodies determination by the modified counterimmunoelectrophoresis test and rapid fluorescent focus inhibition test. *Zbl Bakt Mikrob Hyg (A)*. 1983; 256:1–6.
21. Chauhan A, Sood DK, Saha SM, Kapoor M, Aggarwal RK, Saxena SN. The quantitation of rabies specific antibodies. III. A comparative evaluation of modified counter immunoelectrophoresis, haemagglutination inhibition and serum neutralization titres of human sera. *Biologicals*. 1991; 19:103–6. doi:10.1016/1045-1056(91)90007-7.
22. Albas A, Pardo PE, Rinaldi PLF, Tarumoto MH. [Evaluation of the counterimmunoelectrophoresis method to titrate bovine sera vaccinated against rabies]. *Rev Med Vet Zoot UNESP*. 1995; 7:141–5 (in Portuguese).
23. Oliveira AN, Andrade MCR, Silva MV, Moura WC, Contreiras EC. Immune response in cattle vaccinated against rabies. *Mem Inst Oswaldo Cruz*. 1995; 95:83–8.
24. Queiroz LH, Moreira WC, Moura WC, Silva MV. Demonstration of viral antibodies by the counterimmunoelectrophoresis test. In: Rupprecht C, Nagarajan T, editors. *Current laboratory techniques in rabies diagnosis, research, and prevention*. vol. 2. New York (NY): Academic Press; 2015:175–86.
25. ISO 7870-4. Control charts – Shewhart control charts. Geneva: International Organization for Standardization (ISO 7870-2:2013).
26. Manual for quality control of diphtheria, tetanus and pertussis vaccines. Geneva: World Health Organization; 2013 (WHO/IVB/11.11).

Annex. Composition of media, buffers and solutions for the CIE test

Phosphate buffered saline pH 7.2

- sodium phosphate dibasic (Na_2HPO_4): 2.083 g
- sodium chloride (NaCl): 2.125 g
- potassium phosphate monobasic (KH_2PO_4): 0.273 g
- purified water (clinical laboratory reagent water, CLRW) q.s.: 250 mL

Adjust pH to 7.2 and sterilize at 121 °C for 15 min.

Agarose gel 0.9%

- agarose type II medium EEO: 4.5 g
- veronal buffer q.s.: 500 mL

Prepare the suspension in a water bath below 90 °C. Distribute in aliquots of 10.0 mL and keep at 4–8 °C until use.

0.05 M veronal buffer pH 8.2

- 5,5-diethylbarbiturate acid sodium ($\text{C}_8\text{H}_{11}\text{N}_2\text{NaO}_3$): 5.155 g
- CLRW q.s.: 500 mL

Adjust pH to 8.2.

Borate buffer pH 9.1

- boric acid (H_3BO_3): 12.268 g
- sodium borate ($\text{Na}_2\text{B}_4\text{O}_7$): 19.072 g
- sodium chloride (NaCl): 8.768 g
- CLRW q.s.: 2000 mL

Adjust pH to 9.1.

Glycine 0.01% and sucrose 5% pH 7.2

- glycine ($\text{C}_2\text{H}_5\text{NO}_2$): 0.05 g
- sucrose ($\text{C}_{12}\text{H}_{22}\text{O}_{11}$): 5.0 g
- CLRW q.s.: 500 mL

Sterilize by filtration.

Penicillin solution 100 000 IU and streptomycin 200 mg/mL

- penicillin: 1000 IU
- streptomycin: 2.0 mg

Previously dilute the penicillin to 1000 IU/mL and prepare streptomycin with this solution to contain 2.0 g/mL.

Mineral oil mixture 90% marcol 52 and 10% montanide 888

- marcol 52 (Esso Standard Oil Co.): 90.0 mL
- montanide 888 (Seppic Co. Paris, France): 10.0 mL

Sterilize the mineral oil mixture at 121 °C for 15 min.

Appendix 1

List of contributors

Jacques Barrat

ANSES
Malzéville, France

Helena B. C. Ruthne Batista

Pasteur Institute
São Paulo, Brazil

Emma M. Bentley

University of Westminster
London, United Kingdom

Jesse Blanton

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Juliano Bordignon

Instituto Carlos Chagas
Fiocruz, Curitiba, Paraná,
Brazil

Leonard Both

Medicines and Healthcare
Devices Regulatory Agency
London, United Kingdom

Herve Bourhy

Pasteur Institute
Paris, France

Graciane Maria Medeiros Caporale

Instituto Pasteur
São Paulo, Brazil

Maria Luiza Carrieri

Butantan Institute
São Paulo, Brazil

Luciana Botelho Chaves

Instituto Pasteur
São Paulo, Brazil

Florence Cliquet

ANSES
Malzéville, France

Laurent Dacheux

Pasteur Institute
Paris, France

Scott Dessain

Lankenau Institute for
Medical Research
Wynnewood, Pennsylvania,
USA

Elisa Eggerbauer

Friedrich-Loeffler-Institut
Greifswald-Insel Riems,
Germany

Richard J. Ellis

Animal and Plant Health
Agency
Addlestone, Surrey, United
Kingdom

Hildegund Ertl

The Wistar Institute
Philadelphia, Pennsylvania,
USA

Christine Fehlner-Gardiner

Canadian Food Inspection
Agency
Ottawa, Canada

E.R. Fernandes

Instituto Pasteur
São Paulo, Brazil

Anthony R. Fooks

Animal and Plant Health
Agency
Addlestone, Surrey, United
Kingdom

Richard Franka

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Conrad M. Freuling

Friedrich-Loeffler-Institut
Greifswald-Insel Riems,
Germany

Jinxin Gao

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Donna Gatewood

Edge Consulting
Ames, Iowa, USA

Florian Gohr

Aachen University
Aachen, Germany

Cynthia Goldsmith

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

F. Guedes

Instituto Pasteur
São Paulo, Brazil

Hans de Haard

Argenx
Zwijnaarde, Belgium

Hajime Hatta

Kyoto Women's University
Kyoto, Japan

Daniel J. Hicks

Animal and Plant Health
Agency
Addlestone, Surrey, United
Kingdom

Anna Hultberg

Argenx
Zwijnaarde, Belgium

Satoshi Inoue

National Institute of Infectious
Diseases
Shinjuku, Tokyo, Japan

Takuya Itou

Nihon University Veterinary
Research Center
Kanagawa, Japan

Corinne Jallet

Pasteur Institute of Guinea
Conakry, Guinea

Iana Suly Santos Katz

Instituto Pasteur
São Paulo, Brazil

Ivanete Kotait

Venco Saúde Animal
Londrina, Paraná, Brazil

Ivan V. Kuzmin

USDA
Ames, Iowa, USA

Florence Larrous

Pasteur Institute
Paris, France

Monique Lechenne

University of Basel
Basel, Switzerland

Yu Li

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Julian K.C. Ma

St George's Hospital Medical
School
London, United Kingdom

Anita Mahadevan

National Institute of Mental
Health and Neurosciences
Bangalore, India

Reeta Mani

National Institute of Mental
Health and Neurosciences
Bangalore, India

Daria Llenaresas Manalo

Research Institute for Tropical
Medicine
Muntinlupa City, Philippines

Wilfred E. Marissen

CruceCell Holland
Leiden, The Netherlands

Wanda Markotter

University of Pretoria
Pretoria, South Africa

Denise A. Marston

Animal and Plant Health
Agency
Addlestone, Surrey, United
Kingdom

Lorraine M. McElhinney

Animal and Plant Health
Agency
Addlestone, Surrey, United
Kingdom

Sharon Messenger

California Department of
Public Health
Richmond, California, USA

Susan Moore

Kansas State University
Manhattan, Kansas, USA

Wildeberg C. Moreira

Instituto Nacional de Controle
de Qualidade em Saúde
Rio de Janeiro, Brazil

Wlamir C. Moura

Instituto Nacional de Controle
de Qualidade em Saúde
Rio de Janeiro, Brazil

Thomas Müller

Friedrich-Loeffler-Institut
Greifswald-Insel Riems,
Germany

Thirumeni Nagarajan

Biological E. Limited
Shameerpet, Hyderabad,
India

Louis Nel

University of Pretoria
Pretoria, South Africa

Alejandro Núñez

Animal and Plant Health
Agency
Addlestone, Surrey, United
Kingdom

Lilian Orciari

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Chun-Ho Park

Kitasato University
Higashi, Towada, Aomori,
Japan

Evelyne Picard-Meyer

ANSES
Malzéville, France

Luzia H. Queiroz

Universidade Est. Paulista
Araçatuba, São Paulo, Brazil

Paulo Michel Roehe

Federal University of Rio
Grande do Sul
Porto Alegre, Brazil

Robert Rudd

New York Department of
Health
Albany, New York, USA

Charles E. Rupprecht

LYSSA LLC
Atlanta, Georgia, USA

Claude Sabeta

ARC-Onderstepoort
Veterinary Institute
Onderstepoort, South Africa

Markus Sack

Aachen University
Aachen, Germany

B. K. Chandrasekhar Sagar

National Institute of Mental
Health and Neurosciences
Bengaluru, India

Michael Saunders

Argenx
Zwijnaarde, Belgium

Jean Luc Schereffer

ANSES
Malzéville, France

Karin C. Scheffer

Instituto Pasteur
São Paulo, Brazil

Alexandre Servat

ANSES
Malzéville, France

Wunju Shieh

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Ade C. R. Silva

Instituto Pasteur
São Paulo, Brazil

Sandriana Ramos Silva

Pasteur Institute
São Paulo, Brazil

Marlon V. Silva

Instituto de Tecnologia em
Imunobiológicos
Rio de Janeiro, Brazil

Catelijne Stortelers

Ablynx
Zwijnaarde, Belgium

Tim H. Szeto

St George's Hospital Medical
School
London, United Kingdom

Audrey Y-H. Teh

St George's Hospital Medical
School
London, United Kingdom

Sanne Terryn

Ablynx
Zwijnaarde, Belgium

Noel Tordo

Pasteur Institute
Paris, France

Changchun Tu

Academy of Military Medical
Sciences
Changchun, China

Steven Van Gucht

Scientific Institute of Public
Health
Brussels, Belgium

Peter Vanlandschoot

N-Spire
Aalter, Belgium

Theo Verrips

Utrecht University
Utrecht, The Netherlands

Ryan Wallace

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Marine Wasniewski

ANSES
Malzéville, France

Kimberly Wilkins

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Edward Wright

University of Sussex
Brighton, United Kingdom

Mary Yager

Atlanta Health Associates,
Inc.
Cumming, Georgia, USA

Pamela Yager

Centers for Disease Control
and Prevention
Atlanta, Georgia, USA

Carlos Roberto Zanetti

Universidade Federal de
Santa Catarina
Florianópolis, Brazil

Appendix 2

WHO collaborating centres on rabies, neurovirology, viral zoonoses and zoonoses control

[WHO Collaborating Centre for Reference and Research on Rabies](#), Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris, France
Head, Dr Hervé Bourhy; e-mail: herve.bourhy@pasteur.fr

[WHO Collaborating Centre on Research and Management on Zoonoses Control](#), Laboratory of Rabies and Wild Animals of Nancy, Agricultural and Veterinary Technopole, PO 40009, 54220 Malzéville, France
Head, Dr Florence Cliquet; e-mail: florence.cliquet@anses.fr

[WHO Collaborating Centre for the Characterization of Rabies and Rabies-related Viruses, Animal Health and Veterinary Laboratories Agency](#), Weybridge, Surrey KT15 3NB, United Kingdom
Head, Dr Anthony Fooks; e-mail: tony.fooks@apha.gov.uk

[WHO Collaborating Centre for Rabies Surveillance and Research](#), Friedrich-Loeffler Institut, Federal Research Institute for Animal Health, Sudufer 10, 17493 Greifswald-Insel Reims, Germany
Head, Dr Thomas Müller; e-mail: thomas.mueller@fli.bund.de

[WHO Collaborating Centre for Traveller's Health](#), University of Zurich, Hirschengraben 84, 8001 Zurich, Switzerland
Head, Dr Christoph Hatz; e-mail: christoph.hatz@unibas.ch

[WHO Collaborating Centre for Control and Epidemiology of Rabies in Carnivores](#), Canadian Food Inspection Agency, 3851 Fallowfield Road, Ottawa, ON, Canada
Head, Dr Christine Fehlner-Gardiner; e-mail: christine.fehlner-gardiner@canada.ca

[WHO Collaborating Centre for Neurovirology](#), Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA
Head, Dr Matthias Schnell; e-mail: matthias.schnell@jefferson.edu

WHO Collaborating Centre for Reference and Research on Rabies, Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA
Head, Dr Hildegund Ertl; e-mail: ertl@wistar.upenn.edu

WHO Collaborating Centre for Reference and Research on Rabies, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA
Head, Dr Jesse Blanton; e-mail: asi5@cdc.gov

WHO Collaborating Centre in Rabies, Institut Pasteur of São Paulo, Av. Paulista, 393, São Paulo, Brazil
Head, Dr Lucianna Gomes / Dr Juliana Galera Castilho; e-mail pasteur@pasteur.saude.sp.gov.br / jgcastilho@pasteur.saude.sp.gov.br

WHO Collaborating Centre for Rabies Epidemiology, Division of Zoonosis, National Centre for Disease Control, 22-Sham Nath, Delhi 110054, India
Head, Dr Mala Chabra; e-mail: malachhabra@yahoo.co.in

WHO Collaborating Centre for Reference and Research in Rabies, Department of Neurovirology, National Institute of Mental Health and Neurosciences, PO Box 2900, 560029 Bangalore, India
Head, Professor V. Ravi / Dr Reeta Mani; e-mail: virusravi@gmail.com/ drreeta@gmail.com

WHO Collaborating Centre for Research on Rabies Pathogenesis and Prevention, Queen Saovabha Memorial Institute, Thai Red Cross Society, 1871 Rama IV Road, 10330 Bangkok, Thailand
Head, Professor Visith Sitprija; e-mail: sitprija@yahoo.com; and Dr Pakmanee Narumol; e-mail: npakmanee@yahoo.com

WHO Collaborating Centre for Research and Training on Viral Zoonoses, Department of Medicine, Neurovirology Division, Faculty of Medicine, Chulalongkorn University, Rama IV Road, 10330 Bangkok, Thailand
Head, Professor Thiravat Hemachudha; e-mail: fmedthm@gmail.com; and Dr Supaporn Wacharapluesadee; e-mail: spwa@hotmail.com

ISBN: 978-92-4-151515-3



9 789241 515153