The Immunological Basis for Immunization Series

Module 14: Cholera

Immunization, Vaccines and Biologicals

World Health Organization

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Contents

Abbreviations and acronyms

ADCC	antibody-directed cell-mediated cytotoxicity
ASC	antibody secreting cell
CFU	colony-forming unit
CMRI	cell-mediated immune response
CT	cholera toxin
CTA	cholera toxin A subunit
CTB	cholera toxin B subunit
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
GALT	gut-associated lymphoid tissue
HIV	human immunodeficiency virus
IEC	intestinal epithelial cell
LPS	lipopolysaccharide
MSHA	mannose sensitive haemagglutinin
OCV	oral cholera vaccine
OMP	outer membrane protein
O-SP	oligosaccharide part of LPS
RITARD	removable intestinal tie adult rabbit diarrhoea
sIgA	secretory immunoglobulin A
TCP	toxin-coregulated pilus
TI-1	T-cell independent type 1 cells
TLR4	Toll-like receptor 4
WC	whole-cell
WC-CTB	whole-cell-cholera toxin B
WC-rCTB	whole-cell-recombinant cholera toxin B

Preface

This module is part of the series *The Immunological Basis for Immunization*, which was initially developed in 1993 as a set of eight modules focusing on the vaccines included in the Expanded Programme on Immunization (EPI)[1]. In addition to a general immunology module, each of the seven other modules covered one of the vaccines recommended as part of the EPI programme — diphtheria, measles, pertussis, polio, tetanus, tuberculosis and yellow fever. The modules have become some of the most widely used documents in the field of immunization.

With the development of the Global Immunization Vision and Strategy (GIVS) (2005–2015) (http://www.who.int/vaccines-documents/DocsPDF05/GIVS_Final_EN.pdf) and the expansion of immunization programmes in general, as well as the large accumulation of new knowledge since 1993, the decision was taken to update and extend this series.

The main purpose of the modules — which are published as separate disease/vaccine-specific modules — is to give immunization managers and vaccination professionals a brief and easily-understood overview of the scientific basis of vaccination, and also of the immunological basis for the World Health Organization (WHO) recommendations on vaccine use that, since 1998, have been published in the *Vaccine Position Papers* (http://www.who.int/immunization/documents/positionpapers_intro/en/index.html).

WHO would like to thank all the people who were involved in the development of the initial *Immunological Basis for Immunization* series, as well as those involved in its updating, and the development of new modules.

[1] This programme was established in 1974 with the main aim of providing immunization for children in developing countries.

1. *Vibrio cholerae* and cholera

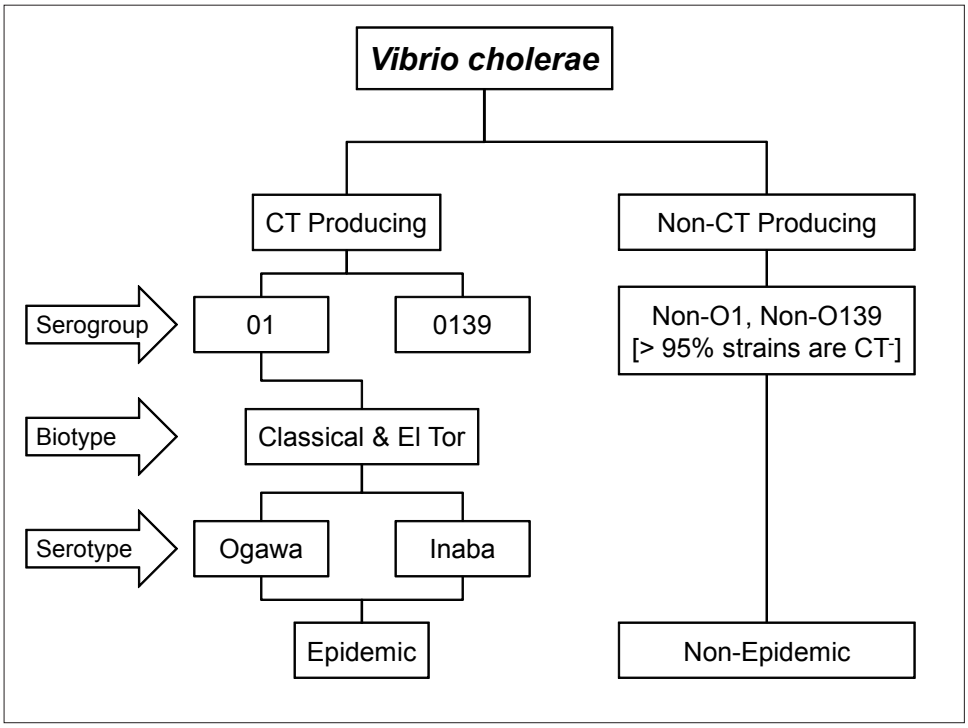
1.1 Classification

Cholera is an acute watery diarrhoeal disease with vomiting caused by the highly infectious facultative anaerobic Gram-negative pathogenic bacterium *Vibrio cholerae* belonging to the family *Vibrionaceae*. Humans are the only natural host for the pathogen. Transmission of pathogenic *V. cholerae* in humans occurs through ingestion of contaminated food and water.

The subspecies classification of *V. cholerae* is based on serogroups, which differ significantly in their antigenic lipopolysaccharide (LPS) composition, with no apparent cross-reactivity between them. LPS comprises three domains: (a) oligosaccharide part (O-SP) composed of glycosidically-linked sugars (15–18 substituted perosamine residues); (b) the core sugars (branched heteropolysaccharides) linking O-SP to lipid A; (c) lipid A (phosphoglycolipid) part anchoring to bacterial membrane (Chatterjee & Chaudhuri, 2003). So far, 206 serogroups have been recognized, and out of these only two serogroups, namely O1 and O139, are the causative agents of cholera. The current classification of *V. cholerae* is shown in Fig. 1. The O-SP and core region distinguish O1 and O139 serogroups, while the terminal sugar of the O-SP differentiates the two O1 serotypes, Ogawa and Inaba (Cox & Perry, 1996; Hisatsune et al., 1996). Apart from these serotypes, Hikojima is the third serotype of *V. cholerae* O1, which agglutinates with both anti-Inaba and anti-Ogawa antisera. Inaba, Ogawa and Hikojima serotypes have been designated by the antigenic formula AB, AC and ABC, respectively, where 'A' antigen is common (Sakazaki & Tamura, 1971). The Hikojima serotype is, in reality, rarely encountered, and is more of an academic curiosity than of epidemiological significance. The O139 serogroup evolved in 1992 and eventually spread to many parts of Asia (Sack et al., 2004). However, the pandemic potential of *V. cholerae* O139 is uncertain.

The O1 and O139 serogroups are distinctly different from others in their ability to produce cholera toxin (CT) as shown in Fig. 1. CT is responsible for most of the manifestation of the disease. Based on some phenotypic differences, the O1 serogroup is further classified into two distinct biotypes, namely, classical and El Tor (Fig. 1) that differ with respect to the severity of their infections (and consequently to their infection-to-case ratios), ability to survive outside the human host, and seasonal patterns. Among the seven cholera pandemics recorded so far, it is recognized that the fifth and sixth pandemics were caused by the classical biotype. The ongoing seventh pandemic, started in 1961 in the Sulawesi islands of Indonesia, was caused by the El Tor biotype which has the capacity to generate variants within a very short period of time.

Figure 1: The current classification scheme of epidemic and non-epidemic strains of *V. cholerae*.
Serogrouping is based on O-SP moiety of LPS.
CT is cholera toxin.
All epidemic *V. cholerae* O1/O139 strains are CT producer.



1.2 Global burden

Cholera is not a public-health problem in developed countries, but the burden in most developing countries is enormous. The disease cholera is highly endemic in Africa and Asia, where it has been estimated that there are over one million cases of cholera annually, with more than 120 000 deaths (WHO, 2001). In spite of the availability of simple rehydration treatments, which have greatly reduced the case-fatality rate, small children and the elderly are still vulnerable to severe dehydration caused by cholera. The disease burden of cholera is steadily increasing, as evident from a 2007 WHO report. For example, in 2006, 52 countries had officially-notified cholera cases (WHO, 2007). Increasing trends since the beginning of this millennium are confirmed when analysed in 5-year periods. The cumulative total of cholera cases during 2004 to 2008 increased by 24% as compared with the 2000 to 2004 period (WHO, 2009). These statistics do not reflect the true burden of cholera because cholera is underreported, especially in Asia, due to unfounded fears of travel and trade embargoes.

1.3 Endemic cholera

Cholera is endemic in many parts of the developing world and especially in the African and Asian continents. Although the exact basis of endemicity of cholera is currently not clear, it is certainly related to the population of some geographic zones being immune or sub-immune to *V. cholerae* infection, this being compounded by other factors, such as inadequate sanitation, poor hygiene, limited access to safe drinking water, dense population and malnutrition. However, from various environmental studies, and based on the nature of the pathogen, it is strongly believed that environmental biotic and abiotic factors also contribute critically to the pathogen becoming endemic in a given area, and to its seasonality, as seen in the Indian subcontinent.

1.4 Epidemic cholera

Epidemic or explosive outbreaks of cholera covering extensive areas and large populations are a common feature in many developing countries. Since 2000, massive cholera epidemics have occurred annually, either in sub-Saharan Africa, or in Asia. The last was recorded in Zimbabwe with more than 98 000 cases occurring within a period of 10 months (WHO, 2009). Since epidemic *V. cholerae* O1 or O139 has a potential to cause outbreaks and the disease has the property of rapid and severe onset, it can easily overwhelm public-health systems in areas with limited clinical and managerial resources. Cholera epidemics may arise during natural disasters, like floods or cyclones, when proper sanitation and supply of safe food and drinking water is heavily compromised. The extension of uncontrolled epidemics can rapidly spread across borders and affect different sub-regions. Since in most developing countries vast populations live in conditions that perpetuate cholera, where minimal standards of living cannot be improved in a short time, use of safe and efficient cholera vaccines can certainly make a difference when used as an additional public-health tool to those control measures usually recommended.

1.5 Imported cholera cases

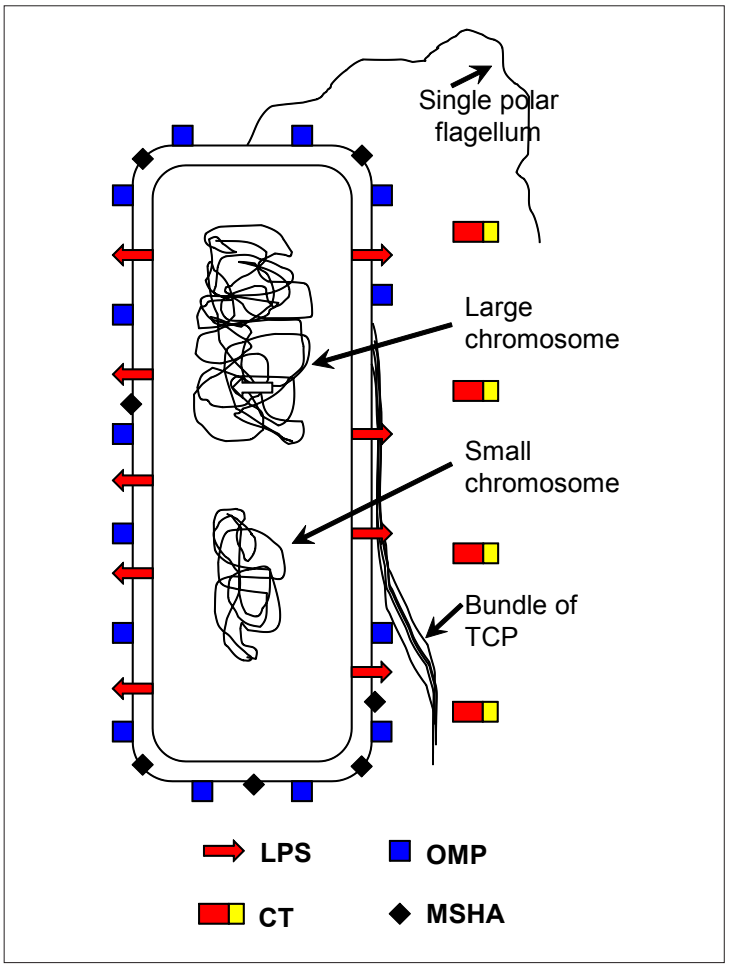
A substantial increase in international travel could raise concerns regarding imported cholera cases (Zuckerman et al., 2007). However, the numbers of such cases are quite low, and there is no evidence that imported cholera has led to epidemics in industrialized countries. In fact the number of imported cholera cases was only 68 during 2005; however, several additional cases could readily be identified from sources other than WHO, as reported by Zukerman et al. (2007).

Even though the exact figures on imported cholera cases are unavailable, their contribution to the global cholera burden is not significant.

2. The nature of immune response against cholera

Before going into details of immune response against *V. cholerae*, the major antigens, identified from a large number of studies involving clinical samples and animal experiments, as well as human volunteers, are shown in Fig. 2.

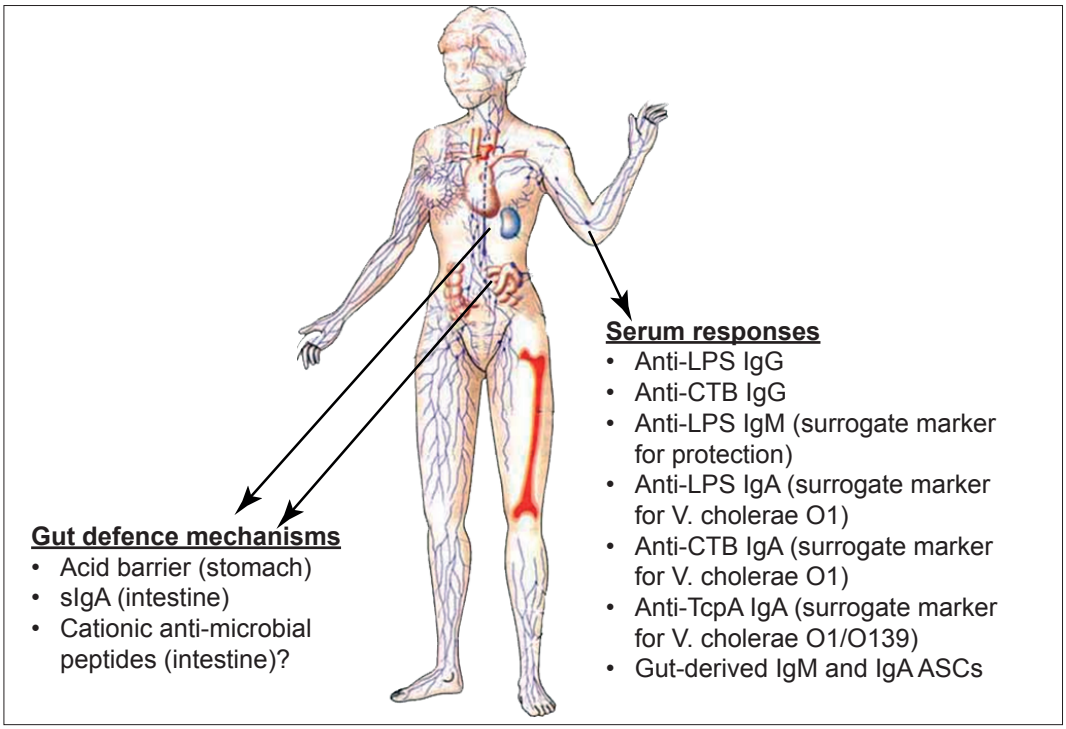
Figure 2: Major antigens of *V. cholerae* are shown schematically. All these antigens are either of cell surface associated or secreted type as shown. The organism also carries two chromosomes, a special feature of the members of the family *Vibrionacae*.



2.1 Immunity acquired from natural infection

The existence of acquired immunity against the disease cholera has been known since very ancient times. Patients recovering from *V. cholerae* infection are either protected against reinfection with the same *V. cholerae* biotype, or the subsequent episodes are less severe. Around 90% to 100% biotype-specific protection lasting for several years has been demonstrated in the volunteer trials with *V. cholerae* O1 in non-endemic areas and in response to natural infection in endemic zones (Levine et al., 1981; Glass et al., 1982). Since *V. cholerae* is a non-invasive pathogen, it is widely believed that protection against cholera is conferred by secretory IgA (sIgA) within the intestinal lumen (Fig. 3). A majority of Bangladeshi patients convalescing from moderate to severe cholera produced high levels of anti-CT as well as anti-LPS sIgA antibodies in the intestine. Elevated sIgA levels were also present in other body fluids, such as breast milk and saliva (Glass et al., 1983). However, sIgA confers only short-term protection, while protective immunity following *V. cholerae* infection is seen in the absence of significantly raised mucosal sIgA. The reason for this long-lasting immunological memory currently remains unknown (Svennerholm et al., 1984a). It is to be noted that the serum titres correlate with protection, but may not be the mechanisms of protection. In addition to clinical disease, repeated natural exposure to *V. cholerae* in the endemic areas may also give rise to significant serum antibody responses (Glass et al., 1985a). Antitoxin antibody levels, however, do not show a similar increase with age. In endemic areas, the age group between two and nine years has the highest risk of developing *V. cholerae* infection, while the younger children may be protected by maternal antibodies acquired through breast-feeding (Glass et al., 1983). Mannose-sensitive haemagglutinin (MSHA) (Fig. 2), a type IV pilus of either O1 El Tor or O139 serogroup could be immunogenic, since both convalescing patients and vaccinees develop anti-MSHA antibody responses. However, the titre is significantly lower compared to that of antibacterial and antitoxin antibodies (Svennerholm et al., 1984b). Another type IV pilus, called toxin-coregulated-pilus (TCP) (Fig. 2), which is a polymer of TcpA subunits, is considered to be a major virulence factor of *V. cholerae*. However, the protective role of anti-TCP antibody is not clear, and long-term immunological memory against TCP has not been studied. A comparative study of immune responses against *V. cholerae* O1 and O139 has shown that, despite the presence of a polysaccharide capsule in the latter serogroup, comparable systemic vibriocidal and antitoxin antibodies, as well as gut-derived antibody (both IgM and IgA) secreting cells (ASCs) in the peripheral blood (Fig. 3), are generated upon natural infection (Qadri et al., 1997b). Further studies are needed to show whether immunological memories against *V. cholerae* O1 and O139 infections are comparable or not.

Figure 3: Schematic diagram showing gut defences and serum immune responses against *V. cholerae* infection and cholera vaccines (see details in text).



2.2 Intrinsic factors influencing the outcome of exposure

Several innate host factors may influence the outcome of exposure to *V. cholerae*. ABO blood group antigens remain the most well studied intrinsic host factors that determine the susceptibility to cholera (Barua & Paguio, 1977; Levine et al., 1979b; Sircar et al., 1981; Glass et al., 1985b). The O blood group antigen has been reported to confer serotype-specific protection against infection with *V. cholerae* O1 (Barua & Paguio, 1977) and O blood group individuals respond to the live attenuated vaccine CVD103-HgR with higher antibody responses compared to the other blood groups (Lagos et al., 1995). On the other hand, patients with O blood group are at increased risk of suffering from more severe symptoms and fatal outcomes when infected with either O1 or O139 serogroup (Faruque et al., 1994; Harris et al., 2005). Hence it has been hypothesized that *V. cholerae* infection may have selected for the low prevalence of blood group O in the Gangetic Delta region (Glass et al., 1985b). Genetic factors other than blood group antigens probably play a role, since the first-degree relatives of cholera patients in Bangladesh were significantly more likely to develop the disease than less closely related members living in the same household, and this association was independent of blood group (Harris et al., 2005). Nutritional status of the host, like levels of zinc or vitamin A supplementation, and recent infections with other organisms, may also influence the outcome of disease development (Tomkins et al., 1993; Roy et al., 2006; Chowdhury et al., 2008). In the same way, microbiological and environmental factors may influence the outcome of exposure (Colwell, 1996 & 2004). Thus, the household contacts of O1, as opposed to O139 index cases, have a higher likelihood of infection, and markedly higher attack rates are seen in individuals living in households that contain more than one clinical case.

2.3 Antibacterial immunity

2.3.1 Anti-LPS immunity

The LPS (Fig. 2) of *V. cholerae* O1 and O139 are the most consistently described antigens associated with protection against cholera. Serologic studies define three O1 antigens associated with B-cell epitopes (A, B and C). It is generally appreciated that LPS of different serotypes possesses unique B-cell epitopes that are protective (Wang et al., 1998; Villeneuve et al., 2000). Although the exact molecular mechanism of how *V. cholerae* LPS elicits immune response is not clear, it has been shown that *V. cholerae* LPS is a T-cell independent type I (TI-1) antigen and does not require T-cell help for antigen-specific antibody production (Jacobs, 1975). LPS activates B-cells by binding to multiple receptors. The lipid A component of LPS binds Toll-like receptor 4 (TLR4) and other TLR-like molecules (Nagai et al., 2002; Peng, 2005). Additionally, complement receptors on B-cells can bind C3d, which may be covalently bound to LPS or to bacterial outer membrane structures. Complement-antigen complex binding to B-cell surface receptors enhances activation by lowering the threshold of cross-linking required (Lyubchenko et al., 2005). In addition, cytokines released from macrophages following LPS binding to their surface TLR4 may modulate B-cell activation (Corbel & Melchers, 1983).

2.3.2 Immunity against non-LPS structural antigens

Systemic and mucosal immune responses against TCP develop in patients infected with *V. cholerae* O1 El Tor and O139 (Fig. 3) and these responses are comparable in magnitude and frequency to those seen with LPS and MSHA (Qadri et al., 1997a; Asaduzzaman et al., 2004). It is to be noted that TCP of classical biotype differs to some extent from that of El Tor (Johnson et al., 1991; Rhine & Taylor, 1994). A volunteer study indicated that the TCP of classical biotype may provide protective immune response in humans (Herrington et al., 1988). Other antigens of *V. cholerae* that are believed to contribute to virulence have also been investigated for induction of antigen-specific immune responses. These include outer membrane proteins (OMPs) like the iron-regulated OMPs, and porin-like proteins that help in colonization of the gut (Sengupta et al., 1992). In particular, an 18 kDa protein that promotes colonization in the infant rabbit model and is present in both biotypes has been suggested as an important protective antigen (Sciortino, 1989). The polar flagellum of *V. cholerae* (Fig. 2) is considered to be a virulence factor and consequently it is believed that anti-flagellar antibodies may exert a protective role by preventing colonization (Yancey et al., 1979; Sinha et al., 1993). However, several non-motile mutants of *V. cholerae* were also evaluated as cholera vaccine, and one such strain called Peru-15 was found to be safe and immunogenic in volunteer trials (Sack et al., 1997; Cohen et al., 2002; Qadri et al., 2005 & 2007). Thus, the role of flageller antigens in immunity against cholera is not clear.

2.4 Antitoxin immunity

CT is one of the most potent oral immunogens ever studied (Lycke & Holmgren, 1986; Williams et al., 1999). Vigorous immune responses are generated, not only against CT administered orally alone, but also against unrelated antigens delivered along with it. Animal studies have shown an indirect correlation between CT-induced fluid secretion and intestinal synthesis of sIgA, as well as the number of antitoxin-producing cells in the intestine (Pierce, 1978). Immune responses against CT are mainly directed against the B-subunit (CTB) and neutralization of CT is primarily provided by anti-CTB. Thus, antibodies against CTB are equally effective as antibodies against holotoxin in the protection against CT-induced fluid accumulation in rabbit ileal loops (Svennerholm et al., 1994). CT subunit A (CTA), on the other hand, possesses strong immunomodulatory properties which have a multifactorial basis, including increased antigen uptake by the intestinal epithelial cells (IECs), enhanced antigen presentation by macrophages, isotype switching of IgA in B-cells, increased antigen-specific CD4 T-cell priming and induction of regulatory T-cells (Lycke, 1997), and are mainly contributed by its ADP-ribosylating activity (Lycke, 1997). Moreover, this immunomodulatory role is also observed when CT is co-administered with unrelated protein antigens like keyhole limpet haemocyanin (Snider, 1995; Glenn et al., 1998). Although it is possible to induce biotype-specific monoclonal antibodies against CT, polyclonal antiserum raised against classical CT are equally effective in neutralizing classical and El Tor toxins. It is notable that immunization with classical or El Tor CTB has resulted in equally high titres of antibodies against both these toxins (Kazemi & Finkelstein, 1990).

2.5 Non-specific defense mechanisms

A number of innate defence mechanisms may act as a first line of prevention against colonization of the gut with *V. cholerae*. Gastric acid milieu is an efficient barrier, and neutralization of the acidity before ingestion of *V. cholerae* drastically reduces the infectious dose in human volunteers (Cash et al., 1974) (Fig. 3). Cationic antimicrobial peptides that are avidly produced by the mucosal epithelial cells of the gut may also significantly contribute to anti-*V. cholerae* defence mechanisms (Fig. 3). The bacteria are susceptible to human cathelicidin in vitro while it may evade the host immune response by transcriptionally downregulating cathelicidin expression in the gut mucosa (Islam et al., 2001; Chakraborty et al., 2008). Although *V. cholerae* was traditionally considered to cause non-inflammatory diarrhoea, several recent studies have reported inflammatory response in the intestine (Silva et al., 1996; Harrison et al., 2008; Qadri et al., 2002 & 2004). Thus, there is neutrophilic infiltration of the gut and increased neutrophils and lactoferrin levels are found in the intestinal lavage fluid. However, the specific role of these neutrophils in the short-term and long-term protection against *V. cholerae* remains to be elucidated. As mentioned earlier, persons with O blood group are somewhat protected against *V. cholerae* infection although the mechanism remains unknown.

2.6 Cell-mediated immune response

Considering the non-invasive nature of *V. cholerae* infection, cell-mediated immune responses like the major histocompatability complex-restricted cellular cytotoxicity, natural killer-cell activity and antibody-directed cell-mediated cytotoxicity (ADCC) probably play a minor role in protective immunity. However, cell-mediated immune response (CMIR) was found to be protective in an infant mouse model (Chaicumpa & Rowley, 1973). Anti-*V. cholerae* immunity may be contributed by increased antigen presentation by different mucosal cells, including enterocytes, as well as enhanced sIgA production (Quiding et al., 1991). Gamma interferon-producing T-cells in the gut mucosa may significantly increase in numbers following antigenic exposure and may play a role in host defence (Quiding et al., 1991). A recent report by Weil et al. (2009) has shown that memory T-cell responses develop at least seven days after *V. cholerae* infection in humans, a time prior to, and concurrent with, development B-cell responses. This result suggests that T-cell responses to *V. cholerae* antigens may be important for the generation and stability of memory B-cell responses. The authors had previously shown that significant numbers of IgG and IgA memory B-cells against protein antigens of *V. cholera,* such as CTB and TcpA, persisted in the gut-associated lymphoid tissue (GALT) after one year of natural infection, while LPS-specific memory B-cells had waned by this time (Harris et al., 2009). As immune response against protein antigens is T-cell dependent, these results further suggest that T-cell help may result in a more durable memory B-cell response to protein antigens (Harris et al., 2009).

3. Protective antibodies to *V. cholerae* antigens

As described above, infection with *V. cholerae* evokes significant anti-LPS and anti-CT antibody responses in both serum and the intestine. Both these antibodies are protective against experimental cholera in animal models and probably work synergistically. Animal studies, e.g. removable intestinal tie adult rabbit diarrhoea (RITARD) and suckling mouse models mimic the disease and, despite limitations, have added valuable tools to study protective immune responses against human infection. Antibodies of multiple isotypes (IgM, IgA, IgG1, IgG3) specific for several *V. cholerae* antigens (LPS, CT, TCP, MSHA and flagella) are protective in the mouse model of cholera (Provenzano et al., 2006; Weil et al., 2009). However, animal models offer little insight into the nature of the protective antibodies in human cholera. Experimental evidence to date suggests that the level of serum vibriocidal antibodies is the best correlate of human resistance to cholera and that the anti-LPS antibodies constitute the main component exerting vibriocidal activity (Mosley et al., 1968; Glass et al., 1985a; Clemens et al., 1991) (Fig. 3). Anti-LPS antibody alone is sufficient to provide protection against human infection (Tacket et al., 1990), although it may be serotype specific, as underscored by the fact that the older people who were immune to *V. cholerae* O1 remained just as equally susceptible to O139 serogroup as younger O1 naive individuals (Qadri et al., 1997b). Curiously, field trials with monovalent killed whole-cell (WC) cholera vaccines (described below) have shown that Inaba serotype is able to induce cross-reactive anti-LPS antibodies that are protective against Ogawa serotype, while immunization with Ogawa fails to induce cross-protection in children but did in adults (Mosley et al. 1970; Mosley, 1973). The serum vibriocidal antibody test (see below) detects antibacterial IgM antibody which may not be the functional antibody in the intestinal secretions because of its protease sensitivity; instead it may serve as a surrogate marker for immunity against cholera. In an urban Bangladeshi population, baseline vibriocidal antibody titre predicted protection from subsequent infection with *V. cholerae* O1 but not from the development of symptomatic disease. By contrast, no correlation of vibriocidal antibody titre with O139 infection was found (Losonsky et al., 1996; Saha et al., 2004). Parenteral vaccination with killed WC *V. cholerae* induces protective anti-LPS antibodies (Azurin et al., 1967; Mosley et al., 1968), suggesting that *V. cholerae* LPS-specific serum IgG may also mount a short-term protective response. A recent discovery showing that human neonatal Fc receptor can transport IgG from intestinal spaces into the gut lumen and retrieve antigen back to the host, provides an explanation as to how a non-invasive infection like cholera induces antigen-specific protective IgG in the serum (Yoshida et al., 2004). Although IgG is extremely susceptible to proteolytic degradation, functional IgG that binds rotavirus has been detected in the intestinal secretions (Watanabe et al., 1978). Given that neither the bacteria nor the toxin penetrate the gut wall, it is widely believed that *V. cholerae*-specific sIgA in the intestinal lumen provides major protection (Fig. 3). sIgA does not fix complement in the classic manner; rather it functions by a mechanism called immuno-exclusion that prevents attachment of the pathogen to the receptor.

Anti-LPS IgA may obstruct colonization by interfering with flagellar functions or the LPS function that render the bacteria more susceptible to bile (Nesper et al., 2001; Brandtzaeg, 2003).

Several studies reported low protection against *V. cholerae* infection in humans by either serum or intestinal (secretory) antitoxin antibodies (Holmgren & Svennerholm, 1977; Clemens et al., 1991; Szu et al., 1994). Parenteral WC inactivated vaccines, but not toxoids, provide significant protection, although only for short periods. In addition, a single oral dose of live JBK70 vaccine strain (CT negative El Tor mutant) to human volunteers conferred ~90% protection against wild-type El Tor challenge (Levine et al., 1979a). However, the immunomodulatory functions of both CT holotoxin and CTB have been exploited to boost the levels of antibacterial antibodies. An oral vaccine (Dukoral®) that combines killed whole *V. cholerae* O1 cells and recombinant CTB (WC-rCTB) conferred excellent short-term protection and significant protection for up to three years (Clemens et al., 1988; Migasena et al., 1989). In addition, antibodies against TCP or MSHA and LPS cooperate, at least additively, and possibly synergistically, in protecting baby mice against experimental challenge with O1 vibrios (Osek et al., 1992 & 1994). *V. cholerae* polar flagellum contributes to pathogenicity and is immunogenic; however, immunization of rabbits with flagellar antigens failed to show protection in the ileal loop model. In a recently published report, serum LPS- and CTB-specific IgA levels were found to be directly correlated to protection from *V. cholerae* O1 infection, while serum anti-TCP IgA correlated with protection against both O1 and O139 infections (Harris et al., 2008) (Fig. 3). Interestingly, neither serum IgG nor faecal sIgA directed against the same antigens as above, could predict protection.

4. Immunological memory

The ultimate goal of all vaccination strategy is to generate effective long-term memory. Antigen-specific memory is defined as a faster response manifested by enhanced antibody titres after secondary exposure to the antigen. It is generally believed that protective memory against *V. cholerae* is determined by follicular B-cells and this requires T-cell help (Maruyama et al., 2000). LPS bound to protein components of *V. cholerae* may drive LPS to a T cell-dependent response. Although a T cell-independent immune response may be generated by the ability of LPS to bind TLR4 and activate nuclear factor-κB (NF-κB), generation of a bona fide, long-term, antigen-independent transferable memory B-cell response has not been reported (Provenzano et al, 2006). Immunological memory depends on the route of vaccine administration as different routes target different B-cell subsets. Parenteral administration of the LPS neo-glycoconjugates may be the fastest and simplest method to induce sIgA recall responses in the endemic zone with the smallest amount of vaccine (Svennerholm et al., 1980), while oral immunization remains the most effective route to induce immunity in immunologically naive populations. Immune status of an individual or the anti-LPS antibody profile may determine the optimal vaccination strategy. It is interesting to note the relationship between the *V. cholerae* serotype and the memory response. Inaba serotype is capable of inducing cross-reactive antibodies to Ogawa serotype, but the reverse does not happen. Priming with Inaba decreases the vibriocidal antibody response and, with Ogawa, results in an additive effect when the boosting is done with the Ogawa serotype. On the other hand, boosting with Inaba increases the vibriocidal response if priming is done with Ogawa serotype. The difference in the serotype-specific immune response may be due to the difference in the immunodominant LPS epitope (Mosley et al., 1970 & 1973).

5. Assays to measure serum and mucosal antibody responses

5.1 Serum vibriocidal antibodies

V. cholerae chiefly colonizes the small intestinal mucosal surface without invading enteric epithelial cells, and protection against infection is expected to be mediated almost entirely by antibodies that reach the mucosal surface. In this respect, serum vibriocidal antibody functions as a surrogate marker and, as already mentioned, the level correlates directly with the protection against *V. cholerae*. Thus, the detection of vibriocidal antibodies is considered 'gold standard' in determining immune responses to *V. cholerae* infection. Serum vibriocidal antibody assay is usually performed with *V. cholerae* O1 or O139 as the target organism, using the methods described earlier (Jertborn et al., 1986; Qadri et al., 1995).

5.2 Antibody secreting cells (ASCs)

Our current understanding of ASCs as a mechanism against cholera infection is not clear. Gut-derived antibody-producing cells in the peripheral blood were measured after oral immunization of human volunteers with CTB (Czerkinsky et al., 1991). Numbers of total and antigen-specific ASCs were measured by two-colour enzyme-linked immunospot (ELISPOT) assay (Czerkinsky et al., 1987; Qadri et al., 2003).

5.3 LPS-specific sIgA antibodies

Increased levels of IgA-specific for LPS of *V. cholerae* can be detected in the convalescent sera as well as sIgA in intestinal secretions of patients recovering from cholera (Levine et al., 1981; Migasena et al., 1989; Qadri et al., 2003). Furthermore, it was shown that *V. cholerae* vaccination helps to boost sIgA levels in individuals living in cholera endemic zones (Svennerholm, 1977 & 1980). Anti-LPS IgA may be measured directly from a variety of samples, such as infected patient's sera, cholera stools (sIgA), etc., and indirectly by assessing the sIgA-specific ASCs in the circulation, which serves as a proxy indicator of the mucosal response (Czerkinsky et al., 1993).

5.4 Anti-CT antibodies

Like LPS, CT is also immunogenic and elicits both mucosal (Svennerholm et al., 1984a) and systemic immune responses. There are distinct CT-specific ASCs of different IgG and IgA subclasses in the circulation of convalescent cholera patients, and the pattern of the response is in the following order: IgG1 > IgA1 > IgG2 > IgA2, with low levels of IgG3 and IgG4 ASCs (Qadri et al., 1999). When anti-CT antibodies are directly measured from sera of convalescent patients, unlike ASCs, the levels of all subclasses of Ig are usually high and include IgG3 and IgG4. In the faecal extracts of convalescent

patients, the levels of sIgA1 and sIgA2 isotypes found were quite high. To measure all these antibody responses, either ELISPOT (for ASCs) or ELISA technique is used (Qadri et al., 1999). Microtitre plates are coated with purified recombinant B subunit of CT and the rest of the procedure is a standard ELISA assay (Svennerholm et al., 1983; Qadri et al., 1999).

6. Nature of immune response following vaccination

6.1 Post-vaccination serum antibody profile

Although over the years several approaches have been taken to develop a safe and effective cholera vaccine, so far only three formulations have been licensed: (i) the killed whole-cell (WC) parenteral vaccine administered intramuscularly; (ii) the oral killed WC preparation containing CTB; (iii) the live oral recombinant vaccine. Overall efficacy of parenterally-administered killed WC vaccine was about 50% and duration of protection was only about six months. Thus, for an as yet, unknown reason, the vaccine had less overall impact on modulation of immunological response in humans, although a few volunteer studies were able to demonstrate induction of anti-LPS sIgA as well as vibriocidal antibodies, particularly in adults.

The most extensively studied oral killed WC vaccine contains non-toxic but immunogenic B subunit of CT (WC-CTB). This vaccine was developed in the 1980s based upon the concept that natural *V. cholerae* infection strongly induces sIgA producing B-cells, which occur essentially at intestinal mucosal surfaces as well as GALT, the site of colonization of the pathogen. The WC-rCTB vaccine Dukoral®, the only cholera vaccine prequalified by WHO for the United Nations to purchase, showed about 85% protective efficacy in the first year, with strong serum vibriocidal antibodies (Clemens et al., 1988) indicating that intestinal mucosal site of vaccination in humans is indeed an excellent immunological basis to evoke *V. cholerae* antigen-specific immune response. However, after three years of surveillance, the vaccine only showed about 63% protective efficacy among individuals aged more than five years of age (Clemens et al., 1990a).

Parallel to WC-CTB vaccines, live oral recombinant vaccine strains were also constructed with a view that, like natural infection, this vaccine strain will colonize the small intestine and express various hitherto unknown factors relating to pathogenesis. On oral immunization, live attenuated vaccines showed more than 90% effectiveness in inducing anti-LPS antibodies (Kotloff et al., 1992; Kenner et al., 1995). However, apart from the demonstration of vibriocidal antibodies upon immunization with each of the three vaccines (parenteral WC-killed, oral killed WC-CTB and oral live) developed so far, information about involvement of various Ig subclasses and B-cell populations responsible for immunity against *V. cholerae* is currently lacking.

6.2 Extent of immunity after vaccination

Long-lasting immunity upon vaccination is a most desirable criterion for a candidate vaccine. Although natural infection by *V. cholerae* induces long-term protection for at least three years against subsequent disease (Levine et al., 1981; Glass et al., 1982 & 1985a; Harris et al., 2009; Weil et al., 2009), immune responses induced by cholera vaccines developed so far are usually short-lived; for example, administration of parenteral WC-killed vaccine in humans induced very short-term protective immunity, i.e. only for 3–6 months (Ryan & Calderwood, 2000), while later generations of vaccines like oral killed WC-CTB showed about a three-year duration of immunity (this is the maximum period of follow-up study done so far) with significant decrease of titre (Clemens et al., 1990a). In the case of live oral vaccines, exact status of long-term immunity in vaccinees is not clear, although a volunteer study revealed protection to infection 6 months after vaccination (Tacket et al., 1992). Thus, it appears that pathogen-derived immunoprotective factors responsible for long-lasting immunity, as seen under natural infection, are currently unknown; discovery of those factors may help in designing improved cholera vaccine. Recently, Weil et al. (2009) have shown that *V. cholerae* haemolysin induces significant T-cell proliferative response compared to that of other known antigens. Since natural infection induces systemic response against LPS and is of IgM class (WHO, 2001), the modulation of IgM memory in long-term protection against *V. cholerae* infection deserves further study.

6.3 Status of immune response in age, sex and socioeconomic groups

One peculiar observation in immune response against WC-rCTB (Dukoral®) is that the level of vaccine-induced protection is age-related, being lowest in young children (a population group which is at high risk of cholera), and highest in adults, indicating that vaccines worked best in immunologically-primed populations by boosting underlying immunity. It is, however, not known whether live oral vaccine (CVD103-HgR) confers any protection in children aged below two years (WHO, 2001). Several field trials conducted with parenteral WC vaccine revealed that, in young children, serum vibriocidal antibody titre is quite low compared to older individuals (Mosley et al., 1968). Oral killed WC-CTB, as well as live vaccine, also suffers with this drawback for children below five years of age. Thus, to maintain considerable serum titre against *V. cholerae* antigens in this lower age group, booster doses are needed. Although there is no clear-cut study conducted on sex-related immune response after cholera vaccination, it appears from various volunteer and field trials that it is likely that the antibody response is not sex independent. Studies conducted in Bangladesh and other developing countries where cholera is endemic did not assess the immune response in different socioeconomic groups. Furthermore, the vast majority of cholera cases occur among those with high poverty making it difficult to assess.

6.4 Placental passage and passive immunity following immunization

Although no information on the nature of passive immunity is available for any oral vaccines, parenterally-administered vaccine induced anti-LPS sIgA antibodies in saliva and breast milk in women living in endemic zones, but not in women in developed countries. Infants who drank breast milk of immunized mothers experienced reduced severity of the disease (Glass et al., 1983), indicating the role of passive immunity in preventing cholera. However, the drawback of the study was that the authors did not control for age of the children. A type of 'passive immunity' was seen in Bangladesh when children of vaccinated mothers had lower rates of cholera, apparently due to reduced infection in the mothers who might otherwise have transmitted the infection to their babies (Clemens et al., 1990b). Thus, the results raise the possibility that vaccinated mothers may provide protection to their young children in cholera- endemic settings. Further studies are needed to understand the role and nature of passive immunity to prevent cholera.

6.5 Role of various adjuvants in potentiation of cholera immune response

Adjuvants play a critical role in enhancing host immune response against a particular antigen. It is well established that CT of *V. cholerae* is a potent immunogen following mucosal or systemic delivery and it may act as an adjuvant in potentiating local and systemic immune responses when co-administered with other antigens (Williams et al., 1999, and references therein). In the course of natural infection it is quite possible that secreted CT of *V. cholerae* plays a critical role in inducing mucosal immunity in humans and, for this reason, B subunit of CT is used as an important component of oral vaccines, whether killed or live. Unfortunately, the exact mechanism of action of CT as an adjuvant is currently not clear. In certain field trials killed WC vaccines were injected with aluminium phosphate as an adjuvant, which prolonged the duration of protection for up to between 12 and 18 months. However, the adjuvant used had serious local side-effects and it was not considered further. In fact, at present we know very little about the role of different adjuvants in inducing mucosal immune response in humans. Since *V. cholerae* infection induces long-lasting potent immune response in humans (Harris et al., 2009; Weil et al., 2009) through activation of mucosal immunological network, a serious effort must be undertaken to explore further in this direction.

7. Efficacy of cholera vaccines

7.1 Parenteral WC-killed vaccines

This was the first vaccine developed and tested in clinical trials (WHO, 2001; Sack et al., 2004; Provenzano et al. 2006). It was initially administered intramuscularly and later subcutaneously or intradermally. The efficacy was 42%–45%, although side-effects, albeit minor in most cases, were common in immunized persons, most likely developed due to LPS toxicity (Benenson et al., 1968; Provenzano et al., 2006). Protective efficacy of this vaccine suggested that serum antibodies might confer protection to cholera. The major reason why this vaccine was discontinued was limited duration (up to six months only) of protection. Parenteral vaccination of Pakistani women induced anamnestic anti-LPS sIgA response in saliva and breast milk, while a similar vaccination of Swedish women showed only serum anti-LPS IgG response (Svennerholm, 1980). As discussed above, adjuvants, particularly alum-absorption, may increase the duration of protection of parenteral vaccine (Pal et al., 1980).

7.2 Oral killed WC vaccines

The concept that sIgA is the predominant isotype that confers protection at the mucosal surfaces encouraged the use of oral cholera vaccines in the 1980s. At least three formulations of WC vaccines, heat- or formalin-killed, have been tested in field trials in many parts of the world (Hill and Lalloo, 2006; Provenzano et al., 2006). The most extensively studied preparations contain killed classical and El Tor biotypes mixed with CTB, WC-CTB. Excellent protective efficacy (85%) was noticed in the first 12 months, which, as already mentioned above, reduced to 50% over the next two years. However, young children, although initially protected, showed a similar response as with the parenteral vaccines with rapid decline in protection after six months. A bivalent (O1 and O139) killed WC vaccine without CTB tested in Viet Nam showed higher antibody titre in children, most probably due to the use of a higher dose, and achieved ~50% protection after 3–5 years (Thiem et al., 2006). The WC-rCTB showed good initial response in Peruvians, but the titre returned to the baseline within a year (Begue et al., 1995). This vaccine was also effective in controlling a seasonal epidemic in Mozambique and, quite surprisingly, was also found to be effective in children less than five years old, and in older persons (Lucas et al., 2005). Booster doses of the killed WC vaccines are needed for continued immunoprotection after two years for adults as well as children over six years of age, and after six months for children in the age group >2 and <6 year (Zuckerman et al., 2007). A retrospective study of vaccinees in Bangladesh indicated that the WC killed vaccines (two or three doses) might confer protection to non-vaccinated neighbours by reducing their risk of becoming infected by 25%. People were less likely to become infected rather than individually protected and this was termed ‘herd protection’ as opposed to ‘herd immunity’ (Ali et al., 2005).

7.3 Oral modified live vaccines

A live oral cholera vaccine was thought to be more immunogenic and better protective owing to several reasons (Bhadra et al., 1994; Levine and Tacket, 1994). Firstly, a single clinical infection confers long-lasting protection and a live vaccine would mimic the temporal expression of antigens that would occur during natural infection. However, the antigens or combination of the antigens that constitutes the protective repertoire still remains to be established. Secondly, the protective antigen dose would increase with pathogen growth and multiplication in the case of a live vaccine. Finally, the critical protective immunity in cholera is mainly antibacterial rather than antitoxin in nature, and the degree of stimulation of serum vibriocidal antibody following a live oral vaccine constitutes the best surrogate marker of immunity (Levine & Tacket, 1994). However, the high correlation between protection and vibriocidal response does not apply to the killed oral vaccines since the vibriocidal response is not as great; however killed vaccines still protect. Protective efficacies of three different versions of oral live vaccines have been tested both in volunteer studies and in field trials (Hill & Lalloo, 2006; Ryan et al., 2006). The CVD vaccines (CVD-103HgR and CVD-110) were very effective in volunteers in developed countries with 97% seroconversion following a single oral dose. The vibriocidal antibodies also appeared significantly earlier compared to the WC killed oral vaccines. However, the titre rapidly declined after two weeks although the individuals remained protected 4–5 weeks after vaccination against experimental cholera (Wasserman et al., 1994). Another improved live oral cholera vaccine strain, Peru-15, was developed based on *V. cholerae* O1 El Tor Inaba strain which has been found to be safe, immunogenic and efficacious in North American volunteers (Kenner et al., 1995; Sack et al., 1997; Cohen et al., 2002). Peru-15 was also safe and immunogenic in Bangladeshi adults, toddlers and infants (Qadri et al., 2005 & 2007). Overall, vibriocidal antibody response in Bangladeshi children (aged between five years and nine months) who received a single high dose ($2x10^8$ CFU) was 77% and serum IgA antibodies to CTB was 40%. The immune responses were dose dependent, and at a reduced dose ($2x10^7$ CFU) responses were also lower (Qadri et al., 2007). Several technical problems prevent us drawing a clear conclusion about the superiority of a particular vaccine when the protective efficacies of WC killed, parenteral or oral and an oral live vaccine were compared in a volunteer study in a cholera endemic area. Furthermore, it is not just the efficacy of the vaccine that makes it better; cost and freedom from side effects, etc. also have to be considered.

7.4. Currently licensed cholera vaccines and doses

Several oral cholera vaccines (OCVs) have been developed and proven to be safe, immunogenic and effective. Four of these vaccines have been licensed in some countries, and one of them (killed WC plus recombinant CTB (WC/rCTB), Dukoral® by Crucel/SBL, Sweden) is used mainly by travellers visiting cholera-endemic countries, but has also been used in demonstration programmes for public-health interventions. This is the only cholera vaccine which has been WHO prequalified. This vaccine has been modified and technology transferred to Viet Nam where it is produced by VaBiotech (killed WC vaccine, ORC-Vax®). This vaccine has recently been licensed in India after South-South technology transfer (Shanchol® by Shantha Biotechnics). These three vaccines are given in a two-dose regimen ten days apart and they elicit protective efficacy eight days after the second dose.

The only licensed single-dose vaccine (recombinant live oral vaccine CVD103-HgR, Orochol® manufactured by Crucel/Berna Biotech, Switzerland, stopped production in 2004. Dukoral® is available in many countries, but is used primarily as a travel vaccine.

8. New cholera vaccines in development

Apart from the several cholera vaccines discussed above, some new candidate vaccines are currently in various stages of development and some of them are discussed here. The vaccine 638, which is a live attenuated single-dose oral cholera vaccine based on *V. cholerae* O1 El Tor Ogawa strain was developed in Cuba and the vaccine has already tested in Phase II trials in Mozambique (Garcia et al., 2005). Bengal-15 and CVD112 are live attenuated strains of *V. cholerae* O139, which have been shown to be safe and immunogenic in human volunteers (Ledon et al., 2003). Recently it has been reported that transgenic rice seeds with expressed CTB when fed to mice elicited serum anti-CTB IgG and mucosal IgA antibodies and protected animals from oral challenge with CT (Nochi et al., 2007).

9. Safety of cholera vaccines

9.1 Safety to humans

Oral cholera vaccines developed in recent times are needle-free and thus safe so far from serious blood-borne diseases, including human immunodeficiency virus (HIV) infection. Although oral vaccine does not always mean that it is safe, oral killed WC cholera vaccines developed so far have shown minimal side-effects (mostly related to gastrointestinal symptoms), and the vaccines are well tolerated. Initial live-attenuated oral recombinant vaccines developed by using *V. cholerae* O1 El Tor strains N16961 were found to be highly reactogenic including mild to moderate diarrhoea, abdominal cramps, anorexia and fever, etc. However, subsequently the live- attenuated oral vaccine strain CVD103-HgR has been constructed using the classical strain 569B, which showed minimal side-effects. CVD103-HgR was found to be genetically stable during preparation and during passage through the intestines of volunteers.

9.2 Environmental safety

While use of oral killed WC vaccines are environmentally quite safe since the cells are of non-replicative type, use of live cells as vaccine candidates does not rule out this possibility. It is particularly important to note that choleragenic *V. cholerae* cells are very proficient in genomic rearrangement, in particular its major virulence locus coding for CT. Furthermore, CT genes are carried by a filamentous phage called CTXΦ (Waldor & Mekalanos, 1996), so release of the live CTX locus attenuated vaccine strain in the environment may be reverted to virulent strain upon acquisition of wild-type CT genes carried by the phage. However, it has been shown that live oral vaccine strain CVD103-HgR dies within 20 days under various environmental conditions (Viret et al., 2004). Therefore, during development of any live vaccine strain all these important environmental hazards should be considered.

10. Post-genomic future generation cholera vaccines

From the ongoing discussion it can be seen that oral cholera vaccines, particularly the combination killed WC-CTB vaccines, are quite effective in inducing solid mucosal immunity as well as systemic immunity. However at present, our knowledge about other potent protective antigens, except LPS of *V. cholerae*, is limited. Since the whole genome sequence of information of the pathogen is available in the public database, a comprehensive genomic approach should be undertaken to identify as yet unknown but immunoprophylactic antigens of *V. cholerae*, so that an improved oral vaccine can be constructed, which will be safe, effective, longlasting, and cheap and easy to administer.

References

Ali M et al. (2005). Herd immunity conferred by killed oral cholera vaccines in Bangladesh: a reanalysis. *Lancet*, 366:44–49.

Asaduzzaman M et al. (2004). The major subunit of the toxin-coregulated pilus TcpA induces mucosal and systemic immunoglobulin A immune response in patients with cholera caused by *Vibrio cholerae* O1 and O139. *Infection and Immunity*, 72:4448–4454.

Azurin JC et al. (1967). A controlled field trial of the effectiveness of cholera and cholera El Tor vaccines in the Philippines. *Bulletin of the World Health Organization*, 37:703–727.

Barua D, Paguio AS (1977). ABO blood groups and cholera. *Annals of Human Biology*, 4:489–492.

Benenson AS et al. (1968). Cholera vaccine field trials in East Pakistan. 2. Effectiveness in the field. *Bulletin of the World Health Organization*, 38:359–372.

Begue RE et al. (1995). Immunogenicity in Peruvian volunteers of a booster dose of oral cholera vaccine consisting of whole cells plus recombinant B subunit. *Infection and Immunity*, 63:3726–3728.

Bhadra RK et al. (1994). Developmental strategies and problems. *Indian Journal of Biochemistry and Biophysics*, 31:441–448.

Brandtzaeg P (2003). Role of secretory antibodies in the defence against infections. *International Journal of Medical Microbiology*, 293:3–15.

Cash RA et al. (1974). Response of man to infection with *Vibrio cholerae*. I. Clinical, serologic, and bacteriologic responses to a known inoculum. *The Journal of Infectious Diseases*, 129:45–52.

Chaicumpa W, Rowley D (1973). Mechanisms of antibacterial immunity against *Vibrio cholerae* in the intestinal tracts of baby mice. *The Journal of Infectious Diseases*, 128:56–62.

Chakraborty K et al. (2008). Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (HBD-1) expression in the intestinal epithelial cells. *Cellular Microbiology*, 10:2520–2537.

Chatterjee SN, Chaudhuri K (2003). Lipopolysaccharides of *Vibrio cholerae*. I. Physical and chemical characterization. *Biochimica Biophysica Acta*, 1639:65–79.

Chowdhury F et al. (2008). A comparison of clinical and immunologic features in children and older patients hospitalized with severe cholera in Bangladesh. *The Pediatric Infectious Disease Journal*, 27:986–992.

Clemens JD et al. (1988). Field trial of oral cholera vaccines in Bangladesh: results of one year of follow-up. *The Journal of Infectious Diseases*, 158:60–69.

Clemens JD et al. (1990a). Field trial of oral cholera vaccines in Bangladesh: results from a three year follow-up. *Lancet*, 335:270–273.

Clemens JD et al. (1990b) Field trial of oral cholera vaccines in Bangladesh: evaluation of anti-bacterial and anti-toxic breast-milk immunity in response to ingestion of the vaccines. *Vaccine*, 8:469–472.

Clemens JD et al. (1991). Field trial of oral cholera vaccines in Bangladesh: serum vibriocidal and antitoxic antibodies as markers of the risk of cholera. *The Journal of Infectious Diseases*, 163:1235–1242.

Cohen MB et al. (2002). Randomized controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. *Infection and Immunity*, 70:1965–1970.

Colwell RR (1996). Global climate and infectious disease: the cholera paradigm. *Science*, 274: 2025–2031.

Colwell RR (2004) Infectious disease and environment: cholera as a paradigm for waterborne disease. *International Journal of Microbiology*, 7:285–289.

Corbel C, Melchers F (1983). Requirement for macrophages or for macrophage- or T cell-derived factors in the mitogenic stimulation of murine B lymphocytes by lipopolysaccharides. *European Journal of Immunology*, 13:528–533.

Cox AD, Perry MB (1996). Structural analysis of the O-antigen-core region of the lipopolysaccharide from *Vibrio cholerae* O139. *Carbohydrate Research*, 290:59–65.

Czerkinsky C et al. (1987). Oral immunization with bacterial antigen induces IgA-secreting cells in peripheral blood in humans. *Advances in Experimental Medicine and Biology*, 216B:1709–1719.

Czerkinsky C et al. (1991). Antibody-producing cells in peripheral blood and salivary glands after oral cholera vaccination of humans. *Infection and Immunity*, 59:996–1001.

Czerkinsky C et al. (1993). Induction and assessment of immunity at enteromucosal surfaces in humans: implications for vaccine development. *Clinical Infectious Diseases*, 16:S106–S116.

Faruque AS et al. (1994). The relationship between ABO blood groups and susceptibility to diarrhoea due to *Vibrio cholerae* O139. *Clinical Infection and Diseases*, 18:827–828.

Garcia L et al. (2005). The vaccine candidate *Vibrio cholerae* 638 is protective against cholera in healthy volunteers. *Infection and Immunity*, 73:3018–3024.

Glass RI et al. (1982). Endemic cholera in rural Bangladesh, 1966–1980. *American Journal of Epidemiology*, 116:959–970.

Glass RI et al. (1983). Protection against cholera in breast-fed children by antibodies in breast milk. *The New England Journal of Medicine*, 308:1389–1392.

Glass RI et al. (1985a). Seroepidemiological studies of El Tor cholera in Bangladesh: association of serum antibody levels with protection. *The Journal of Infectious Diseases*, 151, 236–242.

Glass RI et al. (1985b). Predisposition for cholera of individuals with O blood group. Possible evolutionary significance. *American Journal of Epidemiology*, 121:791–796.

Glenn GM et al. (1998). Skin immunization made possible by cholera toxin. *Nature*, 391:851.

Harris AM et al. (2009). Antigen specific memory B-cell responses to *Vibrio cholerae* O1 infection in Bangladesh. *Infection and Immunity*, 77:3850–3856.

Harris JB et al. (2008). Susceptibility to *Vibrio cholerae* infection in a cohort of household contacts of patients with cholera in Bangladesh. *PLoS Neglected Tropical Diseases*, 2:e221.

Harris JB et al. (2005). Blood group, immunity, and risk of infection with *Vibrio cholerae* in an area of endemicity. *Infection and Immunity*, 73:7422–7427.

Harrison LM et al. (2008). *Vibrio cholerae* flagellins induce Toll-Like receptor 5-mediated interleukin-8 production through mitogen-activated protein kinase and NF-κB activation. Infection and Immunity, 76:5524–5534.

Herrington DA et al. (1988). Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. *The Journal of Experimental Medicine*, 168:1487–1492.

Hill DR, Lalloo DG (2006). Oral cholera vaccines: use in clinical practice. *Lancet Infectious Diseases*, 6:361–373.

Hisatsune K et al. (1996). Lipopolysaccharides of *Escherichia coli* K12 strains that express cloned genes for the Ogawa and Inaba antigens of *Vibrio cholerae* O1: identification of O-antigenic factors. *Microbiology and Immunology*, 40:621–626.

Holmgren J, Svennerholm AM (1977). Mechanisms of disease and immunity in cholera: a review. *The Journal of Infectious Diseases*, 136(Suppl.):S105–S112.

Islam D et al. (2001). Downregulation of bacteriocidal peptides in enteric infections: A novel immuno escape mechanism with bacterial DNA as a potential regulator. *Nature Medicine*, 7:180–185.

Jacobs DM (1975). Structural and genetic basis of the in vivo immune response to TNP-LPS. *The Journal of Immunology*, 115:988–992.

Jertborn M et al. (1986). Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. *Journal of Clinical Microbiology*, 24:203–209.

Johnson G et al. (1991). Epitope differences in toxin-coregulated pili produced by classical and El Tor *Vibrio cholerae* O1. *Microbial Pathogenesis*, 11:179–188.

Kazemi M, Finkelstein RA (1990). Study of epitopes of cholera enterotoxin-related enterotoxins by checkerboard immunoblotting. *Infection and Immunity*, 58:2352–2360.

Kenner DF et al. (1995). Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. *The Journal of Infectious Diseases*, 172:1126–1129.

Kotloff KL et al. (1992). Safety and immunogenicity in North Americans of a single dose of live oral cholera vaccine CVD103-HgR: results of a randomized placebo-controlled, double blind crossover trial. *Infection and Immunity*, 60:4430–4432.

Lagos R et al. (1995). Attenuated live cholera vaccine strain CVD 103-HgR elicits significantly higher serum vibriocidal antibody titres in persons of blood group O. *Infection and Immunity*, 63:707–709.

Ledon T et al. (2003). Construction and characterization of O139 cholera vaccine candidates. *Vaccine*, 21:1282–1291.

Levine MM, Tacket CO (1994). Recombinant live cholera vaccines. Wachsmuth K, Blake PA, Olsvik O, eds. *Vibrio cholerae and cholera: molecular to global perspective*. ASM Press:395–413.

Levine MM et al. (1979a). Immunity of cholera in man: relative role of antibacterial versus antitoxic immunity. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 73:3–9.

Levine MM et al. (1979b). Genetic susceptibility to cholera. *Annals of Human Biology*, 4:369–374.

Levine MM et al. (1981) Duration of infection-derived immunity to cholera. *The Journal of Infectious Diseases*, 143:818–820.

Losonsky GA et al. (1996). Factors influencing secondary vibriocidal immune responses: relevance for understanding immunity to cholera. *Infection and Immunity*, 64:10–15.

Lucas ME et al. (2005). Effectiveness of mass oral cholera vaccination in Beira, Mozambique. *The New England Journal of Medicine*, 352:757–767.

Lyubchenko T et al. (2005). Coligation of the B cell receptor with complement receptor type 2 (CR2/CD21) using its natural ligand C3dg: activation without engagement of an inhibitory signaling pathway. *The Journal of Immunology*, 174:3264–3272.

Lycke N (1997). The mechanism of cholera toxin adjuvanticity. *Research in Immunology*, 148:504–520.

Lycke N, Holmgren J (1986). Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology*, 59:301–308.

Maruyama M et al. (2000). Memory B-cell persistence is independent of persisting immunizing antigen. *Nature*, 407:636–642.

Migasena S et al. (1989). Immunogenicity of two formulations of oral cholera vaccine comprised of killed whole vibrios and the B subunit of cholera toxin. *Infection and Immunity*, 57:117–120.

Mosley WH (1973). Field trials of monovalent Ogawa and Inaba cholera vaccines in rural Bangladesh — three years of observation. *Bulletin of the World Health Organization*, 49:381–387.

Mosley WH et al. (1968). The relationship of vibriocidal antibody titre to susceptibility to cholera in family contacts of cholera patients. *Bulletin of the World Health Organization*, 38:777–785.

Mosley WH et al. (1970). The 1968–1969 cholera-vaccine field trial in rural East Pakistan. Effectiveness of monovalent Ogawa and Inaba vaccines and a purified Inaba antigen, with comparative results of serological and animal protection tests. *The Journal of Infectious Diseases*, 121:1–9.

Nagai Y et al. (2002). Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. *Blood*, 99:1699–1705.

Nesper J et al. (2001). Characterization of *Vibrio cholerae* O1 El tor *galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infection and Immunity*, 69:435–445.

Nochi T et al. (2007). Rice-based mucosal vaccine as a global strategy for cold-chain and needle-free vaccination. *Proceedings of the National Academy of Sciences of the United States of America*, 104:10986–10991.

Osek J et al. (1992). Protection against *Vibrio cholerae* El Tor infection by specific antibodies against mannose-binding haemagglutinin pili. *Infection and Immunity*, 60: 4961–4964.

Osek J et al (1994). Role of antibodies against biotype-specific *Vibrio cholerae* pili in protection against experimental classical and El Tor cholera. *Infection and Immunity*, 62: 2901–2907.

Pal SC et al. (1980). A controlled field trial of an aluminium phosphate-adsorbed cholera vaccine in Calcutta. *Bulletin of the World Health Organization*, 58:741–745.

Peng SL (2005). Signaling in B cells via Toll-like receptors. *Current Opinion in Immunology*, 17:230–236.

Pierce NF (1978). The role of antigen form and function in the primary and secondary intestinal immune responses to cholera toxin and toxoid in rats. *The Journal of Experimental Medicine*, 148:195–206.

Provenzano D et al. (2006). The ABCs (antibody, B cells, and carbohydrate epitopes) of cholera immunity: considerations for an improved vaccine. *Microbiology and Immunology*, 50:899–927.

Qadri F (2004). Acute dehydrating disease caused by *Vibrio cholerae* serogroups O1 and O139 induces increases in innate cells and inflammatory mediators at the mucosal surface of the gut. *Gut*, 53:62–69.

Qadri F et al. (1995). Comparison of the vibriocidal antibody response in cholera due to *Vibrio cholerae* O139 Bengal with the response in cholera due to *Vibrio cholerae* O1. *Clinical and Diagnostic Laboratory Immunology*, 2:685–688.

Qadri F et al. (1997a). Immune response to the mannose-sensitive hemagglutinin in patients with cholera due to *Vibrio cholerae* O1 and O139. *Clinical and Diagnostic Laboratory Immunology*, 4:429–434.

Qadri F et al. (1997b). Comparison of immune responses in patients infected with *Vibrio cholerae* O139 and O1. *Infection and Immunity*, 65:3571–3576.

Qadri F et al. (1999). Lipopolysaccharide- and cholera toxin-specific subclass distribution of B-Cell responses in cholera. *Clinical and Diagnostic Laboratory Immunology*. 6:812–818.

Qadri F et al. (2002). Increased levels of inflammatory mediators in children and adults infected with *Vibrio cholerae* O1 and O139. *Clinical and Diagnostic Laboratory Immunology*, 9:221–229.

Qadri F et al. (2003). Antigen-specific immunoglobulin A antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with cholera: comparison to antibody-secreting cell responses and other immunological markers. *Infection and Immunity*, 71:4808–4814.

Qadri F et al. (2005). Randomized, controlled study of the safety and immunogenicity of Peru-15, a live attenuated oral vaccine candidate for cholera, in adult volunteers in Bangladesh. *The Journal of Infectious Diseases*, 192:573–579.

Qadri F et al. (2007). Peru-15, a live attenuated oral cholera vaccine is safe and immunogenic in Bangladeshi toddlers and infants. *Vaccine*, 25:231–238.

Quiding M et al. (1991) Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses and interferon-gamma production and evokes local immunological memory. *The Journal of Clinical Investigation*, 88:143–148.

Rhine JA, Taylor RK (1994). TcpA pilin sequences and colonization requirements for O1 and O139 *Vibrio cholerae*. *Molecular Microbiology*,13:1013–1020.

Roy SK et al. (2006). Impact of zinc deficiency on *Vibrio cholerae* enterotoxin-stimulated water and electrolyte transport in animal model. *Journal of Health, Population, and Nutrition*, 24:42–47.

Ryan ET, Calderwood SB (2000). Cholera vaccines. *Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America*, 31:561–565.

Ryan ET et al. (2006). Live attenuated oral cholera vaccines. *Expert Review of Vaccines*, 5:483–494.

Sack DA et al. (1997). Evaluation of Peru-15, a new live oral vaccine for cholera, in volunteers. *The Journal of Infectious Diseases*, 176:201–205.

Sack DA et al. (2004). Cholera. *Lancet*, 363:223–233.

Saha D et al. (2004). Incomplete correlation of serum vibriocidal antibody titre with protection from *Vibrio cholerae* infection in urban Bangladesh. *The Journal of Infectious Diseases*, 189:2318–2322.

Sakazaki R, Tamura K (1971). Somatic antigen variation in *Vibrio cholerae*. *Japanese Journal of Medical Science and Biology*, 24:93–100.

Sciortino CV (1989). Protection against infection with *Vibrio cholerae* by passive transfer of monoclonal antibodies to outer membrane antigens. *The Journal of Infectious Diseases*, 160:248–252.

Sengupta DK et al. (1992). Major outer membrane proteins of *Vibrio cholerae* and their role in induction of protective immunity through inhibition of intestinal colonization. *Infection and Immunity*, 60:4848–4855.

Silva TM et al. (1996). New evidence for an inflammatory component in diarrhoea caused by selected new, live attenuated cholera vaccines and by El Tor and O139 *Vibrio cholerae*. *Infection and Immunity*, 64:2362–2364.

Sinha VB et al (1993). Identification of the flagellar antigens of *Vibrio cholerae* El Tor and their role in protection. *Vaccine*, 11:372–375.

Sircar BK et al. (1981). ABO blood group distributions in diarrhoea cases including cholera in Calcutta. *Annals of Human Biology*, 8:289–291.

Snider DP (1995). The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins. *Critical Reviews in Immunology*, 15:317–348.

Svennerholm AM (1977). Boosting of secretory IgA antibody responses in man by parenteral cholera vaccination. *Scandinavian Journal of Immunology*, 6:1345–1349.

Svennerholm AM et al. (1980). Different secretory immunoglobulin A antibody responses to cholera vaccination in Swedish and Pakistani women. *Infection and Immunity*, 30:427–430.

Svennerholm AM (1983). Serologic differentiation between antitoxic response to infection with *Vibrio cholerae* and enterotoxin-producing *Escherichia coli*. *The Journal of Infectious Diseases*, 147:514–521.

Svennerholm AM et al. (1984a). Mucosal antitoxic and antibacterial immunity after cholera disease and after immunization with a combined B subunit-whole cell vaccine. *The Journal of Infectious Diseases*, 149:884–893.

Svennerholm AM et al. (1984b). Weak serum and intestinal antibody responses to *Vibrio cholerae* soluble hemagglutinin in cholera patients. *Infection and Immunity*, 45:792–794.

Svennerholm AM et al. (1994) Immunity to *Vibrio cholerae* infection. Wachsmuth K, Blake PA, Olsvik O, eds. *Vibrio cholerae and cholera: molecular to global perspective*. ASM Press:257–271.

Szu SC et al. (1994). Introduction to serum vibriocidal antibodies by O-specific polysaccharide-protein conjugate vaccines for prevention of cholera. Wachsmuth K, Blake PA, Olsvik O, eds. *Vibrio cholerae and cholera: molecular to global perspective*. ASM Press:381–394.

Tacket CO et al. (1990). Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. *Infection and Immunity*, 58:1620–1627.

Tacket CO et al. (1992). Onset and duration of protective immunity in challenged volunteers after vaccination with live oral cholera vaccine CVD 103-HgR. *The Journal of Infectious Diseases*, 166:837–841.

Thiem VD et al. (2006). Long-term effectiveness against cholera of oral killed whole-cell vaccine produced in Viet Nam. *Vaccine*, 24:4297–4303.

Tomkins A et al. (1993). The role of zinc and vitamin A deficiency in diarrhoeal syndromes in developing countries. *The Proceedings of the Nutrition Society*. 52:131–142.

Villeneuve S et al. (2000). Crystal structure of an anti-carbohydrate antibody directed against *Vibrio cholerae* O1 in complex with antigen: molecular basis for serotype specificity. *Proceedings of the National Academy of Sciences of the United States of America*, 97:8433–8438.

Viret J-F et al. (2004). Biosafety aspects of the recombinant *Vibrio cholerae* vaccine strain CVD 103-HgR. *Vaccine*, 22:2457–2469.

Waldor MK, Mekalanos JJ (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science*, 272:1910–1914.

Wang J et al. (1998). On the antigenic determinants of the lipopolysaccharides of *Vibrio cholerae* O1, serotypes Ogawa and Inaba. *The Journal of Biological Chemistry*, 273:2777–2783.

Wasserman SS et al. (1994). Kinetics of the vibriocidal antibody response to live oral cholera vaccines. *Vaccine*, 12:1000–1003.

Watanabe H et al. (1978). Human rotavirus and its antibody: their coexistence in feces of infants. *Journal of Clinical Microbiology*, 7:405–409.

Weil AA et al. (2009). Memory T-cell responses to *Vibrio cholerae* O1. *Infection and Immunity*, 77:5090–5096.

Williams NA et al. (1999). Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic. *Immunology Today*, 20:95–101.

World Health Organization (2001). Cholera vaccines. *Weekly Epidemiological Record*, 76:117–124.

World Health Organization (2007). Cholera, 2006. *Weekly Epidemiological Record*, 82:273–284.

World Health Organization (2009). Cholera: global surveillance summary, 2008. *Weekly Epidemiological Record*, 84:309–324.

World Health Organization (2009). Global alert and response: cholera in Zimbabwe — update 4. http://www.who.int/csr/don/2009_06_09/en/index.html.

Yancey RJ et al. (1979). Flagella-induced immunity against experimental cholera in adult rabbits. *Infection and Immunity*, 25:220–228.

Yoshida M et al. (2004). Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. *Immunity*, 20:769–783.

Zuckerman, JN et al. (2007). The true burden and risk of cholera: implications for prevention and control. *Lancet Infectious Diseases*, 7:521–530.

The World Health Organization has provided technical support to its Member States in the field of vaccine-preventable diseases since 1975. The office carrying out this function at WHO headquarters is the Department of Immunization, Vaccines and Biologicals (IVB).

IVB's mission is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases. The Department covers a range of activities including research and development, standard-setting, vaccine regulation and quality, vaccine supply and immunization financing, and immunization system strengthening.

These activities are carried out by three technical units: the Initiative for Vaccine Research; the Quality, Safety and Standards team; and the Expanded Programme on Immunization.

The Initiative for Vaccine Research guides, facilitates and provides a vision for worldwide vaccine and immunization technology research and development efforts. It focuses on current and emerging diseases of global public health importance, including pandemic influenza. Its main activities cover: i) research and development of key candidate vaccines; ii) implementation research to promote evidence-based decision-making on the early introduction of new vaccines; and iii) promotion of the development, evaluation and future availability of HIV, tuberculosis and malaria vaccines.

The Quality, Safety and Standards team focuses on supporting the use of vaccines, other biological products and immunization-related equipment that meet current international norms and standards of quality and safety. Activities cover: i) setting norms and standards and establishing reference preparation materials; ii) ensuring the use of quality vaccines and immunization equipment through prequalification activities and strengthening national regulatory authorities; and iii) monitoring, assessing and responding to immunization safety issues of global concern.

The Expanded Programme on Immunization focuses on maximizing access to high quality immunization services, accelerating disease control and linking to other health interventions that can be delivered during immunization contacts. Activities cover: i) immunization systems strengthening, including expansion of immunization services beyond the infant age group; ii) accelerated control of measles and maternal and neonatal tetanus; iii) introduction of new and underutilized vaccines; iv) vaccine supply and immunization financing; and v) disease surveillance and immunization coverage monitoring for tracking global progress.

The Director's Office directs the work of these units through oversight of immunization programme policy, planning, coordination and management. It also mobilizes resources and carries out communication, advocacy and media-related work.

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