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Vibrio cholerae: lessons for mucosal vaccine design

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Abstract

The ability of *Vibrio cholerae* to persist in bodies of water will continue to confound our ability to eradicate cholera through improvements to infrastructure, and thus cholera vaccines are needed. We aim for an inexpensive vaccine that can provide long-lasting protection from all epidemic cholera infections, currently caused by O1 or O139 serogroups. Recent insights into correlates of protection, epidemiology and pathogenesis may help us design improved vaccines. This notwithstanding, we have come to appreciate that even marginally protective vaccines, such as oral whole-cell killed vaccines, if widely distributed, can provide significant protection, owing to herd immunity. Further efforts are still required to provide more effective protection of young children.

Keywords

cholera; classical; El Tor; IgA; protection; vaccine; *Vibrio cholerae*; vibriocidal

Cholera prevention by vaccination

Cholera is mainly a fecal–orally transmitted disease and humans are the only known natural veterbrate host. Cholera has been endemic in southern Asia since recorded history. Cholera has spread globally in seven pandemic waves since 1817, of which the current one began in 1961. In 2008, the WHO reported 190,130 cholera cases worldwide, associated with 5143 deaths (98% in Africa), but cholera is globally under-reported and the true disease burden is estimated to be in the millions [1,2]. In countries such as Bangladesh, cholera is endemic and both the rural and urban population is afflicted with biannual outbreaks [3]. In addition to endemic outbreaks, sporadic outbreaks can occur whenever sanitation and clean water provisions are lacking, such as occurred in Zimbabwe in 2008–2009 following the collapse of infrastructure [2].

A lack of good animal models for immunization and challenge has hampered the study of cholera vaccines. For example, adult mice are refractory to acute cholera, but infant mice up to 1 week of age are permissive for intestinal colonization [4]. Therefore, passive protection models have been developed, either using immunization of adult female mice and challenge of their pups, which are protected by sucking, or mixing antibodies with *Vibrio cholerae in*

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vitro prior to infection [5,6]. Despite the limitations of preclinical models, a number of cholera vaccines have been developed (discussed in detail later).

Since 1999, the WHO has advocated the use of oral cholera vaccines as an adjunct to the control of cholera [7]. Whether cholera vaccines can be used effectively to prevent or contain sporadic cholera outbreaks is unclear; the inherent logistical and financial challenges, especially with vaccines requiring two doses administered weeks apart, may be difficult to overcome [8]. However, there has been some encouraging success in outbreak management or prevention by vaccination using an oral whole-cell killed (WCK) vaccine in emergency settings in Darfur (Sudan) and in Aceh (Indonesia).

An increasing wealth of information about *V. cholerae* pathogenicity and transmission, outlined later, can inform our attempts to prevent cholera with effective control measures and by vaccination. Cholera is an orally acquired infection caused by a noninvasive small-intestinal pathogen, so activation of the gut mucosal immune system is likely to be important for effective vaccination. In this article, we will discuss different approaches taken towards cholera prevention by immunization, with a focus on:

- Ways in which an improved understanding of mucosal immunology, immune response to natural infection and the life cycle of *V. cholerae* have shaped and could improve cholera vaccine development;
- A recent triumph in cholera vaccination implementation;
- Areas where improvements still need to be made, such as protection of children.

Cholera pathogenesis & transmission

In 1854, *V. cholerae*, a Gram-negative bacterium, was isolated and proposed to be the causal agent of cholera by Filippo Pacini, and was rediscovered approximately 30 years later and proven to be the causative agent by Robert Koch. Despite the presence of over 200 lipopolysaccharide (LPS) serogroups of *V. cholerae* in the environment, toxigenic strains of O1 are the major cause of epidemic disease. The classical biotype predominated up until the current pandemic, when the El Tor biotype arose and has since entirely replaced the classical biotype. Both the classical and O1 El Tor biotypes consist of two O1 serotypes: Ogawa and Inaba (explained in the following section). In 1992, a new serogroup, O139, was reported to be causing significant cholera-like disease in India and Bangladesh [9,10]. O139 appears to have arisen from O1 El Tor by the acquisition of a new LPS and capsule-encoding locus [11]. In fact, outside of the LPS locus, O139 shares the majority of traits with O1 El Tor. By contrast, the O1 El Tor and classical biotypes, despite having identical O-antigen loci, exhibit many genotypic and phenotypic differences.

Ingested *V. cholerae* have to survive passage through the stomach, which provides a significant barrier. In volunteer studies, sodium bicarbonate administered prior to and during ingestion of laboratory-grown *V. cholerae* reduced the infectious dose by around 10,000-fold to approximately 10^6 colony-forming units (CFUs) to induce severe cholera, and approximately 10^4 for clinically relevant, but milder, symptoms [12,13]. Ingestion of *V. cholerae* with food also provided sufficient buffering [13]. Infection by *Helicobacter pylori* exceeds 90% by 5 years of age in parts of the developing world, and hypochlorhydria that can occur secondary to this infection may represent a major predisposing factor to cholera infection [14].

Although prevention of cholera is the major concern, there may be a similar number of asymptomatic cases, based on rectal swabs for household contacts of index cases [15]. Asymptomatic infections could aid in local transmission to susceptible individuals and could

contribute to wider geographic spread of cholera. In support of the former, Weil *et al.* found that half of prolonged shedders of cholera were asymptomatic, and contacts sharing a household with a prolonged shedder (≥4 days) were more likely to become infected [16]. Therefore, an ideal cholera vaccine should maintain immunity that is sufficient to prevent both symptomatic and asymptomatic infections, in order to minimize disease transmission.

Vibrio cholerae that survives passage through the stomach will go on to colonize the epithelial surface of the small intestine. The virulence factors that mediate this colonization have been identified and characterized using animal models. In infant mice, *V. cholerae* O1 El Tor colonization requires motility, provided by a single, polar, sheathed flagellum, whereas motility appears to be dispensable in an infant rabbit model [17,18]. Motility does not appear to be essential during human infection according to the ability of a nonmotile vaccine strain (Peru-15) to colonize volunteers, but may be required for optimal levels of infectivity [19,20]. *V. cholerae* utilizes adhesion factors, some of which may remain to be elucidated, but may include: O1 LPS [21]; GlcNAc-binding protein (GbpA) [22]; a protein (TcpF) secreted by the toxin coregulated pilus (TCP) biogenesis apparatus [23]; outer membrane protein OmpU [24]; and cholera toxin (CT), although this has only been implicated in an adult rabbit model [25]. TCP facilitates interbacterial interactions that are important for colonization [26,27]. An effective cholera vaccine could prevent colonization by inducing the production of antibodies that directly neutralize the function of key colonization factors and/or facilitate phagocytosis and killing through bacterial opsinization.

The profuse secretory diarrhea of cholera is due to the action of CT, an A_1B_5 subunit toxin that indirectly disrupts the activity of ion channels in epithelial cells, the genes for which are located within the CTXϕ prophage [28]. Rapid replication within the small intestine and the production of CT is the key to the acute nature of this disease, which if left untreated will lead to dehydration and acidosis, followed by renal shutdown, shock and death [29].

Rice water stool contains high levels of electrolytes (Na^+ K^+, Cl^- and HCO_3^-), is released by acute patients in liter volumes per day and contains between 10^7 and 10^{11} CFU/l [30,31]. These electrolytes are absorbed in parallel with glucose and researchers were surprised to find that this glucose-mediated absorption is still active in the intestines of cholera patients [32–34]. Such information allowed an effective life-saving oral rehydration solution (ORS) therapy to be developed, dramatically reducing the death rate from approximately 30% to 1–3% of hospitalized cholera cases [35]. Antibiotics help to reduce the time course and severity of disease, but are not essential to patient survival [36].

In experimental volunteer infections, the infectious dose of *V. cholerae* required to induce severe symptoms (with neutralized stomach acid) is approximately 10^6 CFUs [12,13]. However, symptomatic cholera infection upon exposure to less than 10^5 culturable organisms per day is suggested from a study of environmental viable counts during an outbreak in rural Bangladesh [37], suggesting a lower infectious dose for naturally transmitted *V. cholerae*. Consistent with this, using an infant mouse model, it was shown that freshly shed planktonic rice water stool bacteria are tenfold more infectious than laboratory-grown *V. cholerae* [38,39]. Hyper-infectious spread of cholera may be a contributing factor to the rapid transmission that is observed during cholera outbreaks [40].

Protection after natural *V. cholerae* infection

Experimental infection of healthy volunteers in most cases leads to protection for at least 3 years, although not for a lifetime [41–43]. Although cholera patient biopsy studies do not show gross changes in gut morphology, some villus blunting, release of mucus from goblet cells, inflammation-associated edema, vascular congestion and signs of innate immune cell

activation have been observed [44–46]. This innate immune system activation is likely to be required for the induction of adaptive responses.

There is great interest in adaptive immune markers that may correlate with protection from subsequent symptomatic cholera infections. The best studied has been the reciprocal vibriocidal antibody titer, which has a positive correlation with protection from acute cholera [47,48]. With increased severity of cholera there is some increase in seroconversion, but even asymptomatic infection can lead to vibriocidal responses [12,48]. Therefore, the aim to generate nonreactogenic cholera vaccines that are able to induce protective vibriocidal responses seems feasible, but as severity of disease increases responses, it may be difficult to make any vaccine as effective as wild-type cholera infection. It was shown that the vibriocidal response was largely against LPS antigens [49,50]. The serology of *V. cholerae* O1 consists of 'ABC' antigens, with serotype Ogawa being AB(C) and serotype Inaba AC [51]. These differences are now understood at the molecular level: the 2-*O*-methyl group in the nonreducing terminal sugar of the Ogawa O-antigen forms the specific B antigen [52], while an antigen dominant in the nonmethylated Inaba LPS, but also detectable for Ogawa, is termed the 'C' antigen. Inaba and Ogawa also have a common LPS antigen formed by a combination of the core and O-antigen polysaccharides, termed the 'A' antigen [53]. Data from both natural infection and immunization/challenge studies in mice and humans show that cross-O1 serotype vibriocidal responses and protection can be substantial, but are only partial [12,41,54–56]. Similar conclusions can be drawn from epidemiological analysis of outbreak serotype cycles [3,57].

If complement-dependent vibriocidal antibodies are actually important for protection, what would this imply about mucosal anticholera immunity? In most individuals, isotype switching from IgM towards IgG and IgA was observed 4–7 days after experimental cholera infection [58]. IgG and IgM fix complement, whereas IgA does not. Treatment of serum with β-mercaptoethanol disrupts IgM, but leaves IgG relatively intact, and shows a major role for both IgG and IgM in vibriocidal activity of serum from cholera patients [59]. Complement components, such as C3 and C4, have been detected in the human gut and could, for instance, enhance phagocytosis, but the attack complex that mediates killing (C5 to C9) has not been observed at lytic concentrations in the gut [60,61]. Serum IgG can enter the small bowel and act at the mucosal surface; for example, in dogs, perfusion with anti-CT IgG provided resistance to intestinal challenge with CT [62]. A neonatal Ig receptor (FcRn) that binds to IgG not only facilitates transport of IgG in uterine (in some species including humans) and from maternal milk into the neonatal blood stream [63], but is also expressed in the human gut throughout life [64]. FcRn could provide a specific mechanism by which IgG could enter the small intestine and protect against cholera. Interestingly, upon sublethal challenge with the colon-restricted mouse pathogen *Citrobacter rodentium*, it was found that IgG and recognition of IgG by the FcγR on professional phagocytes, but not secretory IgA (sIgA) or sIgM, are required for protection from inflammation and bacterial clearance [65–67]. Thus, IgG may be able to the control a gut pathogen such as *V. cholerae*. However, IgG is more readily degraded by proteolytic enzymes than sIgA, so is thought to be less well suited to provide protection at the gut mucosal surface [68]. In addition, sIgA is the dominant immunoglobulin isoform detected in feces, and transport of sIgA into the gut can occur via the polymeric Ig receptor, suggesting that sIgA is likely to provide the majority of protection against *V. cholerae* colonization [68,69]. In infant mice, injection of pure anti-LPS IgA antibody or xenografting of hybridomas making anti-LPS IgA was protective against colonization, although IgG was not tested in this model [70]. Thus, the predominance of IgA, rather than IgG, and the lack of attack complex in the gut, as well as the imperfect correlation between vibriocidal antibody titer and protection, suggest that this phenotype is only a surrogate for protection.

In a large study of cholera index patients and their household contacts containing both O1 or O139 index cases, it was shown that circulating IgA levels for anti-LPS, anti-TcpA (TcpA being the major TCP pilus subunit) and anti-cholera toxin B-subunit (CTB) can be correlated with protection from O1 infection, and that circulating anti-TcpA IgA correlates with protection from O139, whereas circulating IgG showed no such correlations [71]. Anti-TcpA responses could, therefore, be helpful to the generation of cross-O1/O139 protection and IgA may be important to protection from natural cholera infection. As mucosally delivered cholera vaccines induce IgA responses, whereas injected vaccines do not induce IgA in naive individuals, this strengthens the argument for mucosal delivery of cholera vaccines. It was further shown that memory B-cell responses to TcpA and CTB in particular, which last longer than do vibriocidal antibodies or anti-*V. cholerae* serum IgA, correlate more closely with the time course of protection [72].

A parenteral cellular killed vaccine

Parenteral cholera vaccines were first developed and tested in the 1960s. Monovalent WCK O1 Inaba or Ogawa vaccines were administered to Bangladeshi children. During the trial period, only O1 Inaba outbreaks occurred. Inaba WCK achieved almost 100% protection 3 months after immunization [55]. By contrast, the monovalent Ogawa WCK parenteral vaccine was ineffective against Inaba infections for young children (under 5 years of age) and only marginally (48%) protective for older children, leading to the recommendation for development of a bivalent Ogawa + Inaba vaccine [55]. A WCK *V. cholerae* O1 Ogawa + Inaba mixture was tested in a double-blind controlled trial in Bangladesh (then East Pakistan) in 1963–1964. It induced high levels of serum vibriocidal antibody, achieved 76% protective efficacy during the first 1–6 months after immunization and significant protection of adults, but not of children, for up to 18 months, but showed many reactogenic events, mainly fever and pain and swelling at the injection site [73]. A formulation of the bivalent O1 Ogawa + Inaba parenteral vaccine licensed in the USA, but no longer commercially available, administered in two doses 2 weeks apart provided only approximately 50% protection that lasted little more than 6 months, a level and duration of protection that was not considered sufficient by the WHO [7]. IgA reponses to the parenteral vaccine were only observed in non-naive populations, owing to previous mucosal priming [74]. These data may explain why the parenteral vaccine provided longer-term protection in Bangladeshi adults compared with children, as the former are likely to be primed by prior exposure to *V. cholerae*, and suggest that IgA responses are necessary for long-term, but not short-term, protection. The short duration of protection and the reactogenicity of parenteral cholera vaccines, plus the requirement for injection devices and trained staff, make their future use in cholera prevention unlikely, with the possible exception of short-term protection for young children (discussed later).

An oral cellular killed vaccine

Some pros and cons of mucosal, as opposed to parenteral, vaccines are noted in Table 1. Our intestinal mucosa is not designed to induce immune responses against nonliving foreign material or against our commensal flora (termed mucosal tolerance). An immune response is thought to require both ‘I am foreign’ and ‘I am dangerous’ signals. In light of this, it is surprising that a WCK oral cholera vaccine could be effective and that such a vaccine was ever developed back in the 1990s. Against this backdrop, a key discovery was made, that CT itself is a mucosal adjuvant that can elicit an immune response to both itself and coadministered antigens [75]. This exciting realization opened up the possibility of developing nonliving mucosal vaccines combined with an adjuvant. CT is of course toxic, so the discovery that CTB subunit alone also has mucosal adjuvant activity, albeit less effective than CT holotoxin, was an important subsequent discovery [28].

A simple oral mucosal vaccine containing WCK *V. cholerae* with or without CTB was therefore developed. A volunteer study was carried out with WCK and WCK-CTB vaccines, the compositions of which are presented in Table 2, administered as three oral doses 2 weeks apart [76]. Formalin-treated El Tor was included to preserve the heat-sensitive antigen mannose-sensitive hemagglutinin (MSHA) made by El Tor [76,77]. Rises in vibriocidal titer were observed for 89% (WCK-CTB) or 71% (WCK) of volunteers [76]. Volunteers were challenged with 2×10^6 CFU of El Tor Inaba 4–5 weeks after completion of the vaccination schedule. WCK-CTB and WCK provided similar protection of approximately 60% (with pooled control groups) [76]. These data, combined with other studies (discussed later) show that the WCK oral cholera vaccine does not require the adjuvancy properties of CTB for immunity to develop.

Volunteer studies were sufficiently encouraging that a large randomized double-blind efficacy field trial was carried out in rural Bangladesh. A total of 62,285 participants received three doses of either WCK-CTB, WCK or WCK *Escherichia coli* K12 strain placebo. Two doses were almost as significantly protective as three, but one dose was not sufficient [78]. The cumulative protective efficacy at 3 years was 50% for WCK-CTB and 52% for WCK [78]. After 4–6 months, protection in children 2–5 years of age was similar to that for older persons, but it rapidly waned thereafter and was not evident during the third and fourth years of follow-up [78]. By contrast, 3 years after immunization, older vaccinees were protected (40% protective efficacy for WCK-CTB and 62% for WCK), but protection was no longer significant 4 years postimmunization [78,79]. Therefore, the oral vaccine appears to provide protection of similar duration to natural infection and a longer duration of protection than parenteral vaccines, but has a similar efficacy to the parenteral vaccine. These trials led to the licensing of WCK-CTB to be made in Sweden under the trade name Dukoral® (Crucell, Stockholm, Sweden; see Table 2).

How does an orally delivered WCK vaccine without CTB induce immunity? Whether killed *V. cholerae* contain ‘danger signals’ recognized by the innate immune system that are not shared by commensal microbes, and can act as an adjuvant, is currently unclear. Surface molecules used by *V. cholerae* for small intestinal adhesion could contribute to effective delivery as a killed vaccine. Are killed *V. cholerae*, O1 and O139 (also immunogenic in WCK oral vaccines, discussed later), unique in being able to induce a mucosal immune response? Are there other examples of nonliving Gram-negative bacterial vaccines that can induce protective immune responses when mucosally delivered? We know of at least one other example: a *Bordetella pertussis* WCK vaccine delivered intranasally can generate mucosal and serum antibodies and activated T cells in human volunteers [80,81], although whether this is protective or not is not known.

Both classical and El Tor biotypes are included in current WCK vaccines (see Table 2). There are many known differences between the classical and El Tor biotypes that could contribute to differences in antigenicity, including MSHA expression by El Tor, but not by classical, and an antigenic difference in the C-terminus of TcpA [82]. When O139 arose, a shift occurred in the age profile of cases, due to a lack of pre-existing immunity to this serogroup [83]. Are the *V. cholerae* O1 classical and El Tor biotypes sufficiently different to produce a similar pattern? Epidemiological data from rural Bangladesh, before and after El Tor became dominant, suggest that there was not a lack of pre-existing cross-immunity between O1 biotypes [3,84]. This further suggests that ‘hybrid’ (of ambiguous biotype) or ‘altered’ (El Tor biotype, but harboring classical CTXϕ) strains, which are becoming dominant across Africa and Asia, are unlikely to cause more severe disease owing to a lack of pre-existing immunity [85,86]. These observations suggest that if different epidemic serogroups (LPS types) arise, the composition of cholera vaccines may require reformulating to include these serogroups, but that biotype variation is unlikely to pose a

significant problem. The relative contributions of classical and El Tor antigens to WCK vaccine adjuvanticity and protection are unclear.

Live-attenuated cholera vaccines

In general, live-attenuated vaccines are expected to generate more robust mucosal responses compared with WCK vaccines. In support of this, in a rabbit ligated ileal loop model, live radio-labeled *V. cholerae* were taken up by M cells in Peyer's patches, whereas killed bacteria (formalin fixed or heated) were not [87]. Thus, antigen sampling for killed vaccine strains is likely to be reduced compared with a live-attenuated strain. Comparisons of immune responses to killed versus live *V. cholerae* in rabbits [88] and mice [89] support the greater effectiveness of live *V. cholerae* for immunization and the need for higher doses of killed bacteria delivered mucosally. However, to make a live strain appropriately attenuated compared with the wild-type organism, in order for it to be safe (nonreactogenic) yet retain antigenicity, can be a difficult balance to achieve.

Having established the dominant role of CT in cholera pathology, attenuation of live oral cholera vaccines was based on mutation of *ctxA*. Responses of rabbits to oral immunization with live (one dose) or killed (three high doses) *V. cholerae* and protection (removable intestinal tie-adult rabbit diarrhea [RITARD] challenge model) correlate with the mucosal-colonizing capacity of the infecting strain, and are otherwise independent of toxin genotype (A^+B^+ or A^-B^+) or whether the strain is motile or nonmotile [88,90]. TCP is retained in live-attenuated *V. cholerae* vaccine strains, as it is required for colonization.

Texas Star-SR was one of the first live-attenuated cholera vaccines to be developed. It was obtained by chemical mutagenesis of an El Tor biotype Ogawa (strain 3083) and produced no catalytic CT A-subunit, but retained B-subunit expression, and was attenuated in infant rabbits [91]. Eight out of 42 volunteers immunized with Texas Star-SR experienced some diarrhea (0.2–1.5 l total volume) [92]. This level of reactogenicity was not ideal, but the vaccine did induce seroconversion in 96% of volunteers, and in challenge studies with *V. cholerae* El Tor Ogawa or Inaba, vaccine efficacy was 61% (regardless of challenge serotype) [92]. However, the chemical mutagenesis approach produces strains with unknown lesions responsible for the attenuation and relies on point mutations that could revert to wild type.

Using recombinant DNA techniques, an attenuated strain, Center for Vaccine Development (CVD) 103-HgR, was constructed from classical O1 Inaba (strain 569B), with 94% of the *ctxA* gene deleted, but leaving *ctxB* intact, and with a mercury-resistance cassette (*mer*) inserted into the gene for hemolysin, *hlyA*, which is disfunctional in this strain, as an identifying marker [93]. In a trial with 2–5-year-old Indonesian children, CVD 103-HgR was well tolerated, inducing a fourfold or greater rise in serum vibriocidal antibody in 75% of vaccines, was minimally excreted by vaccinees and was not transmitted to unvaccinated controls (0% of rectal swabs were positive for vaccine strain) or family contacts (1 out of 177; 0.6%) [94]. In adult Swiss volunteers, CVD 103-HgR induced stronger vibriocidal responses to the homologous than to the heterologous serotype [95]. However, upon experimental challenge of American volunteers 4–5 weeks postvaccination, a single oral dose of 10^8 CFU of CVD 103-HgR, or the CVD 103 pregenitor, was protective against both Inaba and Ogawa, classical or El Tor, challenge [93,96]. The vibriocidal response in American volunteers immunized with CVD 103-HgR was approximately two-thirds of that of unvaccinated volunteers challenged with *V. cholerae* [93], showing that the vaccine response is not as robust as that induced by natural infection.

An ideal vaccine would provide protective immunity rapidly after immunization, which would be especially important in an epidemic scenario, and would provide lasting immunity.

CVD 103-HgR was found to provide protective immunity from homologous challenge as soon as 8 days after vaccination, and protection persisted for at least 6 months in American volunteers [96]. With volunteers from industrialized countries, a dose of 5×10^8 was immunogenic, whereas a ten-times higher dose was needed to elicit a vibriocidal response in 2–5-year-old Indonesian children [97]. In a randomized placebo-controlled field trial in Indonesia, a single dose of CVD 103-HgR showed 70% seroconversion [98]. Very low numbers of cholera cases were encountered during the first year, negating determination of short-term protection. The trial was extended, but after 4 years of observation, the single dose of CVD 103-HgR could not be shown to confer significant long-term protection [98]. Encouragingly, a single dose of CVD 103-HgR induced protective immunity (79.2% estimated efficacy 10 days after vaccination) rapidly enough after immunization to contain a cholera outbreak in Micronesia [99].

A number of live-attenuated candidate vaccine strains have been developed. Similar mutations in different strain backgrounds appear to result in different levels of reactogenicity. For instance, CVD 110 was developed from O1 El Tor Ogawa strain E7946 by deletion of *ctxAB*, zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), and insertion of *mer* and *ctxB* into *hlyA* [100]. Despite deletion of all of these toxin genes, CVD 110 still induced many reactogenic events, including mild-to-moderate diarrhea in seven out of ten adult volunteers with a dose of 10^8 CFU and lactoferrin excretion, a marker of inflammation [101,102]. CVD 111 was constructed in a similar way to CVD 110, but in El Tor Ogawa strain N16117 [13]. However, CVD 111 only induced mild diarrhea in 12% of volunteers, rather than the 70% seen for CVD 110 [103]. The reason for this difference in reactogenicity is unknown. Comparison (in a mouse lung inflammation model) of O1 El Tor Inaba strain P27459 Δ*ctxAB* with a mutant in which the gene encoding repeats-in-toxin (*rtxA*), is also deleted suggested that inflammation in the absense of CT is due to RTX [104]. However, RTX is not produced by classical strains, due to a deletion of *rtxC* [105], yet some classical strains are also reactogenic in the absense of *ctxAB*, which must be due to other reactogenic factors. Classical CVD 103-HgR, derived from strain 569B, is not significantly reactogenic (at a dose of 5×10^8 CFU), yet CVD 105, with similar mutations in classical strain 395, induced mild diarrhea (at a dose of 10^6 CFU) [93,106]. We note that there may be additional unknown mutations in CVD 103-HgR, as when the strain was rederived (CVD 103-HgR2) it did not behave identically to CVD 103-HgR [107]. These data support the idea that RTX elaboration in El Tor strains may correlate with the reactogenicity of live-attenuated strains.

A single-dose oral cholera vaccine developed by combining two live-attenuated vaccine strains, CVD 103-HgR (classical, Inaba) and CVD 111 (El Tor, Ogawa), represents one of the few Inaba + Ogawa live-attenuated vaccine combinations tested. Of 14 American volunteers immunized, all seroconverted against Inaba and 13 against Ogawa [108]. A ten-times higher dose was given to Peruvian volunteers and, in a minority (≤15%, independent of dose), mild reactogenic events were observed: mild diarrhea, abdominal cramps, vomiting and/or fever [108]. A live-attenuated O139 strain (CVD 112) has also been developed with similar mutations to CVD 110 (Δ*ctxA zot ace cep* core encoded pilin) and was found to be immunogenic and protective in American volunteers (85% efficacy 5 weeks after vaccination) [109].

Peru-15, also known as CholeraGarde® (Celldex Therapeutics, MA, USA), is a live-attenuated vaccine strain made in El Tor O1 Inaba strain C6709, from which the complete CTXϕ and surrounding RS1 and *attB* site for CTXϕ integration has been deleted and *ctxB* then added back as a disruption to the *recA* locus [110]. The region removed (a Hind III fragment) also includes the final 1916 bp of *rtxA*, which is 13,677 bp in total. Whether this would disrupt functional RTX production is not clear. The final step in Peru-15 attenuation

was to isolate a spontaneous nonmotile, nonflagellated clone [110]. This strain is likely to have a reduced colonization capacity compared with its motile parent strain (Peru-3) as was seen for a flagellated, but nonmotile, Peru-14 clone (33-fold lower colonization than Peru-3 in an infant mouse model) [111]. The decision to use a nonflagellated mutant was at least partly based on the slightly higher reactogenicity of Peru-14 compared with the parent strain Peru-3 when tested in volunteers [111]. Peru-15 itself was well tolerated and proved 93% protective upon experimental challenge in American volunteers [110,112]. Recent investigation of the reactogenicity of *ctxAB* mutants in infant rabbits demonstrates that the presence of flagellin can be reactogenic, regardless of whether a flagellum is actually assembled [18]. Comparisons of vibriocidal responses induced in volunteers suggest that the Peru-15 live-attenuated vaccine outperforms WCK-CTB, but is not as immunogenic as a wild-type symptomatic cholera infection [19]. In addition, Peru-15 has proven to be safe and immunogenic in both adult volunteers and toddlers and infants in Bangladesh [113,114].

The Peru-15 blueprint was transferred to O139, resulting in a vaccine that was safe and immunogenic in volunteers and provided 83% efficacy against experimental O139 challenge 1 month after immunization [115]. Without sequencing Peru-15 we cannot know the exact mutations leading to the nonmotile/nonflagellate phenotype to perfectly reproduce this in O139 and, ideally, in O1 Inaba, which could possibly allow a trivalent vaccine mixture to be tested.

The toxin causes the disease, so why not use the toxin as a vaccine?

Although CT is the agent that causes life-threatening secretory diarrhea, vaccines composed of inactivated CT (oral or parenteral) did not induce protective immunity [42]. A wealth of information from animal models and adult human studies now supports the notion that anti-CT immunity is certainly not sufficient, or even necessary, for protection from cholera. For example, the ineffectiveness of anti-CT compared with anti-LPS antibodies for passive protection of mice [70], the lack of cross-serogroup protection despite an identical CT between O1 El Tor and O139 in animal models [116,117] and in humans, and similar protection after *V. cholerae* WCK vaccination with or without CTB all support this concept. Nevertheless, some animal studies suggested that anti-toxin immunity could contribute to protection [118–120]. In an American volunteer study, WCK-CTB induced significant rises in anti-LPS sIgA in a larger proportion of individuals (53%) than in the WCK-immunized group (just one out of 13 [8%]), suggesting that CTB may play a role in enhancing mucosal responses to WCK vaccines, at least in a naive immunized population [76]. It was also found that vaccinated 2–5-year-old Bangladeshi children were significantly protected over the first 2 years after immunization with WCK-CTB, but not WCK alone, whereas no such diference was seen for older people [78]. These data suggesting that more protection may be afforded by anti-toxin immunity in younger populations is consistent with ‘naive’ animal studies where both anti-toxin and antibacterial immunity seemed to play a part.

Novel anticholera vaccine approaches

In 1967, Chatterjee *et al.* observed that outer membrane vesicles (OMVs) were released by *V. cholerae* [121]. OMVs were tested alongside flagellar components as a subcutaneous maternal vaccine and proved protective to suckling neonatal mice born to immunized dams upon challenge [122]. Detergent-extracted OMVs (sometimes termed proteoliposomes) from *Neisseria meningitidis* group B have proven successful as an intramuscular vaccine, delivered with adjuvant [123]. Detergent extraction is used to reduce lipooligosaccharide and capsule components and increase the level of protective proteins in this vaccine. Native *V. cholerae* OMVs can be mucosally delivery as a maternal vaccine that induces antibody responses in milk that passively protect neonates from infection [124,125]. The most robust

protection was afforded by intranasal immunization, although OMVs delivered by both the oral and intraperitoneal routes were also immunogenic and protective [56,124]. A triple O1 Ogawa + O1 Inaba + O139 OMV combination delivered intranasally induced antibody responses against both serogroups and serotypes and protected suckling neonates from colonization upon challenge with O1 or O139 [56]. Similarly, cochleates (phospholipid-calcium precipitates) derived for O1 *V. cholerae* proteoliposomes are also immunogenic in mice when delivered intranasally [126].

Synthetic liposomes also provide a promising oral delivery system for subunit vaccines, by protecting their purified antigenic cargo from degradation in the stomach [127]. Synthetic liposomes loaded with purified *V. cholerae* protein and O1 LPS antigens fed orally to rats were much more immunogenic compared with free cholera antigens [128]. In a small trial with Thai volunteers, three oral doses of liposomes (14 days apart) loaded with *V. cholerae* LPS, classical biotype crude fimbrial extract and heat-treated CT (procholeragenoid), each proceeded by sodium bicarbonate to reduced stomach acidity, were well tolerated [129]. Increases in vibriocidal responses and serum anti-CT IgG and IgA were seen, although high baseline anti-*V. cholerae* antibody titers in the majority of volunteers mean that the study was effectively looking at the ability to boost pre-existing immunity [129].

It is intriguing that oral delivery of *V. cholerae* antigens, even in the absense of CT, in the form of OMVs [124], liposomes [130], ghosts (bacteria from which the cytoplasm has been removed by a phage pore-forming toxin) [131,132] or WCK [78] all appear to provide natural mucosal adjuvanticity that can break mucosal oral tolerance. The possible adjuvant properties and mucosal delivery capability of these preparations are the subjects of much interest and of ongoing studies [127]. These preparations, the OMVs and liposomes in particular, could potentially deliver antigens by direct fusion with cells, professional phagocyes or epithelial cells, including M cells [133–135].

Oral WCK vaccine reformulation

During the 1985 Bangladesh field trial of the WCK and WCK-CTB cholera vaccines, levels of vaccine coverage varied from 4 to 65% and there was little movement of people between regions (called baris) [78,136]. Re-analysis of the trial data showed that protection of nonvaccinated people was proportional to the level of vaccine coverage in each bari [136]. Mathematical modeling of these data suggested that for regions with high prior immunity (low number of susceptible individuals), such as the Bangladesh population where the trial was held, as little as 30% WCK vaccine coverage can provide 76% protection due to herd immunity, while higher vaccine coverage would be required for regions with a higher proportion of naive susceptible individuals; for example, with a 1.5-fold higher proportion of susceptible individuals, 50% coverage would be needed to achieve 80% protection [137]. Thus, cholera vaccines may not need to be much more effective than approximately 50%, as long as there is good vaccine coverage. This observation suggested that an inexpensive *V. cholerae* WCK oral vaccine formulation with even moderate protective efficacy could be more effective than previously appreciated for protection in cholera endemic regions.

The WCK-CTB vaccine is considered unsatisfactory owing to ‘its two-dose regimen, short shelf-life, high cost and need for cold chain distribution’, leaving room for an improved cholera vaccine for use in developing countries [138,139]. The inclusion of recombinant CTB was the most costly component. An important milestone on the road to a more affordable cholera vaccine occurred in Vietnam, where technology transfer to the Company for Vaccine and Biological Production in Hanoi allowed WCK without rCTB to be locally produced at a cost of just US$0.4 per dose [140]. This vaccine is similar to Dukoral (see Table 2), except that the formalin-killed classical Ogawa strain Cairo 50 was replaced with

strain 569B, in order to increase the amount of the putatively protective TcpA antigen, which is more efficiently expressed by strain 569B [141]. Two doses of the Vietnam vaccine were shown to be safe, immunogenic and 66% protective 8–10 months after immunization for children 1–5 years of age and for older vacinees [141].

The appearance of O139 in 1992 stimulated the development of a second-generation bivalent Vietnam vaccine, containing both O1 and O139 serogroups (Vabiotech, Vietnam; see Table 2). If given in two doses 2 weeks apart, the bivalent vaccine induced a rise in anti-O1 vibriocidal response in 60% of recipients (2 weeks after the second dose), which is similar to the response induced by Dukoral [142]. The bivalent vaccine also induced a rise in anti-O139 vibriocidal responses in 40% of the recipients [142]. Lower O139 responses could partly be due to a lack of pre-immunity to O139, removing the boosting effects in adults already exposed to O1 antigens. Significant rises in vibriocidal titer (against O1 or O139) in response to the oral Vietnam bivalent WCK vaccine occurred more often in children than in adults, presumably because they represent a more naive population [142]. In 2003, a major outbreak of cholera due to *V. cholerae* O1 occurred in the vaccine test region, providing the opportunity to measure long-term protection associated with mass cholera immunization campaigns that occurred in 1998 and 2000. The overall vaccine effectiveness 3–5 years after vaccination was, on average, 50% [140], which is very similar to Dukoral (see Table 2).

Some additional changes have been made to the Vietnam bivalent WCK vaccine to bring it in line with WHO recommendations (see Table 2). First, technology transfer from Vietnam to Shantha Biotechnics (Hyderabad, India) is allowing the vaccine to be made in a WHO-approved facility. Second, the classical 569B strain has been removed [143] because, although it makes high levels of the potentially antigenic and protective antigen TcpA, it also produces higher levels of CT than other classical strains when grown *in vitro* and the WHO recommended reduction and/or monitoring of CT levels [144]. This reformulated vaccine has been proven to be safe and immunogenic with two doses in Vietnamese volunteers [143]. A randomized placebo-controlled trial in both adults and children (1–17 years of age) in the cholera endemic area of Kolkata, India, is ongoing. Preliminary results from the Kolkata trial show vibriocidal antibody titer rises of at least fourfold for 80% of children and 53% of adults, a generally lower O139 than O1 response and approximately 67% protection from clinically relevant disease 2 years into the study period, regardless of age [145–147].

Expert commentary & five-year view

Cholera prevention and treatment holds a number of examples of simple solutions that could be put into action and are built upon an understanding of complex underlying knowledge. Thanks to a basic understanding of the action of CT and the composition of rice water stool, the development of effective ORS has saved millions of lives. Similarly, even without expensive water purification systems, simple preventative measures, such as choice of water vessels in the home or simple means of filtration, can reduce the cholera burden [148,149]. It may be the case that providing an effective, affordable cholera vaccine for use in endemic areas is another example where a simple approach can be effective. The reformulation of a bivalent WCK oral vaccine to make it affordable and safe for use in cholera endemic areas is an exciting development. If this can be more widely used in endemic areas and employed during or prior to outbreaks, we may enter an era of reduced cholera burden through vaccination.

One criticism of the WCK-CTB vaccine has been the costly and logistically difficult need for refrigeration [139,150]. Excitingly, the reformulated WCK vaccine being produced in India does not require strict cold-chain storage, although 2– 4°C storage has been used for

all reformulated vaccine trials to date [143,145–147]. Improved product quality-control measures, using amounts of LPS rather than CFU prior to killing, have also been instigated in order to further standardize the product (see formulation in Table 2).

A high burden of cholera in Africa, where many of the population are HIV-positive, leads to concerns over cholera vaccine efficacy in this population. It is promising that a mass vaccination campaign with WCK-CTB in Mozambique, in a population that is 20–30% HIV-positive, reported 78% efficacy [151]. Live-attenuated vaccines could be more of a safety risk in HIV-positive individuals than a killed vaccine. However, immunizations carried out with CVD 103-HgR in sub-Saharan Africa (Mali) had a good safety profile, although vibriocidal responses were significantly lower among HIV-seropositive individuals [152].

Expression of heterologous antigens from other relevant pathogens within a cholera vaccine could provide protection from more than one disease with a single vaccine. Using OMVs as a delivery vehicle, responses to heterologous antigens have been observed in mice for a periplasmic protein delivered intranasally within *V. cholerae* OMVs [125] or a surface-exposed (ClyA toxin-fused) antigen delivered subcutaneously in association with *E. coli* OMVs [153], without the need for additional adjuvants. *V. cholerae* ghosts, delivered intramuscularly, have also been utilized to deliver *Chlamydia trachomatis* antigens and have proved to be immunogenic and partially protective in mice [154]. Live-attenuated *V. cholerae* strains have also been constructed to express heterologous antigens from organisms that include *Shigella dysenteriae* type 1, enterohemorrhagic *E. coli* and *Clostridium difficile* (previously reviewed in [155]). The inclusion within Dukoral of recombinant CTB, which is approximately 80% identical to heat-labile toxin B subunit, also provided 67% short-term protection from enterotoxingenic *E. coli*, particularly strains expressing heat-labile toxin B subunit [156].

Arguably the most important area for improvement of cholera vaccines during the next 5 years is the more effective protection of children. Children are most at risk from cholera due to a lack of pre-existing immunity [47,157] and a poor vibriocidal response has been observed for children to *V. cholerae* vaccines or natural infection. Poor responses to oral vaccines in children from developing nations may partly be due to disturbances in digestion and absorption, broadly termed chronic environmental enteropathy [158]. Zinc supplementation, for example, helps to improve cholera vaccine reponses in small children [159,160]. Attempts have been made to directly protect children from cholera by immunization. Vibriocidal responses of children to the parenteral cholera vaccine were slightly higher and more sustained than responses to natural infection, but were still very poor in children under 5 years of age compared with older children [54]. Dukoral is licensed, and effective for children 2 years of age or older, while the Vietnam vaccine has been approved for children as young as 1 year of age (see Table 2). The live-attenuated vaccine Peru-15 was found to be safe and immunogenic in children of 9 months–5 years of age in Bangladesh [114].

Breastfeeding is protective against cholera for infants in endemic countries [161,162]. It is unclear whether this is due to reduced exposure to *V. cholerae* when infants are breastfed or because breast milk contains protective agents, such as cholera-specific antibodies (for *V. cholerae*-immune mothers) or innate factors such as lysozyme, lactoferrin and oligosaccharides that correlate with reduced diarrhea and/or can inhibit *V. cholerae* hemagglutinins [163,164]. Anti-*V. cholerae* antibodies in a mother's breast milk could certainly mediate passive protection of human infants, as is the case in mice [6,125]. Immunization of mothers with oral WCK or WCK-CTB vaccines did induce protection of breastfed nonvaccinated children; however, there was a negligible level of anti-*V. cholerae*

IgA in the mother's milk that could account for this protection [165]. This is consistent with an earlier Bangladeshi volunteer study reporting very few individuals with anti-*V. cholerae* milk IgA after ingestion of a WCK-CTB cholera vaccine, suggesting that in this regard vaccines may fall short of the protection afforded by natural infection [166]. It could simply be that vaccine-mediated protection of mothers reduces transmission of *V. cholerae* to their children. Alternatively, perhaps IgG gained in the placenta through action of the FcRn is a more important marker for protection and serum IgG in mothers may have correlated better with protection of their children, but this has not yet been tested.

Importantly, the same factors in breast milk, both innate and specific antibodies, which could protect infants from cholera infection, may also inhibit cholera vaccines administered orally to infants that are being breastfed. To circumvent this problem breastfeeding could be temporarily withheld, which did lead to an increase from 57 to 77% seroconversion upon WCK-CTB immunization of infants [160]. Alternatively, a parenteral vaccine or a nonoral, but mucosal, vaccination route, such as intranasal vaccination, could be used for infants. For parenteral vaccines, the use of an adjuvant combined with lower doses of WCK bacteria could enhance responses while reducing adverse events. This approach had some success in children in a cholera endemic area of Indonesia [167]. Therefore, a parenteral nonliving vaccine with adjuvant may be a beneficial tool for cholera prevention in children. Parenteral vaccines composed of *V. cholerae* LPS (O1 or O139) conjugated to protein, unlike LPS alone, can induce T-cell-dependent responses, and have proven immunogenic in mice [168–171]. Such LPS–protein conjugates could provide an alternative to cellular injected vaccines for use in children. If injected vaccines could be delivered without the need for needles, such as by using transcutaneous patches, this would be prefereable (see Table 1) [172]. An initial attempt to deliver a cholera LPS–bovine serum albumin conjugate vaccine transcutaneously did induce an anti-LPS IgG response, but, discouragingly, it did not induce significant vibriocidal or anti-LPS IgA responses and was not protective in a mouse model [173]. Using mouse models, intranasal immunization has proven superior to oral delivery for WCK *V. cholerae* and OMVs [56,89,124]. Intranasal delivery of bacterial antigens has not been extensively studied in humans, but it has been found to be well tolerated and sucessfully immunogenic for WCK *B. pertussis* [80] and native group B *N. meningitidis* OMVs [174]. Higher doses than parenteral vaccines, but lower than oral, appear to be required for intranasal immunization in humans, but the use of a mucosal adjuvant could reduce the dose needed for an equivalent response [175]. However, safety concerns for intranasal immunization, owing to the possibility of antigen delivery into the brain via the olfactory epithelium [176,177], may preclude this route for immunization of children. Furthermore, homing of T and B cells to the human gut in order to produce a local IgA response is less efficient after nasal than oral immunization [172,178]. Thus, despite being a mucosal route of immunization, protection of children by intranasal immunization may be similar to that provided by parenteral vaccines owing to a lack of a local gut IgA response.

As LPS is the dominant cholera antigen, this leads to imperfect cross-serotype protection, and little to no cross-serogroup, protection (discussed previously). Therefore, in order to protect individuals from all clinically relevant cholera challenges, cholera vaccines should include O1 Inaba, O1 Ogawa and O139 LPS antigens, as is the case for the reformulated Shanchol™ (Shantha Biotechnics) and bivalent Vietnam vaccines (see Table 2). Although natural infection is highly protective, the duration of protection is relatively short (3 years has been demonstrated experimentally). This may also relate to the LPS dominance of the immune response, as memory B-cell responses to LPS are shorter-lived than protein responses in cholera patients [72]. In addition, children respond poorly to T-cell-independent carbohydrate antigens, such as LPS, which may contribute to their lack of robust protection after natural infection or WCK cholera vaccination. In order to develop cholera vaccines with better characteristics of cross-serogroup protection, protection of children and longer-

term protective memory, we could try to drive a more robust protein antigen response and/or make use of LPS–protein conjugates. In terms of protein antigens, a number of candidates have been suggested. Antibodies raised to outer membrane proteins and adsorbed with LPS, are immunogenic and protective in a passive protection model [179]. It has been observed that the *V. cholerae* hemolysin HlyA generates a particularly robust and long-term memory T-cell response, making it a potentially useful immunogen that has not yet been extensively investigated [180]. *In vivo*-induced antigen technology has also been used to find immunogenic *V. cholerae* proteins expressed *in vivo*, including the type IV pilus proteins TcpA and PilA [181]. Proteomics of stool bacteria, although not yet carried out in a quantitative manner, may reveal insights into antigens expressed in the 'hyper-infectious' state that may also yield candidate protein antigens for inclusion in future cholera vaccines [182]. TCP is an important *V. cholerae* colonization factor [26,27] and anti-TcpA antibodies are protective in the infant mouse model [183,184]. Prior studies have shown the presence of anti-El Tor TcpA antibodies in pooled human serum and anti-TcpA seroconversion for 93% of patients in Bangladesh [181,185]. As mentioned earlier, anti-TcpA responses have now been correlated with protection after natural infection for both O1 and O139 [71,72]. However, current killed vaccine formulations are made under culture conditions that are not conducive to TCP expression. TcpA-derived peptides have been tested as injected subunit vaccines along with an adjuvant and proved to be immunogenic and protective in mice [186,187].

In summary, an affordable oral cholera vaccine has been developed for use in endemic regions, which has been a major goal over the past 50 years. In the coming years, improvements to current cholera vaccines should focus on more effective protection of children.

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References

Papers of special note have been highlighted as:

• of interest

1. Sack DA, Sack RB, Chaignat CL. Getting serious about cholera. N. Engl. J. Med 2006;355(7):649–651. [PubMed: 16914700]
2. WHO. Cholera: global surveillance summary, 2008; Wkly Epidemiol. Rec. 2009. p. 309-324.
3. Longini IM, Yunus M, Zaman K, Siddique AK, Sack RB, Nizam A. Epidemic and endemic cholera trends over a 33-year period in Bangladesh. J. Infect. Dis 2002;186(2):246–251. [PubMed: 12134262]
4. Klose KE. The suckling mouse model of cholera. Trends Microbiol 2000;8(4):189–191. [PubMed: 10754579]
5. Ujiiye A, Nakatomi M, Utsunomiya A, et al. Experimental cholera in mice. 1. First report on the oral infection. Trop. Med 1968;10:65–71.
6. Ujiiye A, Kobari K. Protective effect on infections with *Vibrio cholerae* in suckling mice caused by the passive immunization with milk of immune mothers. J. Infect. Dis 1970;121:S50–S55.
7. WHO. Cholera vaccines; Wkly Epidemiol. Rec. 2001. p. 117-124.

8. Chaignat CL, Monti V, Soepardi J, et al. Cholera in disasters: do vaccines prompt new hopes? Expert Rev. Vaccines 2008;7(4):431–435. [PubMed: 18444890]
9. Ramamurthy T, Garg S, Sharma R, et al. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet 1993;341(8846):703–704. [PubMed: 8095620]
10. Cholera Working Group ICDDR. Bangladesh. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet 1993;342(8868):387–390. [PubMed: 8101899]
11. Waldor MK, Mekalanos JJ. *Vibrio cholerae* O139 specific gene sequences. Lancet 1994;343(8909):1366. [PubMed: 7910357]
12. Cash RA, Music SI, Libonati JP, Snyder MJ, Wenzel RP, Hornick RB. Response of man to infection with *Vibrio cholerae*. I. Clinical, serologic, and bacteriologic responses to a known inoculum. J. Infect. Dis 1974;129(1):45–52. [PubMed: 4809112]
13. Levine, MM.; Black, RE.; Clements, ML.; Nalin, DR.; Cisneros, L.; Finkelstein, RA. Volunteer Studies in Development of Vaccines Against Cholera and Enterotoxigenic Escherichia coli: A Review. Holme, T.; Holmgren, J.; Merson, MH.; Mollby, R., editors. Amsterdam, The Netherlands: Elsevier/North-Holland Biomedical Press; 1981.
14. Frenck RW, Clemens J. Helicobacter in the developing world. Microbes Infect 2003;5(8):705–713. [PubMed: 12814771]
15. Nelson E, Harris J, Morris JG, Calderwood SB, Camilli A. Cholera transmission: the host, pathogen and bacteriophage dynamic. Nat. Rev. Microbiol 2009;7(10):693–702. [PubMed: 19756008]
16. Weil AA, Khan A, Chowdhury F, et al. Clinical outcomes in household contacts of patients with cholera in Bangladesh. Clin. Infect. Dis 2009;49(10):1473–1479. [PubMed: 19842974]
17. Lee SH, Butler SM, Camilli A. Selection for *in vivo* regulators of bacterial virulence. Proc. Natl Acad. Sci. USA 2001;98(12):6889–6894. [PubMed: 11391007]
18. Rui H, Ritchie JM, Bronson RT, Mekalanos JJ, Zhang Y, Waldor MK. Reactogenicity of live-attenuated *Vibrio cholerae* vaccines is dependent on flagellins. Proc. Natl Acad. Sci. USA 2010;107(9):4359–4364. [PubMed: 20160087]
19. Chowdhury MI, Sheikh A, Qadri F. Development of Peru-15 (CholeraGarde), a live-attenuated oral cholera vaccine: 1991–2009. Expert Rev. Vaccines 2009;8(12):1643–1652. [PubMed: 19943759]
20. Butler SM, Camilli A. Going against the grain: chemotaxis and infection in *Vibrio cholerae*. Nat. Rev. Microbiol 2005;3(8):611–620. [PubMed: 16012515]
21. Chitnis DS, Sharma KD, Kamat RS. Role of somatic antigen of *Vibrio cholerae* in adhesion to intestinal mucosa. J. Med. Microbiol 1982;15(1):53–61. [PubMed: 7143426]
22. Kirn T, Jude B, Taylor R. A colonization factor links *Vibrio cholerae* environmental survival and human infection. Nature 2005;438(7069):863–866. [PubMed: 16341015]
23. Kirn TJ, Taylor RK. TcpF is a soluble colonization factor and protective antigen secreted by El Tor and classical O1 and O139 *Vibrio cholerae* serogroups. Infect. Immun 2005;73(8):4461–4470. [PubMed: 16040956]
24. Sperandio V, Girón JA, Silveira WD, Kaper JB. The OmpU outer membrane protein, a potential adherence factor of *Vibrio cholerae*. Infect. Immun 1995;63(11):4433–4438. [PubMed: 7591082]
25. Pierce NF, Kaper JB, Mekalanos JJ, Cray WC. Role of cholera toxin in enteric colonization by *Vibrio cholerae* O1 in rabbits. Infect. Immun 1985;50(3):813–816. [PubMed: 4066032]
26. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ. Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl Acad. Sci. USA 1987;84(9):2833–2837. [PubMed: 2883655]
27. Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med 1988;168(4):1487–1492. [PubMed: 2902187]
28. Sánchez J, Holmgren J. Cholera toxin structure, gene regulation and pathophysiological and immunological aspects. Cell. Mol. Life Sci 2008;65(9):1347–1360. [PubMed: 18278577]

29. Ryan ET, Dhar U, Khan WA, et al. Mortality, morbidity, and microbiology of endemic cholera among hospitalized patients in Dhaka, Bangladesh. Am. J. Trop. Med. Hyg 2000;63(1–2):12–20. [PubMed: 11357989]
30. Nelson EJ, Chowdhury A, Harris J, et al. Complexity of rice-water stool from patients with *Vibrio cholerae* plays a role in the transmission of infectious diarrhea. Proc. Natl Acad. Sci. USA 2007;104(48):19091–19096. [PubMed: 18024592]
31. Darrow DC, Pratt EL. Disturbances of water and electrolytes in infantile diarrhea. Pediatrics 1949;3(2):129–156. [PubMed: 18111422]
32. Pierce NF, Banwell JG, Rupak DM, et al. Effect of intragastric glucose-electrolyte infusion upon water and electrolyte balance in Asiatic cholera. Gastroenterology 1968;55(3):333–343. [PubMed: 5675364]
33. Hirschhorn N, Kinzie JL, Sachar DB, et al. Decrease in net stool output in cholera during intestinal perfusion with glucose-containing solutions. N. Engl. J. Med 1968;279(4):176–181. [PubMed: 4968807]
34. Nalin DR, Cash RA, Islam R, Molla M, Phillips RA. Oral maintenance therapy for cholera in adults. Lancet 1968;2(7564):370–373. [PubMed: 4173788]
35. Mahalanabis D, Choudhuri AB, Bagchi NG, Bhattacharya AK, Simpson TW. Oral fluid therapy of cholera among Bangladesh refugees. Johns Hopkins Med. J 1973;132(4):197–205. [PubMed: 4698667]
36. Lindenbaum J, Greenough WB, Islam MR. Antibiotic therapy of cholera. Bull. World Health Organ 1967;36(6):871–883. [PubMed: 4865453]
37. Spira WM, Khan MU, Saeed YA, Sattar MA. Microbiological surveillance of intra-neighbourhood E1 Tor cholera transmission in rural Bangladesh. Bull. World Health Organ 1980;58(5):731–740. [PubMed: 6975182]
38. Merrell DS, Butler SM, Qadri F, et al. Host-induced epidemic spread of the cholera bacterium. Nature 2002;417(6889):642–645. [PubMed: 12050664]
39. Butler S, Nelson E, Chowdhury N, Faruque S, Calderwood S, Camilli A. Cholera stool bacteria repress chemotaxis to increase infectivity. Mol.Microbiol 2006;60(2):417–426. [PubMed: 16573690]
40. Hartley DM, Morris JG, Smith DL. Hyperinfectivity: a critical element in the ability of *V. cholerae* to cause epidemics? PLoS Med 2006;3(1):e7. [PubMed: 16318414]
41. Cash RA, Music SI, Libonati JP, Craig JP, Pierce NF, Hornick RB. Response of man to infection with *Vibrio cholerae*. II. Protection from illness afforded by previous disease and vaccine. J. Infect. Dis 1974;130(4):325–333. [PubMed: 4443613]
42. Levine MM, Nalin DR, Craig JP, et al. Immunity of cholera in man: relative role of antibacterial versus antitoxic immunity. Trans. R. Soc. Trop. Med. Hyg 1979;73(1):3–9. [PubMed: 442179]
43. Levine MM, Black RE, Clements ML, Cisneros L, Nalin DR, Young CR. Duration of infection-derived immunity to cholera. J. Infect. Dis 1981;143(6):818–820. [PubMed: 7252264]
44. Gangarosa EF, Beisel WR, Benyajati C, Sprinz H, Piyaratn P. The nature of the gastrointestinal lesion in asiatic cholera and its relation to pathogenesis: a biopsy study. Am. J. Trop. Med. Hyg 1960;9:125–135. [PubMed: 13826297]
45. Qadri F, Bhuiyan TR, Dutta KK, et al. Acute dehydrating disease caused by *Vibrio cholerae* serogroups O1 and O139 induce increases in innate cells and inflammatory mediators at the mucosal surface of the gut. Gut 2004;53(1):62–69. [PubMed: 14684578]
46. Flach C, Qadri F, Bhuiyan T, et al. Broad up-regulation of innate defense factors during acute cholera. Infect. Immun 2007;75(5):2343–2350. [PubMed: 17307946]
47. Mosley WH, Ahmad S, Benenson AS, Ahmed A. The relationship of vibriocidal antibody titre to susceptibility to cholera in family contacts of cholera patients. Bull. World Health Organ 1968;38(5):777–785. [PubMed: 5303331]
48. Glass RI, Svennerholm AM, Khan MR, Huda S, Huq MI, Holmgren J. Seroepidemiological studies of El Tor cholera in Bangladesh: association of serum antibody levels with protection. J. Infect. Dis 1985;151(2):236–242. [PubMed: 3968450]

49. Finkelstein RA. Vibriocidal antibody inhibition (VAI) analysis: a technique for the identification of the predominant vibriocidal antibodies in serum and for the detection and identification of *Vibrio cholerae* antigens. J. Immunol 1962;89:264–271.
50. Neoh SH, Rowley D. The antigens of *Vibrio cholerae* involved in the vibriocidal action of antibody and complement. J. Infect. Dis 1970;121(5):505–513. [PubMed: 4986889]
51. Burrows W, Mather AN. Studies on immunity to Asiatic cholera; the O and H antigenic structure of the cholera and related vibrios. J. Infect. Dis 1946;79(2):168–197. [PubMed: 20273896]
52. Villeneuve S, Souchon H, Riottot MM, et al. Crystal structure of an anti-carbohydrate antibody directed against *Vibrio cholerae* O1 in complex with antigen: molecular basis for serotype specificity. Proc. Natl Acad. Sci. USA 2000;97(15):8433–8438. [PubMed: 10880560]
53. Villeneuve S, Boutonnier A, Mulard LA, Fournier JM. Immunochemical characterization of an Ogawa–Inaba common antigenic determinant of *Vibrio cholerae* O1. Microbiology 1999;145(9): 2477–2484. [PubMed: 10517600]
54. McCormack WM, Chakraborty J, Rahman AS, Mosley WH. Vibriocidal antibody in clinical cholera. J. Infect. Dis 1969;120(2):192–201. [PubMed: 5387567]
55. Mosley WH, Aziz KM, Rahman AS, Chowdhury AK, Ahmed A. Field trials of monovalent Ogawa and Inaba cholera vaccines in rural Bangladesh – three years of observation. Bull. World Health Organ 1973;49(4):381–387. [PubMed: 4604478]
56. Bishop AL, Schild S, Patimalla B, Klein B, Camilli A. Mucosal immunization with *Vibrio cholerae* outer membrane vesicles provides maternal protection mediated by anti-lipopolysaccharide antibodies that inhibit bacterial motility. Infect. Immun 2010;78(10):4402–4420. [PubMed: 20679439]
57. Koelle K, Pascual M, Yunus M. Serotype cycles in cholera dynamics. Proc. Biol. Sci 2006;273(1603):2879–2886. [PubMed: 17015366]
58. Ahmed A, Bhattacharjee AK, Mosley WH. Characteristics of the serum vibriocidal and agglutinating antibodies in cholera cases and in normal residents of the endemic and non-endemic cholera areas. J. Immunol 1970;105:431–441. [PubMed: 5433700]
59. Merritt CB, Sack RB. Sensitivity of agglutinating and vibriocidal antibodies to 2-mercaptoethanol in human cholera. J. Infect. Dis 1970;121 Suppl. 121:S25–S30.
60. Ahrenstedt O, Knutson L, Nilsson B, Nilsson-Ekdahl K, Odlind B, Hällgren R. Enhanced local production of complement components in the small intestines of patients with Crohn's disease. N. Engl. J. Med 1990;322(19):1345–1349. [PubMed: 2325733]
61. Ogundele MO. Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement. Immunol. Cell. Biol 2001;79(1):1–10. [PubMed: 11168616]
62. McCleery JL, Kraft SC, Rothberg RM. Demonstration of antibody in rabbit feces after active or passive parenteral immunization. Digestion 1970;3(4):213–221. [PubMed: 5469636]
63. Simister NE, Mostov KE. An Fc receptor structurally related to MHC class I antigens. Nature 1989;337(6203):184–187. [PubMed: 2911353]
64. Yoshida M, Claypool SM, Wagner JS, et al. Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity 2004;20(6): 769–783. [PubMed: 15189741]
65. Maaser C, Housley MP, Iimura M, et al. Clearance of *Citrobacter rodentium* requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. Infect. Immun 2004;72(6):3315–3324. [PubMed: 15155635]
66. Yoshida M, Kobayashi K, Kuo TT, et al. Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. J. Clin. Invest 2006;116(8):2142–2151. [PubMed: 16841095]
67. Masuda A, Yoshida M, Shiomi H, et al. Fcg receptor regulation of *Citrobacter rodentium* infection. Infect. Immun 2008;76(4):1728–1737. [PubMed: 18227164]
68. Tomasi TB. Structure and function of mucosal antibodies. Annu. Rev. Med 1970;21:281–298. [PubMed: 4192968]
69. Strugnell RA, Wijburg OLC. The role of secretory antibodies in infection immunity. Nat. Rev. Microbiol 2010;8(9):656. [PubMed: 20694027]

70. Apter FM, Michetti P, Winner LS, Mack JA, Mekalanos JJ, Neutra MR. Analysis of the roles of antilipopolysaccharide and anti-cholera toxin immunoglobulin A (IgA) antibodies in protection against *Vibrio cholerae* and cholera toxin by use of monoclonal IgA antibodies *in vivo*. Infect. Immun 1993;61(12):5279–5285. [PubMed: 8225601]

71. Harris JB, LaRocque RC, Chowdhury F, et al. Susceptibility to *Vibrio cholerae* infection in a cohort of household contacts of patients with cholera in Bangladesh. PLoS Negl. Trop. Dis 2008;2(4):e221. [PubMed: 18398491]

72. Harris AM, Bhuiyan MS, Chowdhury F, et al. Antigen specific memory B-cell responses to *Vibrio cholerae* O1 infection in Bangladesh. Infect. Immun 2009;77(9):3850–3856. [PubMed: 19528207] • Demonstration that memory B-cell responses, to TcpA and cholera toxin B subunit in particular, last longer than vibriocidal or anti-*Vibrio cholerae* serum IgA and may be a better correlate to protection after natural infection.

73. Benenson AS, Joseph PR, Oseasohn RO. Cholera vaccine field trials in east Pakistan. 1. Reaction and antigenicity studies. Bull. World Health Organ 1968;38(3):347–357. [PubMed: 5302328]

74. Svennerholm AM, Hanson LA, Holmgren J, Lindblad BS, Nilsson B, Quereshi F. Different secretory immunoglobulin A antibody responses to cholera vaccination in Swedish and Pakistani women. Infect. Immun 1980;30(2):427–430. [PubMed: 7439987]

75. Elson CO, Ealding W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J. Immunol 1984;132(6):2736–2741. [PubMed: 6233359]

76. Black RE, Levine MM, Clements ML, Young CR, Svennerholm AM, Holmgren J. Protective efficacy in humans of killed whole-vibrio oral cholera vaccine with and without the B subunit of cholera toxin. Infect. Immun 1987;55(5):1116–1120. [PubMed: 3552989]

77. Hanne LF, Finkelstein RA. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun 1982;36(1):209–214. [PubMed: 7076294]

78. Clemens J. Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet 1990;335(8684):270–273. [PubMed: 1967730] • Report of a whole-cell killed (WCK) cholera vaccine trial showing approximately 50% overall efficacy in an endemic area over 3 years, but that children who were protected similarly to adults for up to 6 months were no longer signficantly protected after 3 years.

79. van Loon FP, Clemens JD, Chakraborty J, et al. Field trial of inactivated oral cholera vaccines in Bangladesh: results from 5 years of follow-up. Vaccine 1996;14(2):162–166. [PubMed: 8852414]

80. Berstad AK, Holst J, Frøholm LO, et al. A nasal whole-cell pertussis vaccine induces specific systemic and cross-reactive mucosal antibody responses in human volunteers. J. Med. Microbiol 2000;49(2):157–163. [PubMed: 10670566]

81. Berstad AK, Oftung F, Korsvold GE, et al. Induction of antigen-specific T cell responses in human volunteers after intranasal immunization with a whole-cell pertussis vaccine. Vaccine 2000;18(22):2323–2330. [PubMed: 10738087]

82. Jonson G, Holmgren J, Svennerholm AM. Epitope differences in toxin-coregulated pili produced by classical and El Tor *Vibrio cholerae* O1. Microb. Pathog 1991;11(3):179–188. [PubMed: 1724789]

83. Albert MJ. *Vibrio cholerae* O139 Bengal. J. Clin. Microbiol 1994;32(10):2345–2349. [PubMed: 7814463]

84. Glass RI, Becker S, Huq MI, et al. Endemic cholera in rural Bangladesh, 1966–1980. Am. J. Epidemiol 1982;116(6):959–970. [PubMed: 7148820]

85. Nair GB, Faruque SM, Bhuiyan NA, Kamruzzaman M, Siddique AK, Sack DA. New variants of *Vibrio cholerae* O1 biotype El Tor with attributes of the classical biotype from hospitalized patients with acute diarrhea in Bangladesh. J. Clin. Microbiol 2002;40(9):3296–3299. [PubMed: 12202569]

86. Faruque SM, Chowdhury N, Kamruzzaman M, et al. Reemergence of epidemic *Vibrio cholerae* O139, Bangladesh. Emerg. Infect. Dis 2003;9(9):1116–1122. [PubMed: 14519249]

87. Owen RL, Pierce NF, Apple RT, Cray WC. M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. J. Infect. Dis 1986;153(6):1108–1118. [PubMed: 2422297]

88. Pierce NF, Cray WC, Kaper JB, Mekalanos JJ. Determinants of immunogenicity and mechanisms of protection by virulent and mutant *Vibrio cholerae* O1 in rabbits. Infect. Immun 1988;56(1): 142–148. [PubMed: 2447017]

89. Nygren E, Holmgren J, Attridge SR. Murine antibody responses following systemic or mucosal immunization with viable or inactivated *Vibrio cholerae*. Vaccine 2008;26:6784–6790. [PubMed: 18951939]

90. Pierce NF, Kaper JB, Mekalanos JJ, Cray WC, Richardson K. Determinants of the immunogenicity of live virulent and mutant *Vibrio cholerae* O1 in rabbit intestine. Infect. Immun 1987;55(2):477–481. [PubMed: 3804447]

91. Honda T, Finkelstein RA. Selection and characteristics of a *Vibrio cholerae* mutant lacking the A (ADP-ribosylating) portion of the cholera enterotoxin. Proc. Natl Acad. Sci. USA 1979;76(4): 2052–2056. [PubMed: 109838]

92. Levine MM, Black RE, Clements ML, et al. Evaluation in humans of attenuated *Vibrio cholerae* El Tor Ogawa strain Texas Star-SR as a live oral vaccine. Infect. Immun 1984;43(2):515–522. [PubMed: 6693169]

93. Levine MM, Kaper JB, Herrington D, et al. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet 1988;2(8609):467–470. [PubMed: 2900401]

94. Simanjuntak CH, O'Hanley P, Punjabi NH, et al. Safety, immunogenicity, and transmissibility of single-dose live oral cholera vaccine strain CVD 103-HgR in 24- to 59-month-old Indonesian children. J. Infect. Dis 1993;168(5):1169–1176. [PubMed: 8228350]

95. Cryz SJ, Levine MM, Kaper JB, Fürer E, Althaus B. Randomized double-blind placebo controlled trial to evaluate the safety and immunogenicity of the live oral cholera vaccine strain CVD 103-HgR in Swiss adults. Vaccine 1990;8(6):577–580. [PubMed: 2087879]

96. Tacket CO, Losonsky G, Nataro JP, et al. Onset and duration of protective immunity in challenged volunteers after vaccination with live oral cholera vaccine CVD 103-HgR. J. Infect. Dis 1992;166(4):837–841. [PubMed: 1527420]

97. Suharyono, Simanjuntak C, Witham N, et al. Safety and immunogenicity of single-dose live oral cholera vaccine CVD 103-HgR in 5–9-year-old Indonesian children. Lancet 1992;340(8821):689–694. [PubMed: 1355798]

98. Richie EE, Punjabi NH, Sidharta YY, et al. Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. Vaccine 2000;18(22):2399–2410. [PubMed: 10738097]

99. Calain P, Chaine J-P, Johnson E, et al. Can oral cholera vaccination play a role in controlling a cholera outbreak? Vaccine 2004;22(19):2444–2451. [PubMed: 15193408]

100. Michalski J, Galen JE, Fasano A, Kaper JB. CVD110, an attenuated *Vibrio cholerae* O1 El Tor live oral vaccine strain. Infect. Immun 1993;61(10):4462–4468. [PubMed: 8406837]

101. Tacket CO, Losonsky G, Nataro JP, et al. Safety and immunogenicity of live oral cholera vaccine candidate CVD 110, a Δ *ctxA* Δ *zot* Δ *ace* derivative of El Tor Ogawa *Vibrio cholerae*. J. Infect. Dis 1993;168(6):1536–1540. [PubMed: 8245542]

102. Silva TM, Schleupner MA, Tacket CO, et al. New evidence for an inflammatory component in diarrhea caused by selected new, live attenuated cholera vaccines and by El Tor and O139 *Vibrio cholerae*. Infect. Immun 1996;64(6):2362–2364. [PubMed: 8675353]

103. Tacket CO, Kotloff KL, Losonsky G, et al. Volunteer studies investigating the safety and efficacy of live oral El Tor *Vibrio cholerae* O1 vaccine strain CVD 111. Am. J. Trop. Med. Hyg 1997;56(5):533–537. [PubMed: 9180604]

104. Fullner K. The contribution of accessory toxins of *Vibrio cholerae* O1 El Tor to the proinflammatory response in a murine pulmonary cholera model. J. Exp. Med 2002;195(11): 1455–1462. [PubMed: 12045243]

105. Lin W, Fullner KJ, Clayton R, et al. Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. Proc. Natl Acad. Sci. USA 1999;96(3):1071–1076. [PubMed: 9927695]

106. Levine MM, Kaper JB, Herrington D, et al. Volunteer studies of deletion mutants of *Vibrio cholerae* O1 prepared by recombinant techniques. Infect. Immun 1988;56(1):161–167. [PubMed: 3335402]

107. Ketley JM, Michalski J, Galen J, Levine MM, Kaper JB. Construction of genetically marked *Vibrio cholerae* O1 vaccine strains. FEMS Microbiol. Lett 1993;111(1):15–21. [PubMed: 8359676]

108. Taylor DN, Tacket CO, Losonsky G, et al. Evaluation of a bivalent (CVD 103-HgR/CVD 111) live oral cholera vaccine in adult volunteers from the United States and Peru. Infect. Immun 1997;65(9):3852–3856. [PubMed: 9284163]

109. Tacket CO, Losonsky G, Nataro JP, et al. Initial clinical studies of CVD 112 *Vibrio cholerae* O139 live oral vaccine: safety and efficacy against experimental challenge. J. Infect. Dis 1995;172(3):883–886. [PubMed: 7658089]

110. Kenner JR, Coster TS, Taylor DN, et al. Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. J. Infect. Dis 1995;172(4):1126–1129. [PubMed: 7561195]

111. Taylor DN, Killeen KP, Hack DC, et al. Development of a live, oral, attenuated vaccine against El Tor cholera. J. Infect. Dis 1994;170(6):1518–1523. [PubMed: 7995992]

112. Cohen MB, Giannella RA, Bean J, et al. Randomized, controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. Infect. Immun 2002;70(4):1965–1970. [PubMed: 11895960]

113. Qadri F, Chowdhury MI, Faruque SM, et al. Randomized, controlled study of the safety and immunogenicity of Peru-15, a live attenuated oral vaccine candidate for cholera, in adult volunteers in Bangladesh. J. Infect. Dis 2005;192(4):573–579. [PubMed: 16028125]

114. Qadri F, Chowdhury MI, Faruque SM, et al. Peru-15, a live attenuated oral cholera vaccine, is safe and immunogenic in Bangladeshi toddlers and infants. Vaccine 2007;25(2):231–238. [PubMed: 16996172]

115. Coster TS, Killeen KP, Waldor MK, et al. Safety, immunogenicity, and efficacy of live attenuated *Vibrio cholerae* O139 vaccine prototype. Lancet 1995;345(8955):949–952. [PubMed: 7715293]

116. Albert MJ, Alam K, Rahman AS, Huda S, Sack RB. Lack of cross-protection against diarrhea due to *Vibrio cholerae* O1 after oral immunization of rabbits with *V. cholerae* O139 Bengal. J. Infect. Dis 1994;169(3):709–710. [PubMed: 8158063]

117. Albert MJ, Alam K, Ansaruzzaman M, Qadri F, Sack RB. Lack of cross-protection against diarrhea due to *Vibrio cholerae* O139 (Bengal strain) after oral immunization of rabbits with *V. cholerae* O1 vaccine strain CVD103-HgR. J. Infect. Dis 1994;169(1):230–231. [PubMed: 8277193]

118. Svennerholm AM, Holmgren J. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun 1976;13(3):735–740. [PubMed: 1270131]

119. Peterson JW. Synergistic protection against experimental cholera by immunization with cholera toxoid and vaccine. Infect. Immun 1979;26(2):528–533. [PubMed: 546785]

120. Pierce NF, Kaniecki EA, Northrup RS. Protection against experimental cholera by antitoxin. J. Infect. Dis 1972;126(6):606–616. [PubMed: 4664117]

121. Chatterjee SN, Das J. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. J. Gen. Microbiol 1967;49(1):1–11. [PubMed: 4168882]

122. Eubanks ER, Guentzel MN, Berry LJ. Evaluation of surface components of *Vibrio cholerae* as protective immunogens. Infect. Immun 1977;15(2):533–538. [PubMed: 844905]

123. Tan LK, Carlone GM, Borrow R. Advances in the development of vaccines against *Neisseria meningitidis*. N. Engl. J. Med 2010;362(16):1511–1520. [PubMed: 20410516]

124. Schild S, Nelson EJ, Camilli A. Immunization with *Vibrio cholerae* outer membrane vesicles induces protective immunity in mice. Infect. Immun 2008;76(10):4554–4563. [PubMed: 18678672]

125. Schild S, Nelson EJ, Bishop AL, Camilli A. Characterization of *Vibrio cholerae* outer membrane vesicles as a candidate vaccine for cholera. Infect. Immun 2009;77(1):472–484. [PubMed: 19001078]

126. Acevedo R, Callicó A, Campo JD, et al. Intranasal administration of proteoliposome-derived cochleates from *Vibrio cholerae* O1 induce mucosal and systemic immune responses in mice. Methods 2009;49(4):309–315. [PubMed: 19545630]

127. Mann JF, Acevedo R, Campo JD, Perez O, Ferro VA. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? Expert Rev. Vaccines 2009;8(1):103–112. [PubMed: 19093777]

128. Chaicumpa W, Parairo JR, New RC, et al. Immunogenicity of liposome-associated oral cholera vaccine prepared from combined *Vibrio cholerae* antigens. Asian Pac. J. Allergy Immunol 1990;8(2):87–94. [PubMed: 2091663]

129. Kalambaheti T, Chaisri U, Srimanote P, Pongponratn E, Chaicumpa W. Immunogenicity and protective role of three formulations of oral cholera vaccine. Vaccine 1998;16(2–3):201–207. [PubMed: 9607031]

130. Chandrasekhar U, Sinha S, Bhagat HR, Sinha VB, Srivastava BS. Comparative efficacy of biodegradable liposomes and microspheres as carriers for delivery of *Vibrio cholerae* antigens in the intestine. Vaccine 1994;12(15):1384–1388. [PubMed: 7887015]

131. Eko FO, Schukovskaya T, Lotzmanova EY, et al. Evaluation of the protective efficacy of *Vibrio cholerae* ghost (VCG) candidate vaccines in rabbits. Vaccine 2003;21(25–26):3663–3674. [PubMed: 12922096]

132. Jalava K, Eko FO, Riedmann E, Lubitz W. Bacterial ghosts as carrier and targeting systems for mucosal antigen delivery. Expert Rev. Vaccines 2003;2(1):45–51. [PubMed: 12901596]

133. Kadurugamuwa JL, Beveridge TJ. Delivery of the non-membrane-permeative antibiotic gentamicin into mammalian cells by using *Shigella flexneri* membrane vesicles. Antimicrob. Agents Chemother 1998;42(6):1476–1483. [PubMed: 9624497]

134. Fiocca R, Necchi V, Sommi P, et al. Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. J. Pathol 1999;188(2):220–226. [PubMed: 10398168]

135. Bauman SJ, Kuehn MJ. *Pseudomonas aeruginosa* vesicles associate with and are internalized by human lung epithelial cells. BMC Microbiol 2009;9:26. [PubMed: 19192306]

136. Ali M, Emch M, Von Seidlein L, et al. Herd immunity conferred by killed oral cholera vaccines in Bangladesh: a reanalysis. Lancet 2005;366(9479):44–49. [PubMed: 15993232] • Reanalysis of the 1985 oral WCK cholera vaccine field trial data showing that herd immunity is proportional to vaccine coverage, which ranged from 4 to 65%, and can significantly protect nonvaccinees with as low as approximately 30% vaccine coverage in a cholera-endemic area.

137. Longini IM, Nizam A, Ali M, Yunus M, Shenvi N, Clemens JD. Controlling endemic cholera with oral vaccines. PLoS Med 2007;4(11):e336. [PubMed: 18044983] • Use of a mathematical model to show the penetrance of immunization and protective efficacy required to result in approximately 80% protection via direct and herd immunity with various proportions of susceptible individuals, based on data from [136].

138. Lopez AL, Clemens JD, Deen J, Jodar L. Cholera vaccines for the developing world. Hum. Vaccin 2008;4(2):165–169. [PubMed: 18414029]

139. Cumberland S. An old enemy returns. Bull. World Health Organ 2009;87(2):85–86. [PubMed: 19274355]

140. Thiem VD, Deen JL, Von Seidlein L, et al. Long-term effectiveness against cholera of oral killed whole-cell vaccine produced in Vietnam. Vaccine 2006;24(20):4297–4303. [PubMed: 16580760]

141. Trach DD, Clemens JD, Ke NT, et al. Field trial of a locally produced, killed, oral cholera vaccine in Vietnam. Lancet 1997;349(9047):231–235. [PubMed: 9014909]

142. Trach DD, Cam PD, Ke NT, et al. Investigations into the safety and immunogenicity of a killed oral cholera vaccine developed in Vietnam. Bull. World Health Organ 2002;80(1):2–8. [PubMed: 11884967]

143. Anh DD, Canh doG, Lopez A, et al. Safety and immunogenicity of a reformulated Vietnamese bivalent killed, whole-cell, oral cholera vaccine in adults. Vaccine 2007;25(6):1149–1155. [PubMed: 17055622]

144. WHO. WHO Expert Committee on Biological Standardization: Fifty-second report. Annex 3. Geneva, Switzerland: WHO; 2004. Guidelines for the Production and Control of Inactivated Oral Cholera Vaccines. p. 129-149.

145. Mahalanabis D, Lopez AL, Sur D, et al. A randomized, placebo-controlled trial of the bivalent killed, whole-cell, oral cholera vaccine in adults and children in a cholera endemic area in Kolkata, India. PLoS ONE 2008;3(6):e2323. [PubMed: 18523643]

146. Kanungo S, Paisley A, Lopez AL, et al. Immune responses following one and two doses of the reformulated, bivalent, killed, whole-cell, oral cholera vaccine among adults and children in Kolkata, India: a randomized, placebo-controlled trial. Vaccine 2009;27(49):6887–6893. [PubMed: 19761838]

147. Sur D, Lopez AL, Kanungo S, et al. Efficacy and safety of a modified killed-whole-cell oral cholera vaccine in India: an interim analysis of a cluster-randomised, double-blind, placebo-controlled trial. Lancet 2009;374(9702):1694–1702. [PubMed: 19819004] • Report of efficacy for a reformulated more affordable version of the oral WCK cholera vaccine 2 years into the trial is promising, showing protection from clinically relevant disease of around 67%, regardless of age.

148. Deb BC, Sircar BK, Sengupta PG, et al. Studies on interventions to prevent eltor cholera transmission in urban slums. Bull. World Health Organ 1986;64(1):127–131. [PubMed: 3488134]

149. Colwell RR, Huq A, Islam MS, et al. Reduction of cholera in Bangladeshi villages by simple filtration. Proc. Natl Acad. Sci. USA 2003;100(3):1051–1055. [PubMed: 12529505]

150. Chen D, Kristensen D. Opportunities and challenges of developing thermostable vaccines. Expert Rev. Vaccines 2009;8(5):547–557. [PubMed: 19397412]

151. Lucas MES, Deen JL, Von Seidlein L, et al. Effectiveness of mass oral cholera vaccination in Beira, Mozambique. N. Engl. J. Med 2005;352(8):757–767. [PubMed: 15728808]

152. Perry RT, Plowe CV, Koumaré B, et al. A single dose of live oral cholera vaccine CVD 103-HgR is safe and immunogenic in HIV-infected and HIV-noninfected adults in Mali. Bull World Health Organ 1998;76(1):63–71. [PubMed: 9615498]

153. Chen DJ, Osterrieder N, Metzger SM, et al. Delivery of foreign antigens by engineered outer membrane vesicle vaccines. Proc. Natl Acad. Sci. USA 2010;107(7):3099–3104. [PubMed: 20133740]

154. Eko F, Lubitz W, McMillan L, et al. Recombinant *Vibrio cholerae* ghosts as a delivery vehicle for vaccinating against *Chlamydia trachomatis*. Vaccine 2003;21(15):1694–1703. [PubMed: 12639492]

155. Ryan E, Calderwood SB, Qadri F. Live attenuated oral cholera vaccines. Expert Rev. Vaccines 2006;5(4):483–494. [PubMed: 16989629]

156. Jelinek T, Kollaritsch H. Vaccination with Dukoral against travelers' diarrhea (ETEC) and cholera. Expert Rev. Vaccines 2008;7(5):561–567. [PubMed: 18564011]

157. Deen JL, Von Seidlein L, Sur D, et al. The high burden of cholera in children: comparison of incidence from endemic areas in Asia and Africa. PLoS Negl. Trop. Dis 2008;2(2):e173. [PubMed: 18299707]

158. Humphrey JH. Child undernutrition, tropical enteropathy, toilets, and handwashing. Lancet 2009;374(9694):1032–1035. [PubMed: 19766883]

159. Albert MJ, Qadri F, Wahed MA, et al. Supplementation with zinc, but not vitamin A, improves seroconversion to vibriocidal antibody in children given an oral cholera vaccine. J. Infect. Dis 2003;187(6):909–913. [PubMed: 12660937]

160. Ahmed T, Svennerholm AM, Al Tarique A, Sultana GN, Qadri F. Enhanced immunogenicity of an oral inactivated cholera vaccine in infants in Bangladesh obtained by zinc supplementation and by temporary withholding breast-feeding. Vaccine 2009;27(9):1433–1439. [PubMed: 19146904]

161. Gunn RA, Kimball AM, Pollard RA, et al. Bottle feeding as a risk factor for cholera in infants. Lancet 1979;2(8145):730–732. [PubMed: 90813]

162. Clemens JD, Sack DA, Harris JR, et al. Breast feeding and the risk of severe cholera in rural Bangladeshi children. Am. J. Epidemol 1990;131(3):400–411.

163. Newburg D, Ruiz-Palacios G, Morrow A. Human milk glycans protect infants against enteric pathogens. Annu. Rev. Nutr 2005;25:37–58. [PubMed: 16011458]

164. Holmgren J, Svennerholm AM, Lindblad M. Receptor-like glycocompounds in human milk that inhibit classical and El Tor *Vibrio cholerae* cell adherence (hemagglutination). Infect. Immun 1983;39(1):147–154. [PubMed: 6295953]

165. Clemens JD, Sack DA, Chakraborty J, et al. Field trial of oral cholera vaccines in Bangladesh: evaluation of anti-bacterial and anti-toxic breast-milk immunity in response to ingestion of the vaccines. Vaccine 1990;8(5):469–472. [PubMed: 2251873]

166. Jertborn M, Svennerholm AM, Holmgren J. Saliva, breast milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. J. Clin. Microbiol 1986;24(2):203–209. [PubMed: 3528211]

167. Saroso JS, Bahrawi W, Witjaksono H, et al. A controlled field trial of plain and aluminium hydroxide-adsorbed cholera vaccines in Surabaya, Indonesia, during 1973–75. Bull. World Health Organ 1978;56(4):619–627. [PubMed: 365388]

168. Gupta RK, Szu SC, Finkelstein RA, Robbins JB. Synthesis, characterization, and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. Infect. Immun 1992;60(8):3201–3208. [PubMed: 1639490]

169. Kossaczka Z, Shiloach J, Johnson V, et al. *Vibrio cholerae* O139 conjugate vaccines: synthesis and immunogenicity of *V. cholerae* O139 capsular polysaccharide conjugates with recombinant diphtheria toxin mutant in mice. Infect. Immun 2000;68(9):5037–5043. [PubMed: 10948122]

170. Boutonnier A, Villeneuve S, Nato F, Dassy B, Fournier JM. Preparation, immunogenicity, and protective efficacy, in a murine model, of a conjugate vaccine composed of the polysaccharide moiety of the lipopolysaccharide of *Vibrio cholerae* O139 bound to tetanus toxoid. Infect. Immun 2001;69(5):3488–3493. [PubMed: 11292781]

171. Chernyak A, Kondo S, Wade TK, et al. Induction of protective immunity by synthetic *Vibrio cholerae* hexasaccharide derived from *V. cholerae* O1 Ogawa lipopolysaccharide bound to a protein carrier. J. Infect. Dis 2002;185(7):950–962. [PubMed: 11920320]

172. Czerkinsky C, Holmgren J. Topical immunization strategies. Mucosal Immunol 2010;3:545–555. [PubMed: 20861833]

173. Rollenhagen JE, Kalsy A, Saksena R, et al. Transcutaneous immunization with a synthetic hexasaccharide-protein conjugate induces anti-*Vibrio cholerae* lipopolysaccharide responses in mice. Vaccine 2009;27(36):4917–4922. [PubMed: 19563890]

174. Katial RK, Brandt BL, Moran EE, Marks S, Agnello V, Zollinger WD. Immunogenicity and safety testing of a group B intranasal meningococcal native outer membrane vesicle vaccine. Infect. Immun 2002;70(2):702–707. [PubMed: 11796602]

175. Haneberg B, Holst J. Can nonliving nasal vaccines be made to work? Expert Rev. Vaccines 2002;1(2):227–232. [PubMed: 12901561]

176. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J. Immunol 2000;165(9):4778–4782. [PubMed: 11045998]

177. Mutsch M, Zhou W, Rhodes P, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N. Engl. J. Med 2004;350(9):896–903. [PubMed: 14985487]

178. Rudin A, Johansson EL, Bergquist C, Holmgren J. Differential kinetics and distribution of antibodies in serum and nasal and vaginal secretions after nasal and oral vaccination of humans. Infect. Immun 1998;66(7):3390–3396. [PubMed: 9632610]

179. Sengupta DK, Sengupta TK, Ghose AC. Antibodies to outer membrane proteins of *Vibrio cholerae* induce protection by inhibition of intestinal colonization of vibrios. FEMS Microbiol. Immunol 1992;4(5):261–266. [PubMed: 1515155]

180. Weil AA, Arifuzzaman M, Bhuiyan TR, et al. Memory T cell responses to *Vibrio cholerae* O1 infection. Infect. Immun 2009:5090–5096. [PubMed: 19703973] • Demonstration that memory T cells responding to *V. cholerae* protein antigens appear just before memory B cells in convalescent patients and that *V. cholerae* hemolysin HlyA generates particularly robust and long-term T-cell memory.

181. Hang L, John M, Asaduzzaman M, et al. Use of in vivo-induced antigen technology (IVIAT) to identify genes uniquely expressed during human infection with *Vibrio cholerae*. Proc. Natl Acad. Sci. USA 2003;100(14):8508–8513. [PubMed: 12826608]

182. Larocque R, Krastins B, Harris J, et al. Proteomic analysis of *Vibrio cholerae* in human stool. Infect. Immun 2008;76(9):4145–4151. [PubMed: 18591230]

183. Sun DX, Mekalanos JJ, Taylor RK. Antibodies directed against the toxin-coregulated pilus isolated from *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. J. Infect. Dis 1990;161(6):1231–1236. [PubMed: 1971653]

184. Sun DX, Seyer JM, Kovari I, Sumrada RA, Taylor RK. Localization of protective epitopes within the pilin subunit of the *Vibrio cholerae* toxin-coregulated pilus. Infect. Immun 1991;59(1):114–118. [PubMed: 1702758]

185. Asaduzzaman M, Ryan E, John M, et al. The major subunit of the toxin-coregulated pilus TcpA induces mucosal and systemic immunoglobulin A immune responses in patients with cholera caused by *Vibrio cholerae* O1 and O139. Infect. Immun 2004;72(8):4448–4454. [PubMed: 15271902]

186. Wu JY, Wade WF, Taylor RK. Evaluation of cholera vaccines formulated with toxin-coregulated pilin peptide plus polymer adjuvant in mice. Infect. Immun 2001;69(12):7695–7702. [PubMed: 11705950]

187. Meeks MD, Wade TK, Taylor RK, Wade WF. Immune response genes modulate serologic responses to *Vibrio cholerae* TcpA pilin peptides. Infect. Immun 2001;69(12):7687–7694. [PubMed: 11705949]

Table 1

Mucosal cholera vaccination: pros and cons.

Pros	Cons
Mimics natural infection • Protective for at least 3 years	**Mimics natural infection** • Not protective for life, possibly due to dominant short-term B-cell memory responses to lipopolysaccharide • Lipopolysaccharide-dominant response leads to a lack of cross-serogroup protection and may lead to poor protection of children
Can induce IgA responses • Circulating anti-*Vibrio cholerae* IgA, but not IgG, appears to be a correlate of protection	**Difficult to overcome mucosal tolerance** • A problem for oral or nasal immunization with nonliving vaccines • May be overcome with mucosal adjuvants • Unclear how whole-cell killed *V. cholerae* vaccines delivered orally overcome this tolerance
Needle-free • No need for trained medics, clean needles or disposal of contaminated sharps • Particularly important in developing countries with limited resources	**Antigens surviving stomach acid and digestive enzymes** • Problem for oral delivery, making choice of delivery vehicle particularly important **Retaining immunogenicity without reactogenicity** • Especially difficult for live-attenuated vaccines
Mucosal adjuvants • Mucosal adjuvants now in development may reduce levels of antigen required	**Choice of antigen(s), adjuvant(s) and delivery vehicle** • A challenge to find the right combination for success, especially for nonliving mucosally delivered vaccines

Table 2

Examples of licensed, or previously tested, oral cholera vaccine formulations.

Vaccine (manufacturer)	Composition Strain, (CFUs or prekilling CFUs/dose), treatment or attenuation					Doses	Age if licensed	Efficacy (%), time post-vaccination	Ref.
	O1			*O139*	*CTB*				
	Inaba		*Ogawa*						
	Classical	*El Tor*	*Classical*						
Whole-cell killed									
Volunteer study: original WCK-CTB	Cairo 48, (5×10^{10}), heat killed	Phil 6973, (1×10^{11}), formalin inactivated[†]	Cairo 50, (5×10^{10}), heat killed		pCTB	Three		60 4–5 weeks	[76]
Dukoral® (Crucell, Stockholm, Sweden)	Cairo 48, (2.5×10^{10}), heat killed	Phil 6973, (2.5×10^{10}), formalin inactivated	Cairo 50, (5×10^{10}), half heat killed, half formalin inactivated		1 mg rCTB	Two	≥2 years	50 3 years	[78]
Vietnam bivalent (Vabiotech, Vietnam)	Cairo 48, (2.5×10^{10}), heat killed	Phil 6973, (5×10^{10}), formalin inactivated	Cairo 50, (2.5×10^{10}), heat killed 569B[‡], (2.5×10^{10}), formalin inactivated	4260B , (5×10^{10}), formalin inactivated		Two	≥1 year	50 3–5 years	[140,142]
Shanchol™ (Shantha Biotechnics, Hyderabad, India)	Cairo 48, (300 EU LPS), heat killed	Phil 6973, (600 EU LPS), formalin inactivated	Cairo 50[§], (600 EU LPS), half heat killed, half formalin inactivated	4260B, (600 EU LPS), formalin inactivated		Two	≥1 year	Phase III ongoing 67% 2 years	[143,147]
Live-attenuated									
CVD 103-HgR (Orochol® [Crucell])	569B *ctxA* 94% deleted *hlyA*:*mer*					One	≥6 years	87–62 1 month (American volunteers) 14% (NS) 4 years (Indonesia) 79.2 10 days (Micronesia)	[93,98,99,155]
Peru-15 (CholeraGarde® [Celldex Therapeutics, MA, USA])		C6709 ΔCTXϕ *RS1 attRS1 recA*:*ctxB*				One	>9 months	93 3 months (American volunteers) Phase II trial ongoing	[19,112]

[†]Formalin-inactivated to preserve the El Tor heat-labile antigen, mannose-sensitive hemagglutinin.

[‡]Included instead of Cario 50 found in Dukoral®, due to higher expression of TcpA.

[§] 569B included in the Vietnam vaccine replaced by Cairo 50, as was the case in Dukoral®, due to potentially higher expression of CT during vaccine preparation.

CFU: Colony-forming unit; CTB: Cholera toxin B subunit; EU LPS: Endotoxin unit lipopolysaccharide; NS: Not significant; pCTB: Purified CTB, extracted from *V. cholerae* culture supernatant; rCTB: Recombinant CTB; WCK: Whole cell killed.