Live attenuated oral cholera vaccines

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Live, orally administered, attenuated vaccine strains of *Vibrio cholerae* have many theoretical advantages over killed vaccines. A single oral inoculation could result in intestinal colonization and rapid immune responses, obviating the need for repetitive dosing. Live *V. cholerae* organisms can also respond to the intestinal environment and immunological exposure to *in vivo* expressed bacterial products, which could result in improved immunological protection against wild-type *V. cholerae* infection. The concern remains that live oral cholera vaccines may be less effective among partially immune individuals in cholera endemic areas as pre-existing antibodies can inhibit live organisms and decrease colonization of the gut. A number of live oral cholera vaccines have been developed to protect against cholera caused by the classical and El Tor serotypes of *V. cholerae* O1, including CVD 103-HgR, Peru-15 and *V. cholerae* 638. A number of live oral cholera vaccines have also been similarly developed to protect against cholera caused by *V. cholerae* O139, including CVD 112 and Bengal-15. Live, orally administered, attenuated cholera vaccines are in various stages of development and evaluation.

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Vibrio cholerae is a Gram-negative bacillus that causes cholera, a severe, dehydrating diarrheal illness of humans. *V. cholerae* serogroup O1 and O139 organisms produce cholera toxin and are the causative agents of epidemic cholera. There are two biotypes of *V. cholerae* O1 organisms, classical and El Tor, and based on differences in antigenic determinants of the lipopolysaccharide (LPS) O antigen, O1 organisms can be further subclassified into serotypes Inaba and Ogawa. *V. cholerae* O139 was first detected in 1992 and is thought to have arisen from O1 El Tor *V. cholerae*, but has acquired the ability to produce a capsule [1,2]. After its emergence, O139 *V. cholerae* predominated as the cause of clinical cholera in areas of Asia, but was eventually largely replaced by El Tor *V. cholerae* O1, although *V. cholerae* O139 still accounts for a minority of cases in Asia. The El Tor biotype of *V. cholerae* O1 has replaced the classical biotype throughout the world, although hybrid strains with characteristics of both biotypes have been characterized recently [3].

Epidemiology & illness

Many countries do not report the number of their cholera cases accurately. Exact figures are, therefore, unknown; however, it has been estimated that each year approximately 5–7 million cases of cholera occur worldwide, resulting in over 100,000 deaths [4]. Cholera is transmitted by contaminated food or water, and usually results from the ingestion of a large inoculum of organisms ($10^{8–11}$; $10^{4–6}$ in hypochlorhydric individuals) [5]. As infected individuals may excrete as many as 10^8 *V. cholerae* organisms per ml of stool and produce up to 10–20 l of diarrhea per day, individuals with cholera may excrete as many as $10^{12–13}$ *V. cholerae* organisms per day in their stool resulting in ongoing contamination of food or water supplies which leads to rapid dissemination of *V. cholerae* in a population. Recent data suggest that *V. cholerae* excreted from humans expresses a phenotype that confers hyper-infectivity, which may also contribute to epidemic spread [6,7].

Following ingestion of *V. cholerae* organisms, a spectrum of disease may result. Disease severity relates to inoculum size, presence/absence of

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pre-existing immunity, and blood group, among other factors [8–11]. Asymptomatic stool passage or mild disease is most common in individuals who reside in endemic areas and who have complete or partial pre-existing immunity. Nonimmune individuals may develop disease ranging from moderate diarrhea to voluminous diarrhea that kills within hours of onset (cholera gravis). During epidemics, death rates for untreated/poorly treated cholera are often 20–50% (with death rates of 70–100% for individuals with cholera gravis). Appropriate rehydration therapy decreases mortality due to cholera to less than 1% [12]. Cholera may rarely present as travellers' diarrhea, although the risk of travel-related cholera is low (estimated at 0.2 per 100,000 European and North American travellers) [13].

Pathogenesis

The major virulence factor for all toxigenic strains of *V. cholerae* is cholera toxin. Cholera toxin consists of an enzymatically active A subunit noncovalently associated with five B subunits that mediate binding to eukaryotic cells. After internalization, the A subunit elevates cyclic AMP (cAMP), leading to secretory diarrhea [14–16]. Another virulence factor of *V. cholerae* is toxin-coregulated pilus (TCP), a colonization factor essential for colonization and virulence in both animal models and human volunteers [17,18]. TcpA is the major structural protein of TCP. TcpA from El Tor and classical strains are approximately 80% homologous at the protein level, but monoclonal antibodies have demonstrated important epitope differences between these proteins [19–22]. TcpA from El Tor and O139 strains are identical [21].

The genes for cholera toxin (*ctxAB*) are encoded on a larger cholera toxin genetic element, which has 17 bp end-repeat sequences at either end [23]. These end-repeat sequences are homologous with an *attRS1* site on the *V. cholerae* chromosome, which allows this genetic element to integrate into the *V. cholerae* chromosome. The cholera toxin's genetic element is encoded on a filamentous bacteriophage (designated CTXϕ), which utilizes TCP as its receptor [24]. Expression of the genes in the cholera toxin's genetic element and TCP operon is regulated by two pairs of membrane-localized transcription factors, ToxR/ToxS and TcpP/TcpH [25–29].

Immunity after natural cholera infection

Infection with *V. cholerae* produces long-lasting protective immunity against subsequent disease [8,9,30,31]. Among volunteers in nonendemic settings, infection with classical biotype *V. cholerae* O1 provided 100% protection against subsequent challenge with a classical strain and infection with El Tor organisms provided 90% protection from subsequent challenge with the homologous biotype. This protection lasted at least 3 years [32]. In an endemic area, El Tor cholera reduced the risk of clinically apparent re-infection by 90% over the next few years [33]. Despite this evidence of long-lasting immunity, the nature of immune responses that are protective following *V. cholerae* infection is not understood adequately. Serum antibody to cholera toxin increases after cholera, but this response does not protect from subsequent colonization or disease [33]. Furthermore, infection with El Tor *V. cholerae* O1 does not protect against infection with *V. cholerae* O139, even though both strains produce identical cholera toxins [1]. Serum vibriocidal antibodies are perhaps the best characterized of the antibacterial immune responses to *V. cholerae*. Vibriocidal antibodies produce bactericidal activity against *V. cholerae* in the presence of complement. In sero-epidemiological studies in cholera endemic areas, the vibriocidal antibody titer increases with age and correlates with protection from disease. In Bangladesh, for example, vibriocidal antibodies are present in 40–80% of individuals 10–15 years of age [33–35]. In one cross-sectional study, for every twofold rise in the baseline vibriocidal titer there was a 44% decrease in the attack rate of classical biotype cholera. A prospective study in Bangladesh in 1980–1982 also demonstrated that El Tor *V. cholerae* O1 infection and disease were significantly more common in people with lower vibriocidal titers [36]. Despite the correlation between vibriocidal antibody levels and protection against diarrhea in household contacts of individuals with cholera, some contacts with high baseline vibriocidal levels still developed cholera [37]. This observation, the uncertain role of a complement-fixing serum response for protection at a mucosal surface, and the fact that *V. cholerae* does not disrupt the intestinal epithelium, suggests that the serum vibriocidal response may be a surrogate marker for as yet unidentified mucosal immune responses. A large component of the vibriocidal response can be adsorbed by *V. cholerae* LPS [38]; however, there is no correlation between serum anti-LPS immunoglobulin (Ig)G antibody and protection from cholera, suggesting that immune responses to other antigens may also be important.

Mucosal immunity to *Vibrio cholerae*

Intestinal infections, such as cholera, usually result in local immune responses comprised primarily of secretory IgA (sIgA). Surrogate markers of mucosal immune response are often measured since proteolytic degradation of antibodies in feces complicates direct measurement of fecal sIgA and intestinal lavage and endoscopy are invasive. The vibriocidal antibody may be one such surrogate marker of *V. cholerae* infection. Another surrogate marker is the antibody-secreting cell (ASC) assay, which detects activated lymphocytes migrating transiently in peripheral blood after mucosal infection. Levels of these activated lymphocytes are highest approximately 1 week after intestinal infection and these lymphocytes then rehome to intestinal mucosal surfaces [39]. In the ASC assay, circulating lymphocytes are harvested and specific immune responses are detected using an enzyme-linked immunosorbant spot (ELISPOT) procedure [40,41]. In North American volunteers infected with *V. cholerae*, anticholeratoxin (CT) and anti-LPS IgA ASC responses were detected in 50 and 83% of recipients, respectively [42]. If these volunteers were rechallenged within 30 days of primary infection, reduced or absent ASC responses occurred. If rechallenge occurred 6 months after primary infection, increased anti-CT and anti-LPS IgA ASC responses were again detectable. If rechallenge was done within 6 months of primary challenge, cholera did not develop. IgA ASC responses to LPS and CT,

therefore, correlate with protection from disease, although some volunteers were protected without ever developing detectable responses to these antigens.

Another way to measure mucosal immune responses is the antibody in lymphocyte supernatant (ALS) assay, an extension of the ASC assay [43]. In the ALS assay, mucosal lymphocytes that are circulating transiently are harvested and cultured *in vitro* for 48 h. These cells secrete antibodies into the supernatant during culturing and antigen-specific antibodies may then be detected in the supernatant by standard enzyme-linked immunosorbant assay (ELISA). An advantage of the ALS assay is the ability to freeze the lymphocyte supernatants for detection of immune responses at a later point. In a study of 30 adults with cholera in Bangladesh, there were significant increases of anti-LPS and anti-CT IgA antibodies in supernatants of lymphocytes cultured 7 days after the onset of cholera [43]. Significant increases in anti-TcpA ASC and ALS responses have also been demonstrated in patients infected with El Tor *V. cholerae* O1 and *V. cholerae* O139 infection in Bangladesh [44].

Efficacy of current vaccines

Several cholera vaccines have been developed over the past 40 years [13,45]. None of these is yet able to reproduce the level of immunity seen following natural infection. A parenteral whole-cell *V. cholerae* vaccine provided low levels of protection for short periods of time and is no longer available. A killed oral cholera vaccine was developed in the 1980s, consisting of killed whole *V. cholerae* mixed with a purified B subunit of cholera toxin, CtxB (WC-BS) [46]. The original WC-BS vaccine was eventually replaced with a version containing recombinantly produced CtxB. This newer vaccine is referred to as rBS-WC. This oral killed vaccine, in two or three doses, stimulated vibriocidal antibody responses, serum antibody responses to LPS and the B subunit of CT, and antibody-secreting cell responses to CT similar to those found in natural cholera [39,46]. However, there was a rapid decline in these immune responses by 1 year following vaccination [47]. Repeat vaccination with WC-BS 10 months after initial vaccination led to anamnestic serum and mucosal immune responses, suggesting that WC-BS primed immunological memory [48]. In adults in the USA vaccinated with WC-BS, subsequent challenge with wild-type cholera demonstrated 64% protective efficacy [49]. In Bangladesh, protective efficacy of 62% was demonstrated [50]. However, protective efficacy was only 38% in children 2–5 years of age and efficacy declined in all groups by the last 4 months of a 1-year follow-up period. In a volunteer study in Peru, a three-dose rBS-WC vaccination regimen over 10 months produced a protective efficacy of 61% over a 2-year follow-up period. In a recent field trial in an area of Mozambique with yearly cholera epidemics, receipt of one or more doses of the rBS-WC vaccine was associated with 78% protection during the 6 months following vaccination. The vaccine was equally effective in children younger than 5 years of age and in older individuals. Furthermore, recent re-analysis of protection afforded by WC-BS in Bangladesh in the 1980s suggests that rBS-WC conferred herd protection to neighboring nonvaccinated individuals, extending the efficacy of the vaccine from 62 to over 90% at the population level [51], strengthening the possible public-health utility of cholera vaccination [52]. A killed oral cholera vaccine has also been developed in Vietnam and a two-dose regimen of this vaccine against *V. cholerae* O1 in a field trial in Vietnam conferred over 60% protection against El Tor cholera, in both children and adults [53]. Overall, however, because killed cholera vaccines require repetitive dosing and because protection against cholera following immunization with killed whole-cell vaccines is often less prominent and of shorter duration than that following natural cholera, investigators have also developed live, oral, attenuated strains of *V. cholerae* as cholera vaccines.

Live-attenuated oral cholera vaccines

Live, orally administered, attenuated vaccine strains of *V. cholerae* have many theoretical advantages over killed vaccines. A single oral inoculation could result in intestinal colonization, obviating the need for repetitive dosing. Live *V. cholerae* organisms can also respond to the intestinal environment and immunological exposure to *in vivo*-expressed bacterial products could result in better immunological protection against wild-type *V. cholerae* infection.

Many live attenuated *V. cholerae* strains have been produced. One of the first was Texas Star™, a *V. cholerae* O1 El Tor Ogawa strain that was attenuated by nitrosoguanidine-mediated mutagenesis [54]. Although attenuated and immunogenic, Texas Star was found to be too reactogenic and the vaccine was abandoned [55,56]. Subsequently, attenuated *V. cholerae* vaccines have been developed using recombinant molecular biology techniques [13,45]. Many of these initial recombinantly produced vaccines were immunogenic, but also too reactogenic, with adverse events, such as diarrhea, abdominal cramps, fever and anorexia occurring in over half of all recipients [57,58]. Attempts were then made to further lessen reactogenicity by decreasing the ability of attenuated *V. cholerae* vaccine strains to colonize the intestinal surface and by further attenuating vaccine strains by removing various purported toxic elements; many of these newly attenuated strains were either poorly immunogenic or still too reactogenic [18,57,59,60].

CVD 103-HgR

A δ*ctxA* derivative of classical strain *V. cholerae* 569B (CVD 103) was found to be immunogenic and minimally reactogenic [61]. To facilitate identification of the vaccine strain, a mercury-resistance gene (*HgR*) was inserted into the hemolysis A gene (*HlyA*) to generate CVD 103-HgR [61–63]. Intestinal secretion of CVD 103-HgR was markedly diminished (5–25%) compared with the parent strain CVD 103 (>80%) [56]. CVD 103-HgR has been well studied and found to be both safe and immunogenic in volunteers [61,64–74]. Following oral administration, the vaccine has not been isolated from the environment. In North American volunteers, a single dose (5×10^8 colony forming units [cfu]) resulted in

vibriocidal antibody responses in 92% of individuals and these responses were three- to fivefold higher than those seen in individuals immunized with three doses of oral killed vaccine [61]. In volunteer studies, CVD 103-HgR was also associated with significant protection against cholera; giving 94–100% protection against severe and moderate diarrhea by wild-type *V. cholerae* O1 [56,61,67,74]. Overall protection from diarrhea caused by classical *V. cholerae* was 82–100%, and 62–80% for El Tor *V. cholerae* [56,67,74,75]. Protection was evident within 8 days of vaccination and lasted for at least 6 months, the last time measured [42,67,70,74,76].

Vaccination with a 5×10^8 cfu dose resulted in lower seroconversion rates in lower socioeconomic groups in initial field trials in resource poor areas (25% of Thai adult men and 16% of Indonesian children) [61,68,69]. It was hypothesized that this lower response rate may have been related to pre-existing immunity inhibiting intestinal colonization by the vaccine strain or effects of small bowel overgrowth or concomitant helminth infection [42,75–77]. In lower socioeconomic settings, a dose of 5×10^9 cfu was subsequently demonstrated to be required in order to elicit higher rates of seroconversion (75–85%) [68,69,72]. In a large, randomized, placebo-controlled, double-blinded field trial of CVD 103-HgR involving over 65,000 individuals in Indonesia, the vaccine was well tolerated and immunogenic, resulting in vibriocidal responses in 64–70% of vaccines. The incidence of cholera was lower than expected during the study period, especially in the 6 months following vaccination, markedly impeding the ability to assess the short-term efficacy of the vaccine. In this study, CVD 103-HgR had a protective efficacy of 14% [78], although more recently, on a Micronesian island, as part of a response plan, the vaccine contributed to approximately 80% protection during a focused cholera outbreak [79]. Although approved for use in a number of countries, CVD 103-HgR is currently not being commercially manufactured. A bivalent vaccine constituting CVD 103-HgR (classical Inaba) and CVD 111 (an El Tor Ogawa phenotype attenuated O1 strain) has also been developed to broaden the range of protection; however, this combination vaccine has only been evaluated in a limited volunteer study [80].

Peru-15

A live, oral, attenuated *V. cholerae* O1 El Tor Inaba C6709 cholera vaccine strain was constructed in the 1990s by deleting the entire CT genetic element and *attRS1* sites, inserting the gene for the B subunit of CT into recA, and screening for a non motile derivative, to derive the vaccine strain Peru-15 [81]. Peru-15 is well tolerated and immunogenic (90–100% vibriocidal seroconversion rate) as a single dose in volunteer studies [81,82]. In an original small pilot study, 2×10^8 cfu of freshly harvested Peru-15 was administered to 11 volunteers [81]. The vaccine was well tolerated; no vaccinee developed diarrhea and ten out of 11 recipients developed vibriocidal responses [81]. Five vaccinees and five control volunteers were challenged with wild-type *V. cholerae* O1 1 month after vaccination/enrollment; four out of the five controls and two out of the five vacinees developed diarrhea [81]. In another immunogenicity and safety pilot study, 12 inpatient volunteers received freshly harvested Peru-15 vaccine (either 10^7 or 10^9 cfu) and 50 outpatient volunteers received freeze-dried vaccine (either 10^8 or 10^9 cfu) or placebo in a three-cell, double-masked, placebo-controlled trial [82]. Peru-15 was well tolerated at all doses and stimulated high levels of vibriocidal antibodies in most inpatient volunteers and all outpatient volunteers [82]. Approximately 60% of the volunteers excreted the vaccine in their feces; however, fecal excretion did not correlate with immunological responses [82].

In US volunteers, a larger randomized, double-blind, placebo-controlled challenge study involving 59 enrollees receiving either 2×10^8 cfu of reconstituted, lyophilized Peru-15 vaccine diluted in buffer or placebo (buffer alone) demonstrated a vibriocidal response rate in vaccinees of 98% and a protective efficacy in vaccineees of 93% against diarrhea caused by wild-type *V. cholerae* O1 El Tor challenge 3 months after vaccination [83].

Based on these studies, Peru-15 has been studied recently in a staged double-blind, randomized, placebo-controlled Phase I/II field trial in Bangladesh (in three decreasing age-group stages: adults 18–45 years of age, children 2–5 years of age and children 9–23 months of age) [84]. Among adults, Peru-15 ($\sim 2 \times 10^8$ cfu) or placebo (buffer only) was studied for safety, immunogenicity and excretion in Phase I (in-patient) and Phase II (outpatient) studies [84]. The vaccine was safe and well tolerated. *V. cholerae* was isolated from the stool of only one volunteer and was found to be genetically identical to the vaccine strain. Vibriocidal antibody responses were seen in 30 out of 40 (75%) vaccine recipients and in three out of 30 (10%) placebo recipients [84]. Peripheral-blood IgA and IgM ASC responses to *V. cholerae* lipopolysaccharide were seen in 78–88% of vaccine recipients and serum anti-LPS-specific IgA antibodies were detected in 88% of vaccine recipients [84]. Studies in toddlers and infants demonstrated 84 and 70% vibriocidal response rates, respectively, with an overall vibriocidal response rate of 77% in children less than 5 years of age [85]. Detailed analysis of responses in children and infants is ongoing.

V. cholerae *vaccine 638*

In the 1990s, a live, oral, attenuated El Tor Ogawa cholera vaccine was constructed by deleting the entire CT genetic element, CTXϕ, from *V. cholerae* O1 El Tor Ogawa strain C7258 and by insertion of the *Clostridium thermocellum* endoglucanase A gene (*celA*) into the hemagglutinin/protease *hapA* gene. The *celA::hapA* modification does not affect immunogenicity or colonization of the vaccine strain in animals [86]; but does permit rapid identification of the strain through assessment of β-(1–4) endoglucanase activity encoded by *celA* in carboxymethylcellulose indicator agar stained with Congo red, in which the vaccine strain appears as a red colony [86]. In a double-blind, placebo-controlled study among healthy adult volunteers in Cuba, *V. cholerae* 638 was

examined preliminarily for safety and immunogenicity [86]. No significant adverse reactions were observed in volunteers immunized with strain 638. Four out of the 42 volunteers who ingested strain 638 and one out of 14 who received placebo had loose stools. The vaccine strain was recoverable from the stools of 37 out of 42 volunteers. Among vacinees receiving 4×10^7–2×10^9 cfu of *V. cholerae* 638, serum vibriocidal antibody responses occurred in 71–82% and anti-Ogawa LPS IgA ASC responses occurred in 85–100% of patients [86]. *V. cholerae* 638 was then evaluated for protective efficacy in a randomized, double-blind, placebo-controlled trial in volunteers in Cuba [87]. Among 24 of the vaccinees, 96% developed vibriocidal responses, and 50% developed a anti-LPS IgA response in serum [87]. At 1 month after vaccination, 12 vaccinees and nine placebo recipients were challenged with 7×10^5 cfu of virulent strain *V. cholerae* 3008 [87]. None of the 12 vaccinees, but seven volunteers from the placebo group, had diarrhea; two of the latter developed severe cholera [87].

Other live oral cholera vaccines

Other live oral attenuated *V. cholerae* O1 strains have been developed, including VA1.3 in India [88] and IEM108 in China [89]. These strains have been immunogenic in animals, but no human studies have yet been reported.

V. cholerae O139

Since 1992, *V. cholerae* O139 has been reported in 11 Asian countries, accounting for a minority of cholera cases in these locales. However, cholera caused by *V. cholerae* O139 is clinically indistinguishable from cholera caused by *V. cholerae* O1 disease and when *V. cholerae* O139 is introduced into a previously unexposed population, it may cause severe epidemics of cholera. Accordingly, a number of cholera vaccines targeting O139 have been developed, including a number of bivalent vaccines that induce immune responses against both *V. cholerae* O1 and O139. The presence of a capsule around O139 organisms has historically complicated the ability to perform and standardize vibriocidal assays, although a modified method has been optimized recently using a less encapsulated, naturally occurring clinical isolate of *V. cholerae* O139 [90]. Bivalent cholera vaccines that have been developed include a bivalent modified killed oral whole-cell B-subunit vaccine (B-O1/O139 WC), containing formalin-killed *V. cholerae* O139 and O1 organisms. The B-O1/O139 WC vaccine has been found to be safe and immunogenic among Swedish volunteers [48]. Out of the 12 volunteers, ten (83%) developed vibriocidal responses against *V. cholerae* O1 and eight (67%) developed vibriocidal responses against *V. cholerae* O139. The frequencies and magnitudes of the serological responses to the B-subunit and O1 WC components were similar to those induced by the rBS-WC vaccine [48]. Among 30 Bangladeshi volunteers, B-O1/O139 induced vibriocidal responses in 50% [90]. Similarly, a bivalent oral killed cholera vaccine, biv-WC, administered in a two-dose regimen, has been produced in Vietnam. The vaccine has been safe and immunogenic in adults and children. Of the 70 adults, approximately 40% developed anti-O139 vibriocidal responses; and out of 25 children, approximately 70% developed anti-O139 vibriocidal responses [91].

Live cholera vaccines targeting O139 have also been developed. CVD 112 is a derivative of the *V. cholerae* O139 strain AI1837 and contains deletions in genes for CT A subunit (*ctxA*), zonula occludens toxin, accessory cholera enterotoxin and core-encoded pilin, which are within the CTXϕ [24,92]. A total of 12 volunteers have received either 10^6 or 10^8 cfu of CVD 112. No subject who received the 10^6 dose had diarrhea or other severe symptoms after vaccination, and three out of six vaccinees developed mild diarrhea after receiving the higher dose [92,93]. After 5 weeks following vaccination, eight vaccinees and 15 unvaccinated control subjects were challenged with 10^6 cfu of wild-type *V. cholerae* O139 AI1837. One vaccinee (13%) and 12 control subjects (80%) developed diarrhea after challenge (protective efficacy of 84%) [92,93]. In another evaluation, 83–92% of the 24 recipients of 10^8 cfu of CVD 112 developed vibriocidal responses, depending on the target O139 strain used in the vibriocidal assay [94].

An additional attenuated *V. cholerae* O139 vaccine has been made by deleting multiple copies of the cholera-toxin genetic element from the virulent *V. cholerae* O139 strain MO10 and inserting a copy of *ctxB* to *recA* to make Bengal-3 [95]. A stable spontaneous nonmotile derivative of Bengal-3 was isolated and designated Bengal-15. Bengal-15 was evaluated as an oral single-dose cholera vaccine candidate in four volunteers [96]. Bengal-15 produced fewer symptoms and was nearly as immunogenic as MO10 [96]. Bengal-15 was then administered to ten volunteers at a dose of 10^8 cfu. No volunteers developed diarrhea. After 1 month following vaccination, seven vaccinees, buffer recipients and three nonimmunized subjects were challenged with 5×10^6 cfu of *V. cholerae* O139. One out of the seven vaccinees had mild diarrhea; and five out of six controls had cholera-like diarrhea (protective efficacy of 83%) [96].

Use of attenuated strains as vaccine vectors to induce mucosal immune responses to heterologous antigens

Development of live attenuated *V. cholerae* vaccines that are safe and immunogenic in humans has permitted the evaluation of live attenuated *V. cholerae* organisms as vaccine vectors capable of expressing heterologous antigens at mucosal surfaces. *V. cholerae* vaccine strains have been developed that express a number of heterologous antigens, including antigens from enterohemorrhagic *Escherichia coli* (EHEC), *Shigella dysenteriae* type 1, *Clostridium difficile*, *Entamoeba histolytica* and *Coccidioides immitis*; such strains have been shown to be immunogenic and induce protective immune responses in animal models [97–105]. *V. cholerae* vaccine strains have also been engineered that secrete immunoadjuvants *in vivo*, such as the nontoxic mutant of *E. coli* heat-labile enterotoxin, LTR192G, and such expression has been shown to boost immune responses after oral administration [106]. Additional

modifications include improved mechanisms for attaining high-level expression of heterologous antigens in *V. cholerae* vaccine strains using a balanced lethal plasmid expression system [107], the use of optimal promoters to drive the expression of heterologous antigens *in vivo* [108] and the use of the hemolysin A protein export machinery of *E. coli* to achieve secretion of large immunogenic heterologous antigens *in vivo* [98]. Although improved animal models and inoculation schedules have also been developed that permit rapid preliminary evaluation of the *V. cholerae* vaccine and vector strains *in vivo* [109,110], development of vaccine-vector strains of *V. cholerae* is in an early phase; no *V. cholerae* vector vaccine has yet been evaluated in humans.

Difficulties faced with cholera vaccines

Oral killed cholera vaccines require multiple doses and induce relatively short-lived protection compared with wild-type disease. It is not clear why the protective efficacy of CVD 103-HgR in a cholera endemic area was markedly less than in US volunteers. As described above, the currently available live and killed oral cholera vaccines may not generate immune responses to all relevant antigens. Furthermore, all oral cholera vaccines are deleted for the enzymatically active A subunit of CT to reduce diarrhea on administration, and this deletion both removes the immunoadjuvant CT compared with natural disease and may alter the *in vivo* expression of key proteins important to protective immunity following immunization with live attenuated cholera vaccines.

Host factors: efficacy of oral cholera vaccines

Efficacy and immunogenicity of enteric vaccines are often lower in resource-poor areas of the world compared with developed areas, especially among children [111]. Among children, factors that may contribute to such differences include nutrition, breast feeding and concomitant infections. Many children in resource-poor areas are malnourished, and many also suffer from micronutrient deficiencies, including deficiencies in iron, iodine, zinc and vitamin A. Such deficiencies may affect the ability of children to mount immune responses following infection or vaccination [112,113]. For example, in Bangladesh, supplementation with zinc was found to lead to more prominent vibriocidal immune responses following immunization with the rBS-WC cholera vaccine in children 2–5 years of age [114,115]. Breast feeding may also affect the ability of immunized children to respond to enteric vaccines [116,117], and the presence of intestinal infections and/or bacterial overgrowth may modulate an individual's ability to mount effective immune responses [118,119]. Finally, the composition of buffers used in formulating oral vaccines may influence immunogenicity [120].

Practical considerations for use of oral cholera vaccines in developing countries

Cholera vaccines will have their greatest utility in resource-poor areas of the world, either to control epidemic outbreaks or to decrease the burden of endemic disease. A clinically useful vaccine will need to be inexpensive, ideally able to be administered to children without impeding or interacting with routine childhood immunization regimens, and be heat-stable or -resistant. Currently, only one oral cholera vaccine (rBS-WC) is commercially available internationally. Strategies to produce oral cholera vaccines locally may decrease costs and improve availability [53].

Use of live-attenuated oral vaccines in immunocompromised individuals

The use of cholera vaccines among immunocompromised individuals, especially among individuals infected with HIV, has not been studied in detail. Killed oral cholera vaccine rBS-WC has been administered safely to HIV-infected individuals in a number of small studies, although oral rBS-WC administration has been associated with a transient increase in HIV viral load in at least one study [121–123]. In a large field trial, rBS-WC had a protective efficacy against cholera of 78% in Beira (Mozambique), a city where the seroprevalence of HIV infection was 20–30%, although HIV-associated virological and immunological parameters were not measured in this study [124]. CVD 103-HgR has also been administered to a limited number of HIV-infected individuals, specifically in a randomized, placebo-controlled, double-blind, cross-over clinical trial involving 38 HIV-seropositive individuals (without clinical AIDS) and 387 HIV-seronegative adults in Mali [125]. Adverse reactions were equivalent in vaccine and placebo recipients and CVD 103-HgR was not recoverable from the stool of any of the vaccine recipients. Both the magnitude and frequency of vibriocidal responses were lower in HIV-infected individuals than non-HIV infected recipients [125]. Significant rises in vibriocidal antibody were observed in 71% of HIV-seronegative individuals and 58% of HIV-positive individuals (and only in 40% of HIV-positive individuals with CD4 cell counts below 500 per mcl) [125]. After immunization, the peak vibriocidal mean titer in HIV-negative individuals was 1:584 compared with 1:124 in HIV-positive individuals (and only 1:40 in HIV-positive individuals with CD4 cell counts below 500 per mcl) [125]. The effect of vaccination on HIV viral load and CD4 cell counts were not reported in this study. Future field testing of cholera vaccines will need to include evaluation in individuals infected with HIV.

Contraindications

Live oral cholera vaccines should not be administered concurrently with antibiotics.

WHO recommendations

The role of vaccines as a public-health tool to control cholera, especially epidemic disease, has been controversial. An argument has historically been made that cholera-control efforts should focus not on vaccination, but on sanitation and provision of potable water. Following a large cholera outbreak in Goma (Zaire) in 1994, in which approximately

500,000–800,000 individuals were exposed to *V. cholerae* and approximately 50,000 died during a combined cholera and shigellosis outbreak over a 21-day period, this philosophy was re-examined [126,127]. The WHO now recommends that cholera vaccination (especially administered pre-emptively), along with efforts to improve sanitation, provide safe water and efforts to optimally manage cases, could be considered as a public health intervention in emergency situations to control epidemic and/or endemic cholera outbreaks among at-risk populations [4,128,129]. The WHO panels largely considered the three cholera vaccines tested in large trials, including the two formulations of the killed whole-cell vaccine (rBS-WC and WC-O1 (minus CtxB) produced locally in Vietnam) and the live vaccine CVD 103-HgR, which together had been tested in over 300,000 individuals in cholera endemic settings [4,129,130]. The WHO panels recognize that WC/rBS has been shown to be safe and protectively immunogenic in a number of field trials. The panels, therefore, recommended consideration of use of the two-dose killed oral cholera vaccine for control of cholera when an epidemic is expected or when seasonal epidemics can be predicted, such as was demonstrated in Beira (Mozambique) [4,124,129]. The WHO panels also recommended consideration of the use of the single-dose live oral cholera vaccine CVD 103-HgR once an outbreak has started [4,129]. Furthermore, the WHO panels recommended that before oral cholera vaccines be accepted as a standard public-health tool, well designed demonstration projects must be undertaken. The killed oral oral cholera vaccine rBS-WC was subsequently evaluated in refugee populations in Africa [130], and more recently to interrupt a predicted seasonal epidemic in an area of Mozambique with a high prevalence of HIV infection [124]. Following the tsunami of December 26, 2004, the Swedish government also donated 400,000 doses of the rWC-BS vaccine for use in Indonesia and/or for further testing during disaster situations [201]. A feasibility study was also performed using CVD 103-HgR in a retrospective analysis of a mass vaccination campaign in Micronesia during a cholera outbreak, supporting its role if implemented with other public health control measures [79].

Expert commentary

Cholera vaccines can be used to control cholera epidemics and to lessen the burden caused by endemic disease. Live, orally administered, attenuated vaccine strains of *V. cholerae* have many theoretical advantages over killed vaccines. A single oral inoculation results in intestinal colonization, obviating the need for repetitive dosing, and live *V. cholerae* organisms can respond to the intestinal environment. Immunological exposure to bacterial products expressed *in vivo* could therefore result in fuller immunological protection against wild-type *V. cholerae* infection. Currently, *V. cholerae* O1 El Tor strain and O139 are the causes of cholera. A number of live attenuated oral cholera vaccines have been developed and are in various stages of evaluation and development, including CVD 103-HgR, Peru-15 and *V. cholerae* 638. All have been demonstrated to be safe and immunogenic in human volunteer studies. *V. cholerae* O1 classical strain-derived CVD 103-HgR showed 14% protective efficacy in a large field trial in a cholera endemic zone in Indonesia and 80% protective efficacy when used in a response plan during a cholera outbreak in Micronesia. *V. cholerae* O1 El Tor strain-derived Peru-15, which has just completed a Phase I/II field trial in Bangladesh, appears to be safe and immunogenic and directly targets El Tor O1 *V. cholerae*, the predominant global cause of cholera. *V. cholerae* O1 El Tor vaccine strain 638 has been evaluated in pilot studies in Cuba. A number of *V. cholerae* O139 vaccines have also been evaluated in small pilot studies. Safety, immunogenicity and efficacy of orally administered live attenuated cholera vaccines needs to be evaluated in resource-poor areas of the world, especially among children and individuals infected with HIV.

Five–year view

Over the next 5 years, we believe studies will address the role of live oral cholera vaccines in preventing cholera among individuals in the developing world, especially children and in areas of the world endemic for cholera.

We believe there will be an additional evaluation of the role of live oral cholera vaccination to control cholera outbreaks and epidemics.

We believe that studies will be considered to compare the utility of live versus killed oral cholera vaccines. Live oral cholera vaccines may be more immunogenic in immunologically naïve individuals, especially among children in the developing world, while killed oral cholera vaccines may have their maximal efficacy among individuals with pre-existing partial immunity.

The safety and efficacy of live oral cholera vaccines will need to be evaluated among immunocompromised individuals, especially among individuals infected with HIV. Cholera and HIV are both highly endemic in a number of countries, especially in sub-Saharan Africa.

A number of new technologies have been developed that could potentially improve the delivery of attenuated oral bacterial vaccines in the developing world. Such technologies, including those that may result in improved heat stabilization, will need to be evaluated.

The role of micronutrient supplementation, pre-emptive antihelminth therapy and/or probiotic therapy in optimizing immunogenicity following oral cholera vaccination may be explored further.

Whether or not booster immunization (yearly or every other year) improves the efficacy of cholera vaccines will need to be addressed. Also, whether oral priming with a live oral cholera vaccine and boosting with a killed vaccine is efficacious may be addressed.

Whether or not combination immunization regimens, for example, oral priming with an oral cholera vaccine and transcutaneous boosting with pertinent antigens, improve the efficacy of cholera vaccination may need to be addressed.

Whether or not live oral cholera vaccines interact with other vaccines will need to be evaluated.

Key issues

- Live, orally administered, attenuated vaccine strains of *Vibrio cholerae* have theoretical advantages over killed vaccines. A single oral inoculation results in intestinal colonization, obviating the need for repetitive dosing, and live *V. cholerae* organisms can respond to the intestinal environment. Immunological exposure to bacterial products expressed *in vivo* could therefore result in more comprehensive immunological protection against wild-type *V. cholerae* infection.
- Pre-existing antibodies may affect the ability of live oral cholera vaccines to colonize partially immune individuals. Live oral cholera vaccines may be most effective in individuals not exposed to *V. cholerae* previously, including children in cholera endemic areas of the world.
- Strategies to optimally utilize oral cholera vaccines, including as public health tools in resource-poor areas of the world, need to be considered.
- A number of live oral attenuated cholera vaccines targeting *V. cholerae* O1 have been developed and are in various stages of evaluation and development, including CVD 103-HgR, Peru-15 and *V. cholerae* 638. All have been demonstrated to be safe and immunogenic in human volunteer studies.
- *V. cholerae* O1 classical strain-derived CVD 103-HgR demonstrated 14% protective efficacy in a large field trial in a cholera endemic zone in Indonesia, but 80% protective efficacy as part of a response plan during a cholera outbreak in Micronesia.
- *V. cholerae* O1 El Tor strain-derived Peru-15 has just completed a Phase I/II field trial in Bangladesh, appears to be safe and immunogenic and directly targets El Tor O1 *V. cholerae*, the predominant global cause of cholera. Additional Phase II studies are planned.
- *V. cholerae* O1 El Tor vaccine strain 638 has been evaluated in pilot studies in Cuba.
- A number of *V. cholerae* O139 vaccines have been evaluated in small pilot studies.
- The role of live oral cholera vaccination to control outbreaks and epidemics will need to be addressed.
- The role of oral live cholera vaccines in preventing cholera among individuals in the developing world, especially children and in areas of the world endemic for cholera will need to be addressed.
- The role and safety of oral live cholera vaccines in preventing cholera among immunocompromised individuals, especially among individuals infected with HIV, in the developing world will need to be addressed.

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