

Laboratory testing for diphtheria in outbreak settings

Interim guidance

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Key points

- Over the past decade, numbers of significant diphtheria outbreaks have increased, primarily affecting settings with low resources and low vaccine coverage, and with vulnerable and conflict-affected populations.
- In ideal conditions, diphtheria testing is performed using an intensive case-based surveillance approach; however, this may be difficult to maintain in the settings described above. Hence, more detailed guidance is needed that builds on the principles of existing surveillance standards and provides laboratory systems with the information needed to prioritize and rationalize testing in accordance with the epidemiological situation and available resources.
- The development of testing strategies for diphtheria outbreak settings must support the public health response by characterizing the strain (or strains) responsible for the outbreak, and by providing information on those strains to guide public health measures (including antibiotic treatment), reduce further transmission, and monitor changes in strain patterns or epidemiology.
- Many laboratory tests recommended for diphtheria cases rely on the isolation of *Corynebacterium diphtheriae*. Hence, correct sampling of suspected cases and rapid transportation of specimens to the laboratory for testing is critical, to ensure the collection and maintenance of viability of sufficient *Corynebacterium*. Resources to support these activities should be highly prioritized as part of the outbreak response.
- Culture and identification of *C. diphtheriae*, followed by confirmation of toxin production by Elek testing, remains the gold standard of laboratory confirmation for diphtheria. However, methods including automated identification systems, molecular testing and genotyping can also play a role in informing public health decisions, sometimes more quickly and effectively than standard methods in an outbreak setting. Careful consideration of the benefits and limitations of each method is required to ensure the best use of available resources and alignment with public health goals.
- Although evidence remains limited, a growing number of *C. diphtheriae* isolates with antibiotic resistance have been detected. Thus, antimicrobial susceptibility testing is essential, not only to guide the selection of antibiotic treatment but also to contribute to global understanding of resistance mechanisms in *C. diphtheriae*.
- Much of the technical expertise required for laboratory testing for diphtheria is concentrated in the national reference laboratory. During large outbreaks, consideration should be given to the decentralization of some testing procedures and the optimization of laboratories that are in closer proximity to the epicentre of the outbreak.
- Where local resources are absent or insufficient to support the public health response, consideration should be given to referring patient specimens to international expert laboratories to ensure characterization and monitoring of outbreak strains, and to provide rapid feedback to the referring laboratories.

Introduction

Diphtheria is a vaccine-preventable disease caused by toxin-producing *Corynebacterium* species. Diphtheria outbreaks in human populations are primarily caused by *Corynebacterium diphtheriae*, which is spread from person to person, usually through respiratory droplets, secretions or infected skin lesions. Diphtheria may also be caused by *C. ulcerans* or *C. pseudotuberculosis*; however, because these are zoonotic infections, with limited person-to-person transmission, they are rarely associated with large outbreaks (1). Typical diphtheria cases present with upper respiratory tract symptoms including pharyngitis, nasopharyngitis, tonsillitis or laryngitis, or any combination of these symptoms. The toxin produced by the bacterium can cause the formation of a pseudomembrane in the upper respiratory tract and may damage other organs. Severe complications include acute respiratory obstruction, acute systemic toxicity, myocarditis, renal failure, neurologic complications and death. Case fatality rates (CFR) above 10% have been reported in diphtheria outbreaks; with higher CFRs appearing in settings where appropriate treatment options, such as diphtheria antitoxin [DAT], is limited (2).

Following the establishment of the Expanded Programme on Immunization (EPI) in 1974, which included a childhood multivalent vaccine (including diphtheria toxoid), the global incidence of diphtheria decreased dramatically. However, dramatic resurgences of the disease have been seen globally over the past decade, with an average of almost 10 000 cases reported annually – a 50% increase on the decade prior (3). Although most outbreaks have been small and sporadic, larger outbreaks have occurred in areas of low vaccine coverage or in fragile, conflict-affected settings including Bangladesh, Ethiopia, Haiti, India, Indonesia, Venezuela (Bolivarian Republic of) and Yemen; one of the largest outbreaks of the decade began in 2022 in Nigeria and is still ongoing (3, 4).

Current recommendations for diphtheria testing from the World Health Organization (WHO) support intensive case-based surveillance, which may be difficult to maintain in outbreak situations. Hence, WHO has identified the need to provide more detailed guidance on laboratory testing for diphtheria during significant outbreaks or in low-resource settings. This document aims to supplement and build on other existing WHO guidance documents on surveillance standards, diagnostics and research on *Corynebacterium* species (1, 2, 5). It also provides key considerations for laboratories, to allow them to make informed decisions on the rationalization and optimization of testing. The recommendations given here have been prepared by WHO in consultation with, and reviewed by, global experts with experience in laboratory analysis of *Corynebacterium* species and in outbreak settings, or with expertise in developing new technologies for diphtheria research and diagnosis. This guidance document will expire in January 2025, unless a revised version is published before that time.

Context of the guidance

Unless otherwise stated, the considerations provided apply to diphtheria outbreaks caused by toxin-producing *C. diphtheriae* with a classical respiratory diphtheria presentation. Although some of the considerations may be relevant to outbreaks of any size, location or duration, they will be most applicable where:

- outbreaks are of a significant scale (case numbers >100) or duration (>6 months);
- vaccine coverage for diphtheria is low, or affected populations are considered otherwise more susceptible to infection or adverse health outcomes;
- local access to laboratory resources (i.e. reagents, consumables and technologies) is limited by financial, technical or logistical barriers; and
- the outbreak response is compounded by other health emergencies or protracted crises.

Laboratory data in outbreak settings are essential for informing the public health response and for guiding certain clinical decisions; nevertheless, **patient treatment for diphtheria must not be delayed pending laboratory confirmation** (1, 5, 6). Decisions for the rationalization and optimization of laboratory testing in the settings covered by this document should therefore be considered with the following public health goals in mind (1):

- identify the circulating strain or strains of *C. diphtheriae* causing the outbreak, and provide information on clinical and epidemiological characteristics (e.g. antimicrobial susceptibility patterns and virulence factors) to guide public health response efforts;
- monitor the disease burden and confirm ongoing transmission;
- identify changes to the outbreak (i.e. new strains or cases in new geographical areas), to trigger new epidemiological investigations or adjust public health actions to prevent or reduce transmission;
- provide information on antimicrobial susceptibility that may guide clinical case management in the event that resistance patterns are detected; and
- contribute to global data and knowledge on circulating strains of *C. diphtheriae*, including antimicrobial resistance data, virulence factors, transmissibility and infectivity.

Indications for testing

The surveillance standards for diphtheria typically rely on a case-based approach, with the recommendation that all suspected cases be laboratory tested for further case classification (1). Traditional case definitions and further classification of suspected cases can be found in Annex 1. At all stages of an outbreak, **it is always recommended to continue testing as many patient specimens as resources allow**.

In the early stages of an outbreak, testing of all suspected cases should continue until at least 5–10 cases have been characterized, of which 80% arose from the same case cluster (i.e. the same geographical location or bacterial strain, or both). Characterization should include, at a minimum, laboratory confirmation by culture, and identification of species and biotype (biovar), Elek testing and antimicrobial susceptibility testing (AST). Where possible, genotyping using advanced molecular methods is highly recommended, to characterize the outbreak and determine epidemiological linkages to other global strains.

Once the outbreak strain or strains have been characterized, testing may subsequently be rationalized in line with the available resources of the national laboratory system. A reduced testing criteria may be applied by expanding epidemiological linking of cases; that is, testing is not required for cases that can be epidemiologically linked to a laboratory-confirmed case. If further reduction of testing is required, then testing can also be stopped for suspected cases that can be epidemiologically linked to an epidemiologically linked case. Patients with atypical presentations, severe disease or suspected treatment failure should, however, continue to be tested throughout the outbreak as discussed in Table 1 below (1).

To ensure continued monitoring of the outbreak, it is recommended that at least 5–10 patients from each cluster are tested per month, at least five of whom should be positive. Should a new cluster be identified (i.e. a new biotype, geographic location, or antimicrobial resistance pattern), testing of all new suspected cases should resume until such time as the new strain has been well characterized (in 5–10 cases). A summary of all indications for testing and the related rationalization are presented in Table 1 below (2, 5, 7).

Table 1. Indications for patient testing for diphtheria

Indication for testing	Rationale
Early detection on an outbreak	All suspected cases should be tested until at least 5–10 cases have been laboratory confirmed (by culture, identification and Elek testing) and characterized (by AST, biotyping and, where available, genotyping). If multiple clusters (i.e. a group of cases occurring in a circumscribed area or period, caused by the same strain) are detected, characterization of at least 5–10 cases per cluster should be performed.
Case detected in a new geographical location, with no epidemiological link to the existing outbreak (i.e. a new cluster)	All suspected cases should be tested until 5–10 cases have been laboratory confirmed, either as the same strain as the initial outbreak or existing clusters, or as a new circulating strain that has been fully characterized.
Ongoing outbreak monitoring	At least 5–10 patients per cluster should be tested per month, with at least 5 of those cases being positive, to allow confirmation of sustained circulation or detect changes in one or more circulating strains.
Atypical clinical presentations	Any suspected case where the clinical presentation is not consistent with the outbreak of classical respiratory diphtheria, or where clinical symptoms are unclear, should be tested. Examples of unclear clinical symptoms are: <ul style="list-style-type: none"> - a swollen neck or lymph nodes without adherent pseudomembrane; - in patients for whom it is not possible to visualize the upper respiratory tract; and - chronic non-healing ulcers or skin lesions.
Severe disease presentation	Patients requiring airway intervention or where there are clinical signs of significant multisystem disease (e.g. cardiac or neurological complications) should be tested because such symptoms could be attributable to virulence mechanisms in a new bacterial strain.
Suspected treatment failure	Testing should be performed in situations where there is a repeat presentation of a previously treated patient, or where a patient's condition worsens despite that person having been administered antibiotics. The aim of the testing is to detect whether treatment failure could be caused by an antimicrobial resistance mechanism.

AST: antimicrobial susceptibility testing.

Specimen collection, transport and storage

Many laboratory tests recommended for diphtheria cases rely on the isolation of *C. diphtheriae*. Hence, correct sampling of suspected cases and rapid transportation to the laboratory for testing is critical, to ensure the collection and maintenance of viability of sufficient *Corynebacterium*. Resources to support specimen collection and preservation (i.e. transport media), and resources to support collection, handling and transportation, should be highly prioritized as part of the outbreak response.

Specimen type

Where patients present with suspected respiratory diphtheria, multiple swabs should be collected to maximize the possibility of organism recovery. Where available, flocked swabs are preferred because of their higher capture rate and retention of microorganisms. Any universally available swab material may be used (e.g. polyester, Dacron® or nylon); however, cotton swabs should be avoided because they may be toxic to bacteria, or may cause inhibition in molecular-based testing such as polymerase chain reaction (PCR).

Patients should be tested when they first present at a health clinic or facility, before commencing antibiotic treatment. If antibiotics have already been administered, swabs should still be taken, with the treatment course clearly noted on the patient request form. Swabs should be collected under strong lighting to allow clear observation of the airway and pharynx. At a minimum it is recommended to obtain **one nasal swab and one oropharyngeal (throat) swab**.

In addition, swabs should be taken at the edge of, or directly beneath, any pseudomembrane present, because this is where the bacterial concentration is highest. Care should be taken not to dislodge the membrane because this can result in severe bleeding. Where feasible, an appropriately trained health worker may attempt to obtain a small piece of the pseudomembrane and place in a sterile container for transportation. Further information on options and considerations for transport media are presented in Table Two in the section below on specimen handling and transport.

In the event that a patient with a non-typical diphtheria presentation (e.g. wounds or lesions) is to be tested, swabs may be taken and handled in the same way as oropharyngeal swabs. More detailed instructions on specimen collection can be found in Annex 2 (5).

Safety considerations

Health workers collecting specimens should be equipped with appropriate personal protective equipment (PPE) for droplet and contact precautions, including a well-fitted medical mask, gloves, a long-sleeved gown and eye protection (face shield or goggle). In addition, health workers should be vaccinated according to the recommended schedule for health workers published by WHO (8), and should keep their booster vaccines against diphtheria up to date. Hand hygiene should always be performed where indicated by the WHO *Your 5 moments for hand hygiene*; that is, before touching a patient, before a clean or aseptic procedure, after the risk of body fluid exposure, after touching a patient and after touching a patient's surroundings (9).

Health workers must be appropriately trained for the specimen collection procedure, including donning and doffing of the recommended PPE, and storage, packaging and transport of patient samples. Adherence to available standard operating procedures (SOPs) should be ensured. All specimens collected for laboratory investigations should be regarded as potentially infectious and handled with caution (10).

Handling and transport

Once a swab has been taken, it should be placed in an appropriate transport medium, with the most optimal being a semi-solid transport medium such as Amies. Further options for transportation of suspected diphtheria specimens and considerations for their use are discussed below in Table 2.

If transport to the laboratory cannot be immediate (i.e. within 2–8 hours of collection), specimens must be kept at 2–8 °C until tested, especially where there is a delay between collection and transportation, or between transportation and testing. Ideally, all samples should arrive in the laboratory within 24–48 hours (1).

Table 2. Specimen transport options for suspected diphtheria cases

Specimen transport media	Considerations for use
Semi-solid (Amies) transport medium	<ul style="list-style-type: none"> - This is the optimal transport media for swabs from suspected diphtheria cases. - Amies is generally recommended, but other commercially available transport media are also appropriate (e.g. Stuart transport medium). - Semi-solid media, with or without charcoal, may be used. - Helps to maintain viability of the organism, over a reasonably short delay (24–48 hours).
Universal transport liquid medium	<ul style="list-style-type: none"> - Liquid-based collection and transport system for microbiology samples (liquid transport media or liquid Amies). - Can be used with the flocced collection swabs. - Can be used for both culturing <i>C. diphtheriae</i> and for molecular testing of patient samples for <i>C. diphtheriae</i> and its <i>tox</i> gene.
Silica gel packets	<ul style="list-style-type: none"> - Can be used to increase stability of a sample taken on a dry swab. - Is functional at room temperature. - Can maintain viability for more than 1 week, so should be used where large delays in transportation or testing by the laboratory are expected (>48 hours). - Can be purchased commercially, depending on available financial resources and procurement processes. - Requires additional processing before culturing (when compared with other media options) – see Annex 2 of the WHO laboratory manual for diphtheria (5).
Sterile saline	<ul style="list-style-type: none"> - May be used to transport pieces of tissue sampled from the pseudomembrane. - Should only be used where short delays are expected before testing (<24 hours).
No media (dry swab or sterile plastic container for pseudomembrane)	<ul style="list-style-type: none"> - Should be avoided unless no other options are available. - Does not contain any inhibitory substances or any substances that maintain viability and likelihood of negative testing results is high. - Should only be used where transportation to, and testing by, the laboratory is possible with a short turnaround time (<24 hours).

C.: *Corynebacterium*; PCR: polymerase chain reaction; WHO: World Health Organization.

Infectious substances transport

Transport of specimens should comply with any applicable national or international regulations, including the United Nations Model Regulations (11) and any other applicable regulations, depending on the mode of transport being used. Specimens from suspected or confirmed cases of diphtheria, including clinical samples and cultures, should be transported as Category B (UN3373 “Biological Substance, Category B”) using appropriate triple packaging, labelling and documentation. For more detailed information on infectious substances shipment, please see the WHO *Guidance on regulations for the transport of infectious substances 2021–2022* (6).

Specimen and isolate storage

Once received in the laboratory, specimens should be tested immediately and then stored refrigerated (at 2–8 °C) for up to 1 week, in case repeat testing is required. A patient specimen in a liquid-based transport medium may be stored at –20 °C if it is envisaged that additional molecular testing may be performed in future (i.e. more than 1 week later)

Where an isolate has presumptively been identified as positive for *Corynebacterium* species, it should be preserved by subculture on trypticase soy or blood agar medium for no more than 24 hours. Media containing tellurite or antibiotics must not be used for this purpose.

The isolate may then be subcultured for short-term storage (up to 7 days) on an agar slant, incubated at 35–37 °C overnight and then stored in a refrigerator at 4 °C. For long-term storage, growth should be swept off the agar surface and emulsified in glycerol broth (16% v/v), in skimmed milk tryptone glycerol glucose medium (STGG), or in tubes containing STGG cryobeads. Tubes may be stored frozen or cryopreserved at –20 °C to –80 °C. Vials must be correctly labelled with the isolate reference number and date. It is recommended to verify purity and concentration at least once every 2 years.

For strain revival from frozen STGG or cryobeads, it is necessary to work in a biosafety cabinet according to the laboratory’s safety protocol. Cryovials should not be completely thawed and should be returned to the freezer as soon after subculture as possible. More detailed explanations of storage procedures can be found in the WHO laboratory manual for diphtheria (5).

Biological risk management

Before manipulating specimens from suspected or confirmed diphtheria cases, laboratories should conduct a local (institutional) risk assessment, ensuring that all work is conducted using a risk-based approach. At a minimum, core biosafety requirements (similar to those previously referred to as Biosafety level 2) must be met and heightened control measures should be applied based on the outcomes of the risk assessment. More information on the risk assessment process, core biosafety requirements and heightened control measures is available in the WHO biosafety manual (12).

All procedures should be performed in a microbiological biosafety cabinet because laboratory-acquired infections have been reported (5). If sterile disposable loops are available, they are recommended for the spreading of sample material onto culture media. Effective disinfectants include compounds releasing chlorine at 5 g/L (0.5% or 5000 ppm available chlorine), freshly made (12).

All new staff should be appropriately trained and made aware of the risks involved in working in a laboratory before starting work. They must comply with PPE regulations for the specific laboratory and wear suitable protective clothing when handling specimens and pathogens. Also, staff must be competent in the relevant SOPs, safety protocols and risk assessments. All staff who routinely handle cultures of potentially toxigenic *Corynebacterium* should be fully vaccinated (including booster vaccinations) according to the respective national immunization guidelines, or the WHO recommendations for the immunization of health workers (8). Ideally, serum antibody levels should be checked every 3 years to ensure that laboratory staff have adequate immunity (5).

Laboratory testing methods

This section provides an overview of the testing procedures used for the detection and characterization of *C. diphtheriae*. It presents considerations for the use of testing procedures in outbreak settings, including advantages and disadvantages, and the use of the results for public health response. More detailed descriptions of each testing procedure, and its use in diphtheria diagnosis and research outside of outbreak settings, are given in the WHO laboratory manual for diphtheria (5).

Primary culture and isolation

Bacterial culture, followed by identification and toxigenicity testing of suspected colonies, is the current gold standard for laboratory confirmation of diphtheria cases. Isolation of *C. diphtheriae* is important because it allows characterization of the strain or strains causing the outbreak, including antimicrobial susceptibility patterns, genetic sequencing and other molecular analysis. This characterization is essential for understanding the epidemiology of an outbreak, and to inform changes in the outbreak response where changes in strain patterns are detected.

The preparation of bacteriological media for the culture and isolation of *C. diphtheriae* is described in Annex 4 of the WHO laboratory manual for diphtheria (5). For the primary culture of patient specimens, a 5–10% horse or sheep blood agar should be used. Although this medium is easy to prepare and readily available commercially, it is not selective. Patient specimens often contain only a small number of *Corynebacterium* and a

large amount of growth from the patients microbiota; this can make it difficult to identify suspected colonies (grey, entire edge and waxy) especially if laboratory staff are not experienced with the processing of diphtheria isolates. To improve selectivity, fosfomycin may be used as a cost-effective screening tool together with primary blood agar plate. Fosfomycin may be incorporated at 100 ug/mL⁻¹ into the blood agar or added as a disc on the surface of the agar plate (13, 14).

Where resources permit, the use of other primary selective growth media is also recommended – more specifically, a blood agar medium containing tellurite (Hoyle's tellurite) on which suspected colonies appear black and glossy (1). Cystinase medium (Tinsdale) is also useful for the presumptive identification of *C. diphtheriae*, with growth presenting with brown halos. However, use of Tinsdale medium is more appropriate for confirming suspected colonies from the primary culture, rather than as a primary culture itself, owing to its highly selective nature and limited shelf-life (maximum 14 days) (5). If preparing media in-house, keeping the components of Tinsdale medium (Tinsdale base and Tinsdale enrichment) separate until needed can extend the shelf-life to several months.

Primary plates must be examined after 18–48 hours of incubation, to subculture and confirm suspicious colonies as rapidly as possible (5). If a selective media (e.g. Hoyle's) is being used and there is no visible growth after 48 hours, plates should be incubated for a further 24 hours. Prolonged incubation of blood agar plates is not recommended because any *C. diphtheriae* present would probably be overgrown by normal flora. A blood agar culture with no growth (including normal oropharyngeal or skin flora) after 48 hours may indicate suboptimal collection or transport, or that antibiotics were provided to the patient before sampling; in such situations, it may be necessary to request a repeat specimen (1).

Presumptive identification

The presumptive identification of *C. diphtheriae* can be achieved through a combination of microscopic and biochemical analysis on suspect colonies. The purity of suspect colonies on primary culture should first be confirmed by staining using the Gram stain (Gram-positive bacillus or club-shaped rods) or another stain (e.g. Albert, Neisser or Loeffler) (1). The microscopy result alone cannot be used to confirm a diagnosis of diphtheria because both false positives and false negatives can occur (5).

The recommended conventional biochemical tests to confirm a suspected *C. diphtheriae* isolate, and the expected results, are presented in Table 3 below. In an outbreak setting where resources are limited, testing for pyrazinamidase (negative) and cystinase activity (positive) may be used as screening tests before proceeding with toxigenicity testing of the isolate (or referring it for testing) (1, 5).

Table 3. Conventional biochemical testing for *C. diphtheriae*

Test	Test medium	Expected result (<i>C. diphtheriae</i>)
Nitrate reduction	Nitrate broth	Positive ^a
Urea hydrolysis	Urea slope	Negative
Catalase production	Hydrogen peroxide	Positive
Cystinase activity	Tinsdale agar	Positive
Pyrazinamidase activity	Pyrazinamide substrate broth	Negative
Carbohydrate fermentation	Glucose, sucrose, maltose, glycogen or ribose	Glucose – positive Ribose – positive Maltose – positive Sucrose – negative Glycogen – negative (except positive for biovar gravis)

C.: *Corynebacterium*.

^a *Corynebacterium diphtheriae* biovar belfanti, previously differentiated from other strains of *C. diphtheriae* by its inability to reduce nitrate, has now been reclassified as its own species – *Corynebacterium belfantii* (15).

Despite the relative simplicity and inexpensiveness of conventional biochemical testing, the need to produce, inoculate, read and interpret multiple testing media may be challenging in outbreak settings, especially for staff who have limited experience in using these tests. Alternative methods are available as “all-in-one” kits (e.g. API® Coryne, Remel RapID CB Plus System and Rosco Diagnostica), which, when used correctly, can produce more accurate and rapid results. Commercially available kits may, however, be more costly than conventional methods, and in-country procurement may be challenging. Thus, it may be helpful for laboratories to engage in forecasting activities, based on the epidemiological evolution of the outbreak, to inform procurement and stockpiling of kits, or to place requests with donor and partner organizations to support procurement of outbreak supplies.

A number of fully automated identification systems also exist using either biochemical or protein composition techniques to identify *C. diphtheriae* (e.g. MALDI-TOF MS, VITEK® 2 ANC ID card, BD Phoenix™ System and MicroSeq® System). In some cases these systems offer more advanced characterization (e.g. biotyping), perform concurrent AST, produce more rapid and consistent results, and can be integrated for use for the identification of a wide range of other pathogens. However, automated systems can also require large capital investment for installation and ongoing maintenance costs. Therefore, the use of automated systems during an outbreak of diphtheria would be most feasible when such systems are already operational for the identification of other pathogens. If such a system is to be used for *Corynebacterium*, it is important to ensure that the system is equipped with an updated *Corynebacterium* database.

There are various systems that use mass-spectrometry ionization techniques (e.g. matrix-assisted laser desorption/ionization time of flight [MALDI-TOF]). These tests vary in terms of their performance characteristics and ability to differentiate between different clinically significant *Corynebacterium* species. A protein spectrum of a bacterial isolate is compared with those of reference strains on the database to identify the isolate and to determine strain relatedness. The mass protein peaks of each spectrum are compared, and a dendrogram is constructed (5). Such systems cannot differentiate between biovars of *C. diphtheriae*; also, they cannot confirm diphtheria toxin production. Despite their limitations, these systems can be used in clinical laboratories to detect clinically significant *Corynebacterium* species, allowing for rapid and appropriate treatment for infection.

Confirmation of diphtheria toxin production

Isolates identified as *C. diphtheriae* should be tested for the ability to produce diphtheria toxin, coded by the diphtheria toxin (*tox*) gene. The Elek immunoprecipitation test (also known as the modified Elek test) is the current gold standard for confirming toxin production. Elek testing is ideally performed on a pure overnight subculture; however, a few colonies from the primary isolation plate may be used if they are sufficiently pure. The Elek test does require several components (e.g. basal medium, serums and controls) that may be difficult to procure or prepare, or that may have a limited shelf-life. Furthermore, the test requires the use of diphtheria antitoxin (DAT), which is only available from a limited number of suppliers globally. Hence, Elek confirmatory testing is generally only performed by national reference laboratories (NRLs), and it is likely that there would be limited opportunity to expand this testing capacity into other sites during an outbreak, even where demand for testing increases markedly.

Molecular methods – namely PCR, either conventional PCR or real-time quantitative PCR (qPCR) – may also be used for toxigenicity testing through the detection of the *tox* gene. Some *C. diphtheriae* isolates will carry the *tox* gene without active expression (non-toxigenic, *tox* gene-bearing or non-toxigenic *tox* gene-bearing [NTTB]). Therefore, toxigenicity can only be confirmed by the Elek test, and PCR is best used as a screening method, to rationalize the use of Elek reagents with only *tox*-positive isolates being referred for the confirmatory test. A negative PCR result will also be useful in excluding toxigenicity and preventing unnecessary infection control measures (5). However, in the setting of a large outbreak, where the circulating strain or strains have already been confirmed as toxin producing by Elek test, it is considered highly likely that any *tox*-positive isolate would be toxin producing, and PCR may be considered as a standalone confirmation of toxigenicity. Furthermore, the continued presentation of cases with symptoms characteristic of toxin involvement (e.g. pseudomembrane, airway obstruction or multiorgan failure) provides clinical evidence of toxin production and rationalizes the use of PCR techniques only, or a reduction in the number of isolates undergoing toxin testing.

Table 4 below provides an overview of the recommendations for toxigenicity testing in different stages of an outbreak, including the number of isolates to be tested and method to be used.

Table 4. Summary of the recommendations for toxigenicity testing in different outbreak settings

Outbreak setting	Testing recommendations
No outbreak or small number of cases detected	All isolates should be confirmed as toxin producing by the Elek test.
Early outbreak characterization	At least 5–10 isolates should be confirmed as toxin producing, as part of strain characterization using the Elek test.
Shortage of Elek reagents	Consider use of PCR as a screening test and refer only <i>tox</i> -positive isolates for the Elek test.
Ongoing monitoring of outbreak	To confirm continued circulation of toxigenic <i>C. diphtheriae</i> , 3–5 isolates per cluster, per month, should be tested. Where possible, at least 30% of these (1–2 isolates) should be confirmed by the Elek test.

C.: *Corynebacterium*; PCR: polymerase chain reaction.

Molecular detection of *C. diphtheriae*

In addition to the applications of molecular methods for toxigenicity testing described above, qPCR may also be used for the initial identification of *C. diphtheriae* isolates. Benefits of this method include an increase in specimen throughput (especially where automated extraction methods are used) and turnaround time (TAT). Once optimized and validated by the laboratory, the procedure can be completed on the same day.

Furthermore, although the literature is limited, evidence suggests that qPCR can be performed successfully on direct patient specimens, further reducing TAT, with an increased sensitivity in comparison to traditional culture methods (16–18).

Challenges of qPCR include difficulty in procuring reagents, controls, primers and probes; which may not be widely available commercially, and they carry a high cost. Once components are sourced, sufficient technical knowledge is required to optimize and validate methods before routine use. Consideration may be given to collaborating with international expert laboratories, both for the sourcing of necessary components, and for technical guidance in implementing qPCR protocols. Various protocols for qPCR have been used successfully, typically using a multiplex approach to differentiate the presence of *C. diphtheriae* from that of *C. ulcerans* or *C. pseudotuberculosis* (by *rpoB* genes or *dtxR* genes), and to detect the presence of the *tox* gene in any of the three species. A variety of protocols are available (16, 19–23).

The most significant drawback of using qPCR for the confirmation of cases is that it cannot detect changes in strain characteristics. Therefore, qPCR would ideally only be implemented for laboratory confirmation after the strain or strains responsible for the outbreak have already been characterized using gold-standard methods (i.e. culture, identification, Elek and an AST of 5–10 isolates per cluster). Once qPCR is implemented, a subset of isolates (at least 3–5 per cluster, per month) should still be characterized by gold-standard methods to confirm the continued circulation of the same strain or strains. Should new strain characteristics be identified (i.e. a new biotype or new antimicrobial resistance pattern), the laboratory should return to the simultaneous or preferential use of culture methods until that strain has been well characterized. Based on the qPCR cycle threshold (Ct) value, strong positive *C. diphtheriae* samples can be considered for culturing to increase the probability of isolating the bacteria.

Antimicrobial susceptibility testing

Clinical management of diphtheria cases typically prioritizes the administration of DAT, where available, to bind and neutralize the circulating toxin produced by toxigenic *C. diphtheriae*. In parallel, antibiotic treatment is administered to eradicate the bacteria, further reducing toxin production and facilitating more rapid clearance of the organism, thus reducing or preventing onwards transmission. Systematic review of available literature suggests that the use of antibiotic treatment has a critical role in breaking chains of transmission during an outbreak, especially for asymptomatic carriers (24).

Penicillin, erythromycin and amoxicillin have historically been recommended as front-line antibiotic treatment for both diphtheria treatment and prophylaxis of case contacts. Prompt administration of the recommended antibiotic is vital, and it should not be delayed while awaiting laboratory results. Nonetheless, AST remains essential as a growing body of evidence demonstrates reduced susceptibilities in *C. diphtheriae* to a variety of antibiotics including penicillin, cefotaxime and erythromycin, as well as tetracycline, sulphonamide or other agents (5, 25). In the 2022 outbreak of *C. diphtheriae* in Nigeria, a high level of resistance to benzyl penicillin was reported (4).

Currently, two international guidelines are available for AST methods and interpretation of results – Clinical and Laboratory Standards Institute (CLSI) M45, last updated in August 2016 (26) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (27), which was specifically updated for *C. diphtheriae* and *C. ulcerans* through expert consultation in 2022 (28). Standardized methods and breakpoints for microdilution in broth (minimum inhibitory concentrations [MICs]) are described in both standards, and this is considered the gold standard for AST. The CLSI standards provide a slightly more comprehensive list of antibiotic breakpoints (17 antibiotics), although these have been developed for all *Corynebacterium* species (including *C. diphtheriae*) and related coryneform genera. In contrast, EUCAST provides a smaller number of antibiotic breakpoints (11 antibiotics) but these are specific for the species *C. diphtheriae* and *C. ulcerans*. Microdilution broth is always recommended as the gold-standard method where it is feasible and the reagents are available; however, disc diffusion is an accessible alternative for diagnostic laboratories in many countries. A standardized disc diffusion method, and associated breakpoints, are described in the EUCAST 2023 guidelines (27). Genomic sequencing of isolates can be used to detect the presence of resistance genes but cannot confirm whether these genes are actively expressed; therefore, it should not be used as a replacement technique to conventional AST.

AST should be performed on all isolates in the early stages of an outbreak as part of the characterization of circulating strains (i.e. until at least 5–10 isolates per cluster have been characterized) and should be used to guide the selection of appropriate antibiotics for the public health response. As the outbreak progresses, continued AST on a subset of isolates (1–3 per cluster, per month) will help to confirm continued circulation of the same strain, or to detect the presence of new resistance patterns. If a new resistance pattern is detected, a return to AST for all isolates is recommended until the new strain has been fully characterized. Additional performance of AST should also be conducted on all positive isolates where a patient:

- has an atypical case presentation (including wounds or ulcers);
- has particularly severe disease (as defined in Table 1);
- has a suspected treatment failure (i.e. worsening of condition following treatment or return presentation of a patient who previously recovered); and
- is receiving an antibiotic treatment for which antibiotic sensitivity data are not yet available.

Data on antimicrobial resistance patterns in *C. diphtheriae* remain limited globally. Hence, all profiles gathered in the context of an outbreak will be extremely valuable in accelerating knowledge on antimicrobial resistance in *Corynebacterium* species, and in driving further advances in research and development of alternative treatment options. Thus, where resources allow, undertaking AST of as many isolates as possible is encouraged. Where it is not feasible to perform AST on all isolates during an outbreak, consideration should be given to the storage of isolates to perform AST later, either in-country or through referral to an expert international reference laboratory.

Genotyping

Genotyping of circulating strains can provide a rich source of information about strain characteristics, including knowledge on the molecular epidemiology of diphtheria, to predict the evolutionary relationships among strains and to infer the global relatedness of the pathogen (5). The two methods most commonly employed for genotyping are multilocus sequence typing (MLST) and whole-genome sequencing (WGS). MLST, which is used to investigate genetic diversity, involves analysing the nucleotides within the seven or more housekeeping genes, providing efficient high-resolution data suitable for epidemiological and surveillance studies (29). MLST enables the analysis of sequence types and clonal complexes of the organism, and thus helps in the understanding of a specific clone that is widely spreading in the region or during the outbreak (5). WGS can provide even more in-depth information on isolates, including the characterization and monitoring of virulence factors such as the diphtheria toxin gene, mobile gene elements, phages, resistance genes and MLST types. Analytical methods include core genome MLST (cgMLST), whole-genome MLST (wgMLST) and single nucleotide polymorphisms (SNP).

Although these methods are highly technically advanced, and often require significant financial means to be executed, it is highly recommended to ensure that at least a small subset of isolates are submitted for advanced genotyping by MLST or WGS. Most importantly, isolates from index cases (5–10 isolates) should be sequenced as early as possible in the outbreak, to inform the public health response, especially in identifying the

outbreak source. An additional selection of isolates from the duration of the outbreak should also be sequenced to monitor genetic traceability of strains. A statistical selection of isolates may also be considered for research purposes. If it is not possible to test immediately, consideration should be given to storing isolates for genotyping once resources are available in-country or through an international expert laboratory. It is also highly encouraged to publish any raw sequence reads in international sequence repositories, to enable comparisons of the genomes within the outbreak and contribute to global genomic surveillance.

Laboratory network and specimen referral

The detection and characterization of *C. diphtheriae* can include technically advanced methods or require the use of specialized reagents with limited global supply, meaning that testing is often restricted to a designated NRL and relies on specimen referral from within the national laboratory network. Hence, in the event of an outbreak, the NRL should assume responsibility for providing technical and logistical support on diphtheria testing and associated activities to the rest of the national laboratory network. Responsibilities may include:

- training laboratory scientists on diagnostics for diphtheria; for example, through on-site support, mentoring activities, ad-hoc webinars, workshops (online or face-to-face) or dedicated face-to-face training events;
- providing SOPs or testing protocols for testing methods or sample collection, handling and transportation;
- developing or validating new technologies (e.g. optimization of qPCR protocols);
- assuring the provision of transport media and reagents; for example, through national forecasting and stockpiling of reagents and consumables, or preparation of media in-house;
- monitoring of the quality of testing; for example, through the implementation of a national proficiency testing programme, or other external quality assessment methods (e.g. specimen re-testing or on-site quality assessment); and
- participating in national emergency operations teams, providing relevant national laboratory data, or communicating and advocating for the resource needs of the laboratory network.

Initial prioritization of national resources for the laboratory response should focus on specimen collection, handling and transportation, given the importance of obtaining viable bacteria for subsequent testing procedures. Subsequent priorities should include provision of reagents and consumables for primary culture and presumptive identification to subnational laboratories, with a particular focus on laboratories close to the epicentre of the outbreak.

As the outbreak continues, it may be necessary to consider further decentralization of testing to subnational laboratories, to relieve demands on the NRL to both support the national network and provide confirmatory testing. This is especially relevant in settings where the NRL is located far from the epicentre of the outbreak, leading to greater challenges in maintaining specimen viability during prolonged or suboptimal transportation conditions. The type of testing that could be decentralized will depend on the epidemiological features of the outbreak, and the resources available in the country, but may include (in addition to primary culture and presumptive biochemical identification) Elek testing, AST or PCR techniques for *tox* gene detection of identification. However, decentralization should only be considered for a limited number of subnational laboratories that are capable of supporting the outbreak response owing to their proximity or available expertise.

Other mechanisms that may be used either to support decentralization of testing, or to reinforce the capacities of the existing laboratory network for diphtheria testing include:

- the deployment of personnel from the NRL to subnational level;
- the engagement of international laboratory experts in a mentoring or collaborative capacity;
- the referral of specimens to regional or international reference laboratories (e.g. to WHO collaborating centres [CCs] or other laboratories with specialized *Corynebacterium* expertise); and
- the deployment of mobile laboratories.

Finally, although no validated lateral flow tests for *C. diphtheriae* are currently available, international partners for diphtheria diagnostics are engaged in the ongoing research and development of target product profiles for such testing (30), which may allow rapid or point-of-care testing options to be incorporated into national testing algorithms for the diphtheria outbreak response in the near future.

Global laboratory networking

Access to timely and accurate laboratory testing of samples from cases under investigation is an essential part of the diagnosis and surveillance of this re-emerging infection. All countries should have access to reliable testing, either nationally or through referral to laboratories in other countries that are willing and able to perform laboratory confirmation of diphtheria and undertake characterization studies such as molecular typing and WGS. WHO, through its regional offices, can assist Member States to access testing through referral. Countries are encouraged to share any pathogen sequence data, for a better understanding of the current outbreak. The UK Health Security Agency (UKHSA) in the United Kingdom of Great Britain and Northern Ireland (United Kingdom) is the WHO CC for Diphtheria and Streptococcal Infections, which provides support in terms of capacity-building and training in laboratory diagnostics. It is also the WHO CC responsible for isolate referral from other countries if needed for specialized characterization studies.

Reporting of cases and test results

Laboratories should follow national reporting requirements, with all test results immediately reported to national authorities. Reported cases and incidence of diphtheria are collected annually through the WHO/United Nations Children's Fund (UNICEF) Joint Reporting Form on Immunization (JRF). In addition, States Parties to the International Health Regulations (IHR) (2005) have an obligation to share with WHO relevant public health information for events for which they notified WHO, using the decision instrument in Annex 1 of the IHR (2005), for more timely detection of, and response to, diphtheria outbreaks (31).

Laboratories are also encouraged to collect, and report on, indicators that may help in monitoring and evaluating their contribution to the outbreak response. A selection of suggested indicators are given in Table 5 below. Countries should establish their own targets, considering epidemiology of the outbreak and available resources.

Table 5. Attributes and indicators for monitoring and evaluating the outbreak response

Attribute	Indicator
Timeliness of specimen transport	% of specimens received at the laboratory in 48 hours or less / total specimens received
Sample referral	% of specimens referred to NRL / total specimens processed by subnational laboratories % of isolates (or samples) referred to international reference laboratories
Sample acceptability	% of specimens processed / all specimens received
Culture positivity	% of positive cultures of all specimens received
Molecular detection	% of patient specimens testing PCR positive / total patient specimens tested by PCR % of isolates testing PCR positive / total isolates PCR tested
Specimen characterization	Number (or %) of isolates tested for: <ul style="list-style-type: none"> - toxigenicity (using Elek testing or PCR) - antimicrobial susceptibility - WGS
Timeliness of reporting of laboratory results	% of specimens tested by culture with results reported within 3 days of specimen receipt

NRL: national reference laboratory; PCR: polymerase chain reaction; WGS: whole genome sequencing.

Summary

Table 6. Key considerations for laboratory testing in outbreak settings

Outbreak Setting	Key Laboratory Testing Recommendations
Early Outbreak Detection	<ul style="list-style-type: none"> - Testing all suspected cases - Reinforce capacities for specimen referral (collection, handling and transport) to maximize recovery of viable bacteria - Laboratory confirmation must be done using gold-standard methods (culture, identification, and Elek toxin testing) - Continue in this way until outbreak strain or strains can be fully characterized (culture, identification, Elek toxin testing, AST, bio typing and genotyping) - Minimum of 5 – 10 cases per cluster should be characterized - Where possible, genetic sequencing of index cases should be performed (in-country or by referral)
Duration of Outbreak	<ul style="list-style-type: none"> - Consider reducing testing demands by expanding epidemiological linking. - Always maintain testing for unusual or severe cases and reinfections (see table one), ideally using gold-standard methods. - Focus on increasing testing throughput and TAT by: <ul style="list-style-type: none"> o Using all-in-one (API) or automated identification methods (e.g. Vitek, Microscan, Phoenix, MALDI-TOF etc) o Decentralizing some testing into sub-national laboratories (for presumptive identification at a minimum, though AST and PCR capacities may also be considered) o Using PCR molecular methods for the presence of <i>tox</i> gene or multiplex PCR for <i>tox</i> gene presence and identification of isolates. Where possible, optimize PCR for use directly on patient specimens.

	<ul style="list-style-type: none"> - PCR positive results may be used for laboratory confirmation of most patient samples. However, maintain a minimum level of laboratory-confirmation of cases using gold-standard methods (e.g. 3 – 5 cases per month, per cluster) and perform further characterization testing where possible (e.g. 1 – 3 isolates per month, per cluster using AST, bio typing, genotyping) to monitor genetic variability of strains. - If a new cluster is identified, return to testing all suspected cases until the strain is fully characterized. - Store isolates and samples under appropriate conditions to allow for research or further post-outbreak analysis to be performed if needed. - Where possible, share or publish any available data, antimicrobial resistance patterns or sequences from outbreak strains to aid global data and research on <i>C.diphtheriae</i> and diphtheria in outbreak settings.
Post-Outbreak	<ul style="list-style-type: none"> - Consider performing (or referring samples for) genomic sequencing on any available isolates to provide more data about the outbreak strains. - Unless already done, share or publish data and/or genomic sequences in a public forum to aid global data and research on <i>C.diphtheriae</i> and diphtheria in outbreak settings.

Process and methodology

This document was developed in consultation with global external experts in the field of microbiology and *Corynebacterium* research, and other laboratory expertise. Also consulted were WHO staff across the organization with expertise in laboratory diagnosis, vaccine-preventable diseases, epidemiology and clinical management. This version of the guidance incorporates the latest understanding and epidemiology of diphtheria outbreaks, and addresses questions and issues received from WHO's country and regional offices and other channels.

Limitations

This guidance serves to provide interim recommendations for the diagnosis of respiratory diphtheria infection in the operational context of an outbreak setting. The content does not apply to the routine surveillance for diphtheria cases, nor to small outbreak situations where national resources can adequately support the use of case-based surveillance methods. WHO will issue further updates to this interim guidance as necessary. For information applicable to routine surveillance, laboratory diagnosis and research on toxigenic *Corynebacterium* species, please refer to the relevant WHO guidance (1, 5).

Plans for updating

WHO works with experts around the world and continues to monitor the situation closely for any changes that may affect this interim guidance. WHO will issue a further update. Otherwise, this interim guidance will expire 1 year after the date of publication.

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Declaration of interests

Experts in the network completed a confidentiality agreement and declaration of interest. The declaration of interest forms were reviewed, and no conflicts regarding the support of this guidance document were identified.

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Annex 1 – Diphtheria case definitions and final classification

This annex is based on the World Health Organization (WHO) surveillance standards for diphtheria (1).

For case finding, the definition of a suspected case of diphtheria is an illness of the upper respiratory tract characterized by the following:

- pharyngitis, nasopharyngitis, tonsillitis or laryngitis;

AND

- adherent pseudomembrane of the pharynx, tonsils, larynx and/or nose.

A diphtheria pseudomembrane is an exudate that is greyish, thick, firmly adherent and patchy to confluent. Dislodging the pseudomembrane is likely to cause profuse bleeding.

Some countries choose to expand the suspected case definition to include the following:

- mild cases without a pseudomembrane; and
- non-healing ulcers in a person with a history of travel to countries with endemic disease or countries with diphtheria outbreaks.

Final case classification

Laboratory-confirmed case

A laboratory-confirmed case is a person with *Corynebacterium spp.* isolated by culture and positive for toxin production, regardless of symptoms. Toxigenicity must be confirmed by the phenotypic Elek test in all instances. Polymerase chain reaction (PCR) can complement surveillance, and may qualify as laboratory confirmed after reviewing the epidemiology and clinical manifestations of the case. Laboratory-confirmed cases may be further classified into three subcategories based on the type of surveillance occurring in the country.

- Laboratory-confirmed classic respiratory diphtheria cases meet the suspected case definition and are laboratory confirmed as defined above.
- Laboratory-confirmed mild respiratory or asymptomatic diphtheria cases have some respiratory symptoms such as pharyngitis and tonsillitis, but no pseudomembrane, or no symptoms (usually identified via contact tracing).
- Non-respiratory laboratory-confirmed diphtheria cases have a skin lesion or non-respiratory mucosal infection (e.g. eye, ear or genitalia) from which *Corynebacterium spp.* is isolated by culture and tests positive for toxin production.

Epidemiologically linked case

An epidemiologically linked case meets the definition of a suspected case and is linked epidemiologically to a laboratory-confirmed case. In this situation, a person has had intimate respiratory or physical contact with a laboratory-confirmed case within the 14 days before onset of sore throat.

Clinically compatible case

A clinically compatible case is one that meets the definition of a suspected case but lacks both a confirmatory laboratory test result and epidemiological linkage to a laboratory-confirmed case.

Discarded case

A discarded case is a suspected case that meets either of these criteria:

- *Corynebacterium spp.* but negative by the Elek test (non-toxigenic *Corynebacterium*);

OR

- negative PCR for the diphtheria toxin (*tox*) gene.

Asymptomatic or mild case

Sometimes, during outbreak investigations in which household contacts are investigated, a person may be identified as having *Corynebacterium* and may have evidence of toxigenicity, but may not meet the suspected case definition because the person is asymptomatic or has only mild disease. Such people should still be reported as laboratory-confirmed cases, because their treatment and the public health response is the same as for other laboratory-confirmed cases.

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Annex 2 – Collection of clinical specimens

This annex is based on the World Health Organization (WHO) laboratory manual for the diagnosis of diphtheria (1).

A1 Procedures for collecting samples for the laboratory diagnosis of diphtheria

Ideally, two samples should be collected from each suspected case – a nasal and oropharyngeal swab – and placed into the appropriate transport media. Other swabs may also be considered based on the patient's clinical presentation (A1.4–A1.6).

A1.1 Materials required for clinical sampling

The materials required for clinical sampling are:

- strong light source for illuminating the pharynx
- Dacron®, nylon or polyester flocked swab
- Amies transport medium or other suitable transport medium
- sterile tongue depressor
- saline solution
- skin punch or scalpel
- Eppendorf tube
- gloves
- surgical mask
- goggles.

A1.2 Oropharyngeal or throat swabs

To take an oropharyngeal or throat swab:

1. Ensure the pharynx is clearly visible and well illuminated.
2. Depress the tongue with a tongue depressor, then swab the throat without touching the tongue, uvula or inside of the cheeks.
3. Rub vigorously over any membrane, white spots or inflamed areas; apply slight pressure with a rotating movement to the swab.
4. Place the swab in a routine semi-solid transport medium or into a silica gel sachet.

A1.3 Nasal swabs

To take a nasal swab:

1. Insert the swab into the nose through one nostril beyond the anterior nares.
2. Gently introduce the swab along the floor of the nasal cavity.
3. Place the swab in a routine semi-solid transport medium or into a silica gel sachet.

A1.4 Nasopharyngeal swabs

To take a nasopharyngeal swab:

1. Insert the swab into one nostril, beyond the anterior nares.
2. Gently introduce the swab along the floor of the nasal cavity, under the middle turbinate until the pharyngeal wall is reached, rotating the swab two or three times. Do NOT use force to overcome any obstruction.
3. Place the swab in a routine semi-solid transport medium or into a silica gel sachet.

A1.5 Cutaneous lesions

To take a swab from a cutaneous lesion:

1. Moisten the lesion with sterile normal saline and remove crusted material.
2. Press the swab firmly into the lesion.
3. Place the swab into a routine semi-solid transport medium or into a silica gel sachet.

A1.6 Pseudomembrane

The procedure for taking a swab from a pseudomembrane should, where possible, be undertaken by an infectious disease specialist because there is a considerable risk of severe bleeding. To take a pseudomembrane swab:

1. If a pseudomembrane is present, lift the edge and swab beneath it.
2. Using sterile forceps, gently lift the pseudomembrane where possible and aseptically remove pieces of the membrane.
3. Place the membrane into either Amies transport medium or a small volume (2 mL) of sterile broth or saline.

Reference for Annex 2

- 1 WHO laboratory manual for the diagnosis of diphtheria and other related infections. Geneva: World Health Organization; 2021 (<https://apps.who.int/iris/handle/10665/352275>).

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