Development of vaccines against cholera and diarrhoea due to enterotoxigenic <i>Escherichia coli</i>: Memorandum from a WHO meeting*

This Memorandum summarizes current knowledge on the epidemiology of cholera and diarrhoea due to enterotoxigenic Escherichia coli (ETEC) and outlines the results of recent research to develop an effective oral vaccine against cholera. The meeting reviewed current research on the protective antigens of ETEC and made a number of recommendations with the aim of stimulating further efforts towards the development of vaccines against disease caused by ETEC.

Introduction

Although parenteral cholera vaccines have existed for a century, they have not played a significant role in the control of cholera since they induce only a low level of immunity over a short period. Research is under way to develop effective oral vaccines based on nonliving organisms and antigens, or on live, attenuated strains of <i>Vibrio cholerae</i> O1. The meeting discussed the results that have recently emerged from such studies, including a field trial of two formulations of an inactivated oral cholera vaccine in Bangladesh; research on protective antigens of <i>V. cholerae</i> O1 that might lead to improved inactivated vaccines; and studies of the safety, immunogenicity, and efficacy of live cholera vaccines in volunteers.

Recommendations for further research in these areas were also made.

Diarrhoea due to enterotoxigenic <i>Escherichia coli</i> (ETEC) is an important problem in most developing countries, but progress towards the development of vaccines for this disease has been slow. However, important recent results on the virulence antigens of ETEC have led to the development of possible candidate vaccines, and the first of these has reached the stage of initial evaluation in humans.

Cholera vaccines

Epidemiological aspects

Currently, cholera is reported to WHO by about 35 countries. Left untreated, it is still one of the most dangerous infectious diseases, with case-fatality rates of up to 40%. An estimated 5.5 million cases of cholera occur in the world each year, causing about 20,000 deaths in Africa and 100,000 in Asia. About one-third of the deaths involve children under 5 years of age; one-quarter, children aged 5–14 years; and the remainder, adults.

Epidemics of cholera that occur in previously uninfected areas with seronegative populations affect all age groups equally and are often associated with a single mode of spread. Such epidemics are characterized by a relatively low rate of asymptomatic infection and there is usually no environmental reservoir of infection. In contrast, with endemic cholera, disease incidence is highest for 2–15-year-olds and declines with increasing age, except for

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women of child-bearing age, for whom the incidence is higher. Yearly seasonal outbreaks are prominent and transmission is associated with the following: an environmental, aquatic reservoir; multiple modes of spread (contaminated water or food, occasionally person-to-person contact); and frequent asymptomatic infections, leading to a high prevalence of antibody among 20-year-olds.

In endemic areas, risk factors for severe cholera include: absence of low titres of vibriocidal antibodies; close contact with the family of a cholera patient; low gastric acidity (whether naturally acquired, surgically induced, or resulting from the use of antacids); absence of breast-feeding (among children under 3 years of age); and O blood group. The existence of natural immunity to \( V. \) cholerae O1 in endemic settings is suggested by the declining attack rate with increasing age, the lower attack rates in persons with elevated levels of vibriocidal antibody, and epidemiological evidence that one episode of cholera evokes substantial protection against a second. In Bangladesh, the prevalence of vibriocidal antibody is 40% among 5-year-olds and 80% among 20-year-olds. It is estimated that every person in Bangladesh is exposed to \( V. \) cholerae O1 at least once every 10 years, and that some people may be exposed annually; immunity appears to be boosted with each infection, including asymptomatic infections.

Groups that may benefit from effective cholera immunization include children and adults in endemic areas, refugees in camps where the sanitary conditions are poor, and populations in non-endemic areas that are threatened by nearby epidemics. International travellers experience a very low risk of cholera, which would probably not be changed measurably by vaccination.

Even today, many people in Africa and Asia live in areas that experience a high incidence of cholera and where effective treatment is unavailable. In such areas, mortality due to cholera remains high and epidemics cause panic and severe social and economic problems. Hence, there is continuing need for an effective vaccine against the disease. Based on the concept that natural protection against cholera is conferred by intestinal antibacterial and antitoxic antibodies, research in the 1980s focused on the development of oral vaccines that would induce protective immunity by stimulating an intestinal immune response against one or more relevant antigens of \( V. \) cholerae O1.

**Important antigens of Vibrio cholerae O1**

Studies in animals and humans have shown that \( V. \) cholerae cell-wall lipopolysaccharide (LPS) and cholera toxin (CT) both evoke protective immune responses. Antibodies to these substances protect synergistically in the gut. Antibacterial immunity is mainly afforded by antibodies to LPS, but antibodies to other cell-associated protein antigens may also be important in this respect. Antitoxic immunity is primarily directed against the B subunit (BS) of cholera toxin.

Colonization of the human intestine by \( V. \) cholerae O1, which is a prerequisite for developing diarrheal disease, is complex and requires the coordinated expression of chemotactic and motility functions, proteolytic enzymes, haemagglutinins, colonization pili, and finally, production of CT. The prevention of bacterial adherence would block the pathogenesis of cholera at its earliest stage. Recent studies of infant mice and volunteers have indicated that a pilus elaborated by both the classical and the El Tor biotype of \( V. \) cholerae O1 is probably required for colonization. This pilus has been designated TCP (toxin coregulated pilus) because the growth conditions that modulate its level of expression also modulate toxin production. In addition, the major pilus structural gene (\( tcpA \)) and the toxin genes are part of a virulence regulon (\( toxR \)). Because of their critical roles in the pathogenesis of cholera, \( toxR \)-regulated surface and secreted proteins (in particular TCP and cholera toxin B subunit (CT–BS)) are potential immunogens for use in combination vaccines, in addition to LPS.

Rabbit polyclonal antiserum directed against TCP has been found to be protective in passive immunization experiments in infant mice challenged with either Ogawa or Inaba strains of \( V. \) cholerae O1. The protective activity of anti-TCP serum is lost after it has been absorbed with wild-type \( V. \) cholerae O1, but is retained after absorption with a pilus-negative mutant. This suggests that protection is associated with the anti-TCP antibodies. The sequences of the \( tcpA \) genes from two classical strains of \( V. \) cholerae O1 (Ogawa 395 and Inaba 569B) are identical. The sequence of the \( tcpA \) gene from an El Tor strain of Ogawa serotype (E7946) shows less homology: about 80% with respect to the predicted amino acid sequence. However, the predicted secondary structure, distribution of charged residues, and potential antigenic epitopes are conserved to a high degree between the two biotypes, which indicates that functional domains, as well as epitopes that induce protective antibodies, may be shared by the pilus of both biotypes.

Genetic analysis has identified several additional gene products besides TcpA that are required for TCP biogenesis and function. One of these, TcpG, may also
function as an adhesin, and is therefore a candidate for immunization studies. In addition to the genes involved in TCP synthesis, those that encode the outer membrane proteins and the production of an accessory colonization factor (ACF) are toxR-regulated. Thus, TcpA could perhaps be used as an indicator of the presence of other potentially important toxR-regulated antigens during the preparation of whole-cell (WC) vaccines.

The killed oral vaccines recently tested in Bangladesh (see below) contain no detectable TCP by Western blot analysis, possibly because the growth conditions of the bacteria when the vaccine was prepared were not optimal for the expression of these antigens. Neither formalin nor heat (as used to inactivate the bacteria in the WC vaccine) seem to affect adversely the immunoreactivity of TCP in Western blot analyses. Thus, it might be possible to improve the oral WC vaccine by using bacterial strains that produce TCP and by employing production methods that ensure the expression and preservation of TCP and other toxR-regulated antigens.

**Candidate oral vaccines**

**Killed WC and WC+BS (WC/BS) vaccines.** Killed whole-cell vaccines with or without the B subunit of cholera toxin have recently been developed and tested. The oral WC/BS vaccine contains purified B subunit from cholera toxin and formalin- or heat-inactivated classical and El Tor cholera vibrios of the Inaba and Ogawa serotypes. Purified BS is completely nontoxic and gives rise to levels of neutralizing antibodies comparable with those evoked by holotoxin; the capacity of this subunit to bind to cell membranes may contribute to its immunogenicity. The heat-killed organisms in the vaccine provide Inaba and Ogawa LPS antigens, and the formalin-killed organisms provide heat-labile antigens. Because the BS pentamer is acid labile, the vaccine is administered dissolved in a buffer solution of sodium bicarbonate and citric acid.

**Studies with volunteers.** The safety of the WC/BS vaccine was first demonstrated in small-scale clinical trials in Bangladesh, Sweden, and the USA. In Bangladesh, an evaluation of the vaccine's ability to stimulate mucosal antibacterial and antitoxic immune responses showed that two or more oral doses evoked anti-LPS and antitoxin IgA antibody responses in intestinal lavage fluid that were comparable with those induced by natural disease. These responses were considerably higher and longer lasting than those induced by two intramuscular doses of the same vaccine. Oral WC/BS vaccine also induced significant antibacterial and antitoxic antibody responses in Swedish volunteers, though they were less intense than those induced in Bangladeshis of similar age. Furthermore, the BS component of the vaccine given orally to Bangladeshis or Swedish volunteers induced or boosted mucosal (IgA) immunological memory for at least 15 months in the Bangladeshis and 5 years in the Swedes.

In volunteers in the USA, three doses of WC/BS vaccine afforded 63% protection, and the WC component alone 56% protection, against a subsequent ED_{100} challenge with virulent V. cholerae O1 of the El Tor biotype. Protection against diarrhoeal illness with a stool output of at least 2 litres was 100% for the WC/BS vaccine and 56% for the WC vaccine.

**Field trial in Bangladesh.** In 1985, the International Centre for Diarrhoeal Disease Research (Bangladesh), in collaboration with the Government of Bangladesh, and supported by WHO and other agencies, initiated a randomized, double-blind, placebo-controlled field trial of killed oral cholera vaccines at its Matlab field area. In this trial, a total of 63 000 persons aged 2–15 years and females over 15 years received three doses at 6-week intervals of WC/BS vaccine, WC vaccine, or a placebo consisting of killed E. coli strain K12. Each dose of WC/BS contained 1 mg of BS plus 1 × 10^{11} killed V. cholerae O1 whole cells, consisting of heat-killed classical Inaba (Cairo 48), heat-killed classical Ogawa (Cairo 50), formalin-killed El Tor Inaba (Phil 6973), and formalin-killed classical Ogawa (Cairo 50) serotype, in equal proportions. The WC vaccine had the same cellular constituents, but lacked the B subunit. The vaccines were liquid preparations that were stored at +4 °C.

Each vaccine elicited an approximately twofold increase in serum vibriocidal antibody titre and the WC/BS vaccine also evoked a four- to sixfold increase in serum IgG anti-cholera-toxin titre. No side-effects could be attributed to either vaccine during the follow-up of vaccinees.

The data on vaccine efficacy against cholera were derived from surveillance of persons who attended the three diarrhoea treatment centres that serve the population in Matlab. The major findings of the trial are summarized in Table 1. During 3 years of follow-up of the recipients of three doses, the WC vaccine conferred 52% protection and the WC/BS vaccine 50% protection against culture-proven cholera for all vaccinees (P < 0.0001 for each vaccine), including children and adults.

For both the WC and WC/BS vaccines, the protective efficacy was lower in children who were aged
2–5 years when vaccinated: 31% and 24% for WC, and 38% and 47% for WC/BS vaccine in the first and second year of follow-up, respectively. During the third year of follow-up, such children were not protected. In contrast, for persons who were aged 6 years or older when vaccinated, the efficacy was significant during each of the 3 years of follow-up. For this age group the WC vaccine conferred 68% protection and the WC/BS vaccine 63% protection during the entire 3-year period. In the placebo group, 65% of all cases of cholera occurred in persons aged 6 years or older.

During each year of surveillance, disease caused by both the classical and the El Tor biotype of V. cholerae O1 was observed, and most isolates were of the Ogawa serotype. Throughout the period of follow-up, protection was greater against disease caused by the classical biotype (Table 1). Each vaccine appeared to confer a similar degree of protection against cholera episodes that were associated with severe dehydration or milder disease. However, the protective efficacy against the former appeared to be lower in persons of O blood group than in persons of blood groups A, B, or AB. WC/BS was more protective than WC against cholera during the first 8 months after vaccination; this was a nonepidemic season during which V. cholerae biotype El Tor was prevalent. WC/BS was also associated with short-term (approximately 3 months) cross-protection against diarrhoea caused by strains of E. coli that produce heat-labile toxin (LT); presumably this is due to the antigenic similarity of the B subunits of CT and the LT of ETEC.

Although the study had not been designed to examine the efficacy of different doses, two doses of vaccine appeared to be as protective as three. One dose, however, did not provide any protection.

Live, attenuated V. cholerae O1 vaccine strain CVD 103.

During the past 10 years, a considerable amount of research has been devoted to developing attenuated mutants of V. cholerae O1 for possible use as live oral vaccines. Particular attention has been given to producing mutant strains that are nontoxigenic (A−B+ toxin phenotype) or produce only the B subunit of cholera toxin (A−B+). Several candidate strains have been produced that are immunogenic and protective in volunteers; however, they are not suitable for use as vaccines because 25–40% of the volunteers experienced mild diarrhoea. Recent research has sought to determine how these strains cause diarrhoea and to develop new mutants that lack this side-effect but, nevertheless, remain immunogenic.

The live vaccine candidate that is currently of greatest interest is V. cholerae O1 strain CVD 103. This is an A−B+ mutant prepared by recombinant DNA techniques that delete the genes encoding the A (toxic) subunit of CT from the pathogenic classical Inaba strain 569B, leaving intact the production of immunogenic, nontoxic B subunit. Neither the parent strain nor CVD 103 produce Shiga-like toxin, which is generated by many strains of V. cholerae O1 and is considered to be a possible contributor to diarrhoea caused by A−B+ or A−B− strains.

Studies of volunteers. Healthy adult volunteers in the USA who were given a dose of 10⁸ viable organisms of CVD 103 experienced no serious adverse reactions; however, 5 of 46 volunteers (11%) developed diarrhoea that was not accompanied by other symptoms such as malaise, nausea, cramps, or anorexia. Furthermore, 45 of 46 volunteers (98%) developed significant increases in serum vibriocidal antibody, with a geometric mean reciprocal titre of 1339; and 93% had significant rises in serum antitoxin antibody.

In three separate challenge studies, 26 vaccinees who had ingested a single 2×10⁸ dose of CVD 103 were challenged 1 month later with a dose of pathogenic V. cholerae O1 that caused diarrhoea in 24 of 25 unimmunized controls. In each study, the vaccine provided statistically significant protection against illness. Overall in the three studies, which used challenge strains that differed from CVD 103 either in biotype or in serotype, a single dose of CVD 103 conferred 80% protection against any diarrhoea, and 94% protection against severe diarrhoea (>2 litres of stool).

CVD 103 was subsequently modified by inserting a gene that encodes mercury resistance into the hlyA locus of the chromosome, and renamed CVD 103-HgR. This marker allows the vaccine strain to be differentiated from wild strains. A potentially practical, lyophilized formulation of CVD 103-HgR was given to 90 volunteers in the USA in a dose of 5×10⁸ viable organisms, while 15 others received 5×10⁷ organisms. These doses were well tolerated, although three of the volunteers experienced loose stools. Significant in-

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<th>Group and period of follow-up:</th>
<th>Vaccine efficacy (%)</th>
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<tr>
<td></td>
<td>WC</td>
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<tr>
<td>Individual follow-up years:</td>
<td></td>
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<tr>
<td>First year</td>
<td>53</td>
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<td>Second year</td>
<td>57</td>
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<tr>
<td>Third year</td>
<td>43</td>
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<td>Followed up all 3 years:</td>
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<tr>
<td>2–5 years of age</td>
<td>23</td>
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<td>≥6 years of age</td>
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<td>All ages</td>
<td>52</td>
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<td>Etiology:</td>
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<td>Classical biotype</td>
<td>60</td>
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<tr>
<td>El Tor biotype</td>
<td>40</td>
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creases in vibriocidal antibody occurred in 91% of volunteers, the mean titre being three- to fourfold greater than that of similar volunteers who were given three doses of WC/BS vaccine. CVD 103-HgR was recovered from the stool cultures of approximately 30% of vaccinees, in contrast to a recovery rate of 90% from recipients of the parent strain, CVD 103. A single dose of CVD 103-HgR also conferred 65% protection against challenge with pathogenic *V. cholerae* O1 of the heterologous biotype, El Tor Inaba.

In a subsequent study in Thailand (carried out in a containment facility), 12 healthy adult volunteers who ingested a dose of $5 \times 10^8$ CVD 103-HgR organisms experienced no adverse reactions; 11 of the 12 vaccinees had significant increases in serum vibriocidal antibody and 9 had significant increases in serum antitoxin. However, additional outpatient studies of military recruits in Thailand, which were designed to evaluate the safety and immunogenicity of the vaccine, showed much poorer serological responses; the reason for this is currently being investigated.

**Vaccines using live carrier bacteria.** Since bacterial antigen can be more immunogenic when it is produced by a carrier organism within the intestine than when it is given orally in nonliving form, a bacterial hybrid has been created which contains genes from *V. cholerae* O1 that specify the synthesis and assembly of cholera LPS on the surface of the live, attenuated oral typhoid vaccine *Salmonella typhi* Ty21a. This hybrid strain has been evaluated for immunogenicity and safety in about 500 volunteers, and only very minor side-effects were observed. After three doses of $2 \times 10^{10}$ live bacteria, serum antibody responses to *V. cholerae* LPS were detected for about 50%, and vibriocidal antibody responses for about 35% of the recipients. By contrast, 90-100% of the volunteers showed responses to *S. typhi* LPS. In a volunteer challenge study, three doses of $10^{10}$ live organisms of a freeze-dried preparation induced only marginal overall protection (25%) against clinical cholera, but the severity of diarrhoea was significantly reduced compared with controls.

These studies support the concept of a hybrid cholera vaccine, but have not yielded a practical vaccine with sufficient protective efficacy. Work is under way to develop an improved hybrid strain of Ty21a with enhanced expression of *V. cholerae* LPS. Other questions about hybrid bacterial vaccines that still need to be answered include:

—Would another vector be more effective than Ty21a, but still non-reactogenic?

—Can a single carrier be used sequentially in the same individual to deliver different antigens?

Some of these issues are discussed below in relation to the development of candidate vaccines against enterotoxigenic *E. coli*.

**Vaccines against ETEC**

**Epidemiology and acquisition of natural immunity**

Strains of enterotoxigenic *E. coli* (ETEC) cause disease worldwide, but are especially common in developing countries. In hospital- and clinic-based studies of acute diarrhoea in developing countries, the proportion of cases in which ETEC were identified has ranged from 10% to 50%, with an average of about 20% in children under 5 years of age. Prospective community-based studies in Bangladesh, Brazil, Peru, and several other countries indicate that the incidence of ETEC diarrhoea is highest among under-2-year-olds. Overall, during the first 5 years of life, children in areas with high rates of diarrhoea experience 1–2 episodes caused by ETEC per year.

To cause disease, ETEC must be able to colonize the small intestine and elaborate enterotoxins. ETEC may produce heat-stable toxin (ST), heat-labile toxin (LT), or both; the proportion of ETEC isolates that produce only ST, only LT, or both enterotoxins varies somewhat from country to country, but each of these toxin phenotypes usually accounts for at least one-quarter of all ETEC. Several studies suggest that strains of ETEC that produce both ST and LT are restricted to relatively few O groups, and within these groups to a few O:H serotypes. Strains that produce only ST or only LT occur in a wider range of serotypes. Although all ETEC serotypes are found worldwide, there is geographical variation in the relative importance of specific serotypes, and also some temporal variation for individual locations. The prevalence of other important determinants of virulence, such as colonization factors, may also vary geographically, but at present there are only limited data on this aspect.

The illness caused by ETEC ranges from mild diarrhoea without dehydration, which is the most characteristic clinical picture, to cholera-like disease; in endemic areas, at least two-thirds of infections appear to be asymptomatic. In Bangladesh, the highest incidence occurs in young children and decreases with increasing age; the proportion of ETEC infections that are symptomatic also declines as age increases. Both these observations are indicative of naturally acquired immunity. These age-related changes have been observed with strains of ETEC that produce ST/LT or only ST, but have not been sufficiently studied for those strains that produce only LT. Although high titres of serum antibodies to LT are found in young children in endemic areas, such children remain susceptible to diarrhoea caused by LT- or ST/LT-producing ETEC. This suggests that the decline in rates of ETEC infection and illness with age is due to mechanisms that are not dependent on the presence of antibody to LT.

Studies of travellers' diarrhoea offer a few addi-
tional insights into the epidemiology of ETEC and the development of natural immunity. Travellers who journey from industrialized to developing countries experience a high attack rate for diarrhoea (usually at least 30%) during the first few weeks, which appears to diminish with repeated travel or prolonged residence in the developing country. This suggests acquired protection. ETEC strains are usually the predominant enteric pathogen in travellers' diarrhoea. In 19 studies in Latin America, a median of 46% (range, 28–72%) of episodes of diarrhoea were associated with ETEC; in eight studies in Asia, ETEC strains were found in 14% (range, 0–37%) of episodes; and in three studies in Africa, in 36% (range, 31–75%) of episodes. The proportion of isolates that produced ST, LT, or colonization factors varied from study to study.

**Important antigens of enterotoxigenic E. coli**

To produce diarrhoea, ETEC must adhere to the gut epithelial surfaces by means of colonization factors and produce enterotoxin(s). Since ETEC strains possess these common mechanisms, consideration has been given to the feasibility of incorporating the relevant antigens in a vaccine that would be effective against a range of ETEC serogroups.

**Colonization factors.** The best characterized human colonization factor antigens (CFAs) are CFA/I, CFA/II, and CFA/IV (formerly called PCF8775). CFA/I is a single fimbrial antigen, whereas CFA/II and CFA/IV are both antigen complexes. *E. coli* strains that produce CFA/II possess either the CS1 or the CS2 (coli-surface-associated) fimbrial antigens and a fibrillar antigen CS3, or may possess CS3 alone. ETEC-producing CFA/IV have either the fimbrial antigens CS4 or CS5, as well as the antigen CS6, which is probably nonfimbrial. ETEC strains that produce only CS6 have also been identified. These factors are all encoded by plasmids that usually also encode enterotoxins (Table 2).

The CFA/I, CS1, CS2, and CS4 fimbriae are all rigid and rod-shaped with a diameter of 6–7 nm. The terminal amino acid sequences have been elucidated for residues 1–20 and are very similar.

A number of surveys have been carried out to determine the prevalence of these colonization factors in ETEC strains from different geographical areas. The reported combined prevalence of CFA/I, CFA/II, and CFA/IV varied from 29% to 79% of the ETEC isolated in a particular area. CFAs were mainly identified on ST/LT and ST-only strains, which is as expected since plasmids that code for colonization factors usually encode these enterotoxins.

<table>
<thead>
<tr>
<th>CFA*</th>
<th>Coi-surface-antigen combinations</th>
<th>Associated enterotoxin</th>
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<tr>
<td>CFA/I</td>
<td></td>
<td>ST or ST/LT</td>
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<tr>
<td>CFA/II</td>
<td>CS1 + CS3</td>
<td>ST/LT</td>
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<td></td>
<td>CS2 + CS3</td>
<td>ST/LT</td>
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<td></td>
<td>CS3</td>
<td>ST</td>
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<tr>
<td>CFA/III</td>
<td></td>
<td>ST or LT</td>
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<tr>
<td>CFA/IV</td>
<td>CS4 + CS6</td>
<td>ST</td>
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<tr>
<td></td>
<td>CS + CS6</td>
<td>ST or LT</td>
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<tr>
<td></td>
<td>CS6</td>
<td>ST</td>
</tr>
<tr>
<td>PCFO159:H4</td>
<td>ST/LT</td>
<td>ST</td>
</tr>
<tr>
<td>PCFO166</td>
<td>ST or ST/LT</td>
<td>ST or ST/LT</td>
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</table>

* CFA = colonization factor antigen. Other possible colonization factors include 334, 2230, PCFO148, INT407, CFA/VI. These have been reported but are not well characterized.

CS = coli-surface-associated antigen.

Because it has not proved possible to detect colonization factors on many ETEC strains, efforts have been made to identify new factors. The putative colonization factors CFA/III, PCFO159:H4, and PCFO166 are plasmid-encoded fimbrial antigens found, respectively, on *E. coli* serotypes O25:H16 and H--; O159:H4 and H20; and serogroups O71, O78, O98, and O166. The following colonization factors have also been described: 334 was reported on a strain of serotype O15.H11; 2230 on a strain of serotype O25.H16; and CFA/VI on a strain of serotype O9.H—. Their occurrence on other serotypes has not been examined. PCFO148 and INT407 are factors that occur on strains of the O148 or O27 serogroups, respectively (Table 2). The role of these various factors in the pathogenesis of diarrhoea has not been examined in animal experiments.

Studies of animals and volunteers have shown that ETEC strains that produce CFA/I, CFA/II, and CFA/IV, when given orally or intraintestinally induce protective immunity. This suggests that they should be included in vaccines. In passive protection systems in animals, anti-CFA antibodies protect against challenge with ETEC strains that express the homologous CFA. Furthermore, anti-CFA is synergistic with anti-LT in conferring protection against LT diarrhoea caused by ETEC. For rabbits, prior exposure to organisms that express CFA/I, or the different CS components of CFA/II or CFA/IV, confers significant protection against disease, intestinal colonization, or both following challenge with *E. coli* that carry the homologous CFA/CS factor. Such protection was not induced by colonization-factor-negative, toxin-negative mutants.

**Enterotoxins.** Studies of both humans and animals have shown that ETEC infection evokes significant antitoxic as well as antibacterial immune responses in the
intestine. Antitoxic immunity is directed only against LT; ST is a small polypeptide which is not immunogenic in its natural form. The anti-LT response is directed mainly against the B subunit of the molecule, which cross-reacts immunologically with the B subunit of cholera toxin.

Although STa (the form of ST produced by ETEC strains that infect humans) is not naturally immunogenic, it can give rise to neutralizing antibodies when coupled to a protein carrier. Such an approach is being used to develop ST antigens for possible use in vaccines. Both chemical coupling and recombinant DNA techniques have been used to link STa to a variety of carriers; however, chemical conjugates of STa to the B subunit of CT, bovine serum albumin, or STb (a form of ST produced by ETEC strains that infect piglets) have remained toxic. Several synthetic full-length, or shorter, STa peptides have been produced in an attempt to identify nontoxic antibody-binding STa epitopes. By replacing one or two of the cysteines in STa by alanine, a nontoxic STa has been produced which binds monoclonal antibodies that neutralize STa.

Based on these results, research is under way to produce ST-protein conjugates through genetic manipulation of bacteria. A synthetic oligonucleotide has been constructed and fused to the structural gene for the CT–BS in V. cholerae O1. This gene construct directs the expression of high concentrations of an STa–CT–BS fusion protein with substantially reduced residual toxicity. Immunization of experimental animals with this protein evoked detectable, but non-neutralizing anti-STa antibodies. Work is in progress to develop other nontoxic STa-fusion proteins, which, it is hoped, will stimulate production of STa-neutralizing antibodies.

Over the past few years recombinant antigens of STa with the B subunit of LT (LT–BS) (STa–LT–BS) have also been created. Portions of the native STa gene, as well as synthetic oligonucleotides that encode 18 or 19 amino acids of STa, have been inserted at different sites within the gene encoding LT–BS. By means of this approach, STa–LT–BS recombinant hybrids have been generated that are stable and show high-affinity binding to GM1-ganglioside (the receptor for LT), immunoreactivity with monoclonal antibodies that neutralize ST and LT, and the capacity to induce anti-LT and anti-ST serum responses in rabbits immunized with a partially purified fusion protein. In evaluating the potential of these recombinant STa–LT–BS proteins as oral immunogens for protection against ETEC diarrhoea, it will be necessary to ascertain whether they have any residual toxicity and to determine the best mode of delivery (either as components of a killed vaccine or as the products of live, attenuated bacterial vectors).

Determinants of Immunity to ETEC as observed in volunteer studies. Studies in the USA have shown that after an initial experimental infection with an ST/LT strain of ETEC, adult volunteers exhibited significant protection against re-challenge with the homologous strain, but not with a heterologous strain. In one set of investigations, protected volunteers excreted the challenge organism, but failed to develop diarrhoea. In contrast, volunteers who developed diarrhoea after ingesting an LT/ST strain of serotype O148:H28, and who manifested significant rises in LT antitoxin, were not significantly protected against subsequent challenge with an LT-only strain of a heterologous serotype (O25:NM). In another study, volunteers who were immunized with a single dose of an oral attenuated cholera vaccine (CVD 103-HgR) that stimulated strong cholera antitoxin responses were not protected when challenged 1 month later with an ST/LT ETEC strain. Although data from the field trial of the WC/BS cholera vaccine in Bangladesh suggest that anti-CT provides short-term protection against diarrhoea caused by LT-producing ETEC, this has not been observed in volunteer studies.

Other volunteer studies have indicated that protective immunity may be evoked by fimbrial colonization factor antigens. For example, volunteers immunized once with an attenuated strain of ETEC (5 × 10¹⁰ live bacteria) that expressed CS1 and CS3 fimbriae, but lacked LT and ST genes, exhibited marked rises in sIgA anti-CS1/CS3 antibody in their jejunal fluids. When the vaccinees were challenged with an ST/LT strain of a heterologous O:H serotype that also expressed CS1 and CS3, highly significant protection against diarrhoea was observed. The vaccinees demonstrated significantly diminished colonization of the proximal small intestine (as assessed by counts in duodenal fluid specimens) compared with controls, which suggests that the vaccine induced immune mechanisms in the small bowel that interfered with mucosal colonization.

In related studies, passive administration of a cow’s milk immunoglobulin concentrate that contained high levels of antibody to CFA/I and LT (as well as to other ETEC antigens) provided 100% protection against challenge with ETEC strain H10407 (078:H11, ST/LT, CFA/I) in a randomized, placebo-controlled, double-blind trial in volunteers. Although this approach would not be of practical public health value, the demonstration of the efficacy of orally administered anti-ETEC antibodies provides further encouragement to seek ways of achieving active immunity with oral vaccines.

Candidate vaccines

Inactivated whole-cell purified antigen vaccine. Research is being carried out on an oral vaccine against ETEC.
that would consist of killed \( E. coli \) bacteria representing the major O-groups associated with ST/LT production and expressing the key CFAs in immunogenic form, combined with the B subunit of LT or CT, or a nontoxic STa-B-subunit conjugate. The treatment of bacteria with mild formalin causes complete killing, but retention of 50–100% of the antigenicity of the different colonization factors. The CFAs of inactivated organisms are stable at 4 °C for at least 8 months and when incubated in acidic gastric juice. An evaluation of the safety and immunogenicity of this vaccine in volunteers is planned for the near future.

**Colicin-E\(_2\)-treated whole-cell vaccine.** A novel method of killing ETEC bacteria has been developed that does not damage the protein antigens associated with them. This involves treatment with colicin E\(_2\), an endonuclease that enters the cell by means of receptors on sensitive strains of \( E. coli \). Killed bacteria exhibit no change in their antigenicity or concentrations of LT enterotoxin, CFA/I, or flagellar antigens. Colicin-E\(_2\)-treated ETEC preparations have been tested as candidate vaccines in animals and volunteers.

Two oral doses of \( 3 \times 10^{10} \) freshly prepared colicin-E\(_2\)-killed \( E. coli \) strain H10407 given 1 month apart induced both serum IgG and intestinal IgA antibody responses to LT enterotoxin and CFA/I in 29 of 32 adult volunteers. None of the 22 placebo-immunized controls showed increased levels of these antibodies. To evaluate the protection induced by colicin-E\(_2\)-killed organisms, groups of 9 or 10 subjects, consisting of approximately equal numbers of vaccinees and placebo-treated controls, were challenged with virulent ETEC strains 6–8 weeks after immunization. The vaccinees showed 75% protection against diarrhoea when challenged with either homologous or heterologous strains of ETEC. The heterologous challenge was with a strain that differed in both serotype and CFA type, which suggests that other protective antigens may be important. In another challenge study, protection was demonstrated 6–8 months after vaccination. Methods of preserving colicin-E\(_2\)-killed \( E. coli \) have not yet been developed.

**Avirulent salmonellae as carriers for ETEC antigens.** With strain Ty21a as the vector, a potential multivalent live oral vaccine that expresses LT–BS has been constructed. The \( S. typhi \) derivative (strain SE12) induced a significant serum anti-LT response when injected parenterally into mice or guinea-pigs. The potential of this strain as a live oral vaccine for ETEC has not been tested in animals owing to the limited range of hosts for \( S. typhi \).

More recently, an avirulent \( aroA^- \) mutant mouse strain of \( S. dublin \) (SL1438) has been used as the vector strain. This strain produces an infection in orally inoculated mice analogous to that produced in humans by Ty21a. The strain was transformed with a plasmid that carried genes for the production of LT–BS; the derivative strain produced LT–BS and induced high levels of serum IgG antitoxin and intestinal sIgA antitoxin in orally inoculated mice. These mice also developed progressively increasing mucosal and serum antibody responses to the LPS of the vaccine strain. Only one strain with a single \( aroA \) mutation has been able to colonize significantly, invade, and persist in tissues.

In accordance with the latter results, recent studies in humans have shown that recipients of \( aroA^- \), \( purA^- \) \( S. typhi \) mutants develop only low serum antibody responses to the O-polysaccharide of the vaccine strain. These observations suggest that the \( purA \) defect, which creates a requirement for exogenous adenine, reduces the efficacy of the attenuated *Salmonella* species as a live oral vaccine, and would probably limit the effectiveness of salmonellae as vectors for heterologous antigens. As an alternative to the \( aroA \) single-deletion mutant, strains with two mutations in the \( aroA \) pathway should be considered as potential vectors for ETEC antigens. Two mutations are considered desirable to ensure that the strain does not revert to virulence.

An important consideration concerning the use of *Salmonella* species or other live bacteria as vectors for heterologous antigens is whether they remain effective when used repeatedly, especially for the delivery of unrelated antigens. It is possible that pre-existing immunity to the vector that arises from its initial use, or from natural exposure, may limit the immunological response to the heterologous antigen produced by it. Preliminary evidence indicates that mice immunized with a vector (Salmonella SL1438) and then re-immunized with the same vector which expresses a heterologous antigen (LT–BS) develop lower serum IgG and mucosal IgA anti-LT–BS responses than mice not previously immunized with the vector.

**Research recommendations**

*Cholera vaccines*

**Killed oral whole-cell vaccine.** Further research on cholera WC vaccine should seek to increase its efficacy and duration of protection, especially against disease caused by *V. cholerae* O1 biotype El Tor, and in young children. Approaches to improving the efficacy include: incorporating in the vaccine an El Tor strain from the current pandemic; increasing the quantity of bacteria per dose; ensuring full expression of TCP antigen in the vaccine; and including strains that
produce or hyper-produce the B subunit. A practical vaccine formulation, possibly a capsule or soluble tablet that is stable at ambient temperatures, should be developed.

**Live oral cholera vaccine.** The objective should be to develop a genetically defined live vaccine strain that colonizes the intestine efficiently, is nonreactogenic, and contains a stable genetic marker to distinguish it from wild-type strains. Prior to field trials, candidate strains should be studied to determine the extent and duration of their efficacy in adults and their safety and immunogenicity in adults and children. The efficacy of killed whole-cell vaccine and live strains should be compared in volunteers. The transmissibility to non-vaccinated persons and dispersion into the environment of any candidate live oral cholera vaccine should also be evaluated.

Further research is required to define the mechanism by which nontoxigenic mutants of *V. cholerae* O1 cause diarrhoea, in order to develop candidate vaccine strains that lack this capacity but retain the other qualities required for efficient induction of protective immunity.

**Alternative designs for cholera vaccine trials.** Research should be carried out to develop novel designs and new geographical sites for future trials of cholera vaccines. If possible, the designs should be more efficient than those for traditional large-scale prospective trials. Research designs are also required that address specific issues, such as vaccine efficacy in epidemic and family settings, and in young children.

**Cost-effectiveness evaluation of selected vaccines.** Cost-effectiveness analyses should be carried out on vaccines that have been evaluated by field trial and found to be at least moderately effective. The analysis should be based on the conditions in the country where the trial was performed, but may also attempt to predict cost-effectiveness in other settings.

**Enterotoxigenic E. coli vaccines**

**Potential vaccine antigens.** 

(a) **Colonization factor antigens.** The prevalence of specific colonization factor antigens (CFAs) among ETEC strains isolated from young children with acute diarrhoea in developing countries, especially in Africa, needs to be better defined. The following CFAs should be identified: CFA/I, CFA/II, CFA/III, CFA/IV, PCF O159:H4, and PCF O166. The prevalence of CFAs should be determined in prospective, population-based epidemiological studies. Research to identify and characterize new CFAs should also be continued.

To facilitate epidemiological studies, standardized reagents (e.g., polyclonal or monoclonal antibodies, and DNA probes) should be developed for the identification of CFAs.

(b) **STa-toxoids.** Research to develop safe and immunogenic STa-toxoids, based either on STa-protein conjugates or chimeric proteins (e.g., STa–LT–BS) synthesized by genetically manipulated bacteria, should continue. Candidate conjugates or chimeric proteins should be evaluated for residual toxicity and immunogenicity. Using such antigens, the potential protective role of anti-ST should be evaluated in animals and, if possible, volunteers. Practical approaches to the inclusion of safe and protective STa toxoids in candidate oral ETEC vaccines should be explored.

(c) **Possible common protective antigens.** The possibility that antigens may evoke cross-protection against ETEC strains that differ in serogroup, CFA type, and production of LT requires further study in volunteers and animal models. If such cross-protection is confirmed, its extent should be defined and an attempt made to identify and characterize the responsible antigen(s). Killed whole-cell vaccines should be prepared by methods that are known to preserve such antigens in immunogenic form.

**Killed oral whole-cell vaccines.** Research to develop a safe and effective killed oral WC vaccine for ETEC diarrhoea should continue. Bacteria that represent the most important ETEC serogroups and produce the most prevalent CFAs should be included. The benefit of including a nontoxic STa-protein conjugate (if available) or LT–BS, or both, should be evaluated. For antigens that are presumed to be important, particular care should be taken to use methods of killing and preserving bacteria that retain their immunogenic activity. Candidate vaccines should be tested for safety, immunogenicity, and efficacy in adult volunteers. Those that prove to be promising should also be evaluated for safety and immunogenicity in children.

For oral WC vaccine based on colicin-E2-treated bacteria, further evidence of protective efficacy against heterologous challenge strains should be sought and the range of such protection determined in volunteer studies. If this approach proves effective, the efficacy of other vaccine strains (different serogroups and CFA types) should be evaluated. This may require a search for appropriate strains that are sensitive to colicin E2.

**Live, attenuated ETEC vaccines.** Efforts are needed to develop and evaluate the safety and immunogenicity of ETEC strains that express selected CFAs and possibly LT–BS or an STa–LT–BS conjugate. The safety and
efficacy of such strains given singly and in combination should be tested in volunteers.

**Research relevant to both cholera and ETEC vaccines**

*Preservation of live bacterial vaccines.* Research is required to develop methods of preserving live bacterial vaccines that ensure maximum viability and uniform bacterial characteristics, particularly as regards antigenic composition, with a minimum of variation between production lots. This may require an evaluation of preservation methods other than lyophilization.

*In vitro correlates of immunity.* Practical measures for predicting the protective value of candidate vaccines or immunizing regimens are required. In some instances, serum antibody responses may prove satisfactory as proxy measures of protective mucosal immune responses; in others, a simple and accurate measure of the local immune response will probably be required.

*Improved bacterial vectors.* Research to identify improved bacterial vectors for the delivery of selected antigens of *V. cholerae O1* and ETEC should continue. Ideally, vectors should evoke a vigorous mucosal immune response after a single dose, without significant side-effects; this will no doubt require that the vector colonizes or penetrates the small bowel mucosa, at least transiently. The Ty21a strain of *S. typhi* should be regarded as a prototype vector; however, further research is needed to optimize the expression of one or several foreign antigens by this vector.

*Mucosal adjuvants.* The development of efficient killed oral vaccines for cholera and ETEC diarrhoea, as well as other oral or topical vaccines, may require the use of adjuvants that enhance the mucosal (sIgA) immune response. Research is required to identify such adjuvants and optimize their efficacy.