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# Emerging Concepts in Cholera Vaccine Design

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## Keywords

*Vibrio cholerae*, cholera, vaccines, vaccinology

## Abstract

Cholera is a severe diarrheal disease caused by the bacterium *Vibrio cholerae* and constitutes a significant public health threat in many areas of the world. *V. cholerae* infection elicits potent and long-lasting immunity, and efforts to develop cholera vaccines have been ongoing for more than a century. Currently available inactivated two-dose oral cholera vaccines are increasingly deployed to both prevent and actively curb cholera outbreaks, and they are key components of the global effort to eradicate cholera. However, these killed whole-cell vaccines have several limitations, and a variety of new oral and nonoral cholera vaccine platforms have recently been developed. Here, we review emerging concepts in cholera vaccine design and implementation that have been driven by insights from human and animal studies. As a prototypical vaccine-preventable disease, cholera continues to be an excellent target for the development and application of cutting-edge technologies and platforms that may transform vaccinology.

## Contents



## INTRODUCTION

Vaccines are among the most commonly used and effective tools in global efforts to combat infectious diseases. Although the technology underlying successive generations of vaccines has evolved, the guiding principle of immunization—to provoke protective adaptive immunity before exposure to a virulent pathogen—has remained unchanged (105). Contemporary vaccine development has been informed by ever-increasing efforts to understand the basic biology of pathogens and the human immune system. Both domains of investigation have been propelled by the adoption of emerging high-throughput methods and refinement of nonhuman platforms for streamlined preclinical studies. Nevertheless, vaccine development remains a highly iterative and largely empiric process. In the case of cholera, more than a century of research has led to deep understanding of cholera pathogenesis, but a single-dose, highly effective vaccine for all age groups remains elusive. Recent advances in our understanding of immunity to cholera and *Vibrio cholerae* pathogenesis have led to the creation of several new vaccines that show promise for advancing toward this goal.

### *V. cholerae* and Cholera

Cholera is a severe and potentially fatal diarrheal disease caused by the gram-negative bacterial pathogen *V. cholerae* (37). Cholera is transmitted by the fecal-oral route and through contaminated food or water. The characteristic "rice-water" diarrhea of severe cholera can lead to lethal dehydration in less than a day. The disease frequently spreads in explosive epidemics that often follow natural or social disasters when public health and sanitation infrastructure become compromised. There have been six recognized cholera pandemics in the past; the seventh began in 1961 and is ongoing (37) (**Figure 1**). An estimated four million clinical cases occur globally per year, and underreporting remains a major concern (10). Major cholera outbreaks in recent history, including those in Haiti (2010–2019, more than 820,000 cases and almost 10,000 deaths) and Yemen (2016–present, more than 2.5 million cases), highlight the devastating effects of cholera on fragile health care systems.

*V. cholerae* is unusual among microbial pathogens in its capacity to robustly colonize the host small intestine. The most critical *V. cholerae* intestinal colonization factor is the toxin coregulated

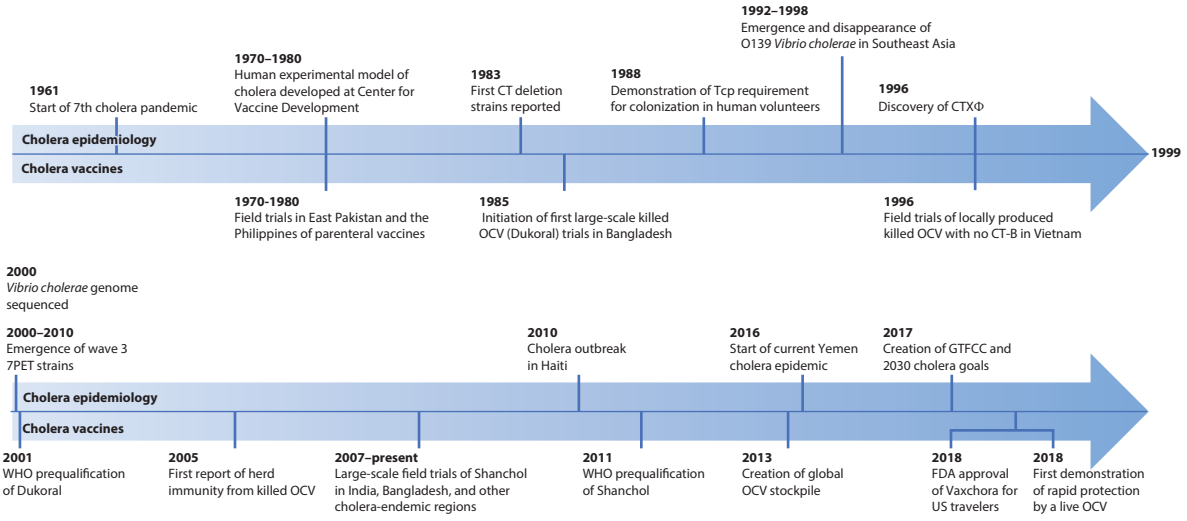


**Figure 1**

Key events in cholera epidemiology (*top rows*) and in the development of cholera vaccines (*bottom rows*). Abbreviations: 7PET, seventh-pandemic El Tor; CT, cholera toxin; FDA, US Food and Drug Administration; GTFCC, Global Task Force on Cholera Control; OCV, oral cholera vaccine; WHO, World Health Organization.

pilus (TCP), a surface-exposed, bundle-forming pilus that promotes formation of *V. cholerae* microcolonies and may promote pathogen adherence to the host intestinal epithelium (61, 79) (**Figure 2*a***). Recent transposon-insertion sequencing studies have fairly comprehensively identified the many genes, pathways, and processes that are required for *V. cholerae* intestinal colonization in animals (51, 69, 108). Within the small intestine, the pathogen secretes its hallmark virulence factor, cholera toxin (CT). CT is an $AB_5$-type toxin composed of a catalytic A subunit (CT-A) and receptor-binding B subunit (CT-B) (125). CT is not strictly required for intestinal colonization but is almost solely responsible for the secretory diarrhea characteristic of cholera. CT is endocytosed by intestinal epithelial cells, where it disrupts cAMP signaling and induces massive $H_2O$ efflux into the intestinal lumen, leading to diarrhea (**Figure 2*a***). Interestingly, CT is encoded on an integrative filamentous bacteriophage (CTXΦ) whose receptor is TCP, suggesting that in the evolution of pandemic *V. cholerae*, nontoxigenic ancestral strains first acquired the pathogenicity island containing the operon encoding the proteins required for TCP assembly and were subsequently lysogenized by CTXΦ (144).

*V. cholerae* is classified according to several genetic and phenotypic schemes (**Figure 2*b***). *V. cholerae* serogroups are determined by the structure of the O-antigen polysaccharide moiety of lipopolysaccharide (LPS) that coats the bacterial surface. While there are more than 200 recognized *V. cholerae* serogroups, only the O1 and O139 serogroups have caused large and sustained cholera outbreaks (37). The O1 serogroup has caused all seven cholera pandemics. The O139 serogroup led to large cholera epidemics in Southeast Asia in the early 1990s but is currently rarely isolated. O1 serogroup strains are further classified into two principal serotypes, Ogawa and Inaba, based on the methylation status of the terminal perosamine of the O-antigen. Ogawa (methylated) and Inaba (unmethylated) serotype strains both cause severe disease and can cocirculate and interconvert during epidemics (7). A rare third O1 serotype, Hikojima, expresses both methylated and unmethylated O-antigen.

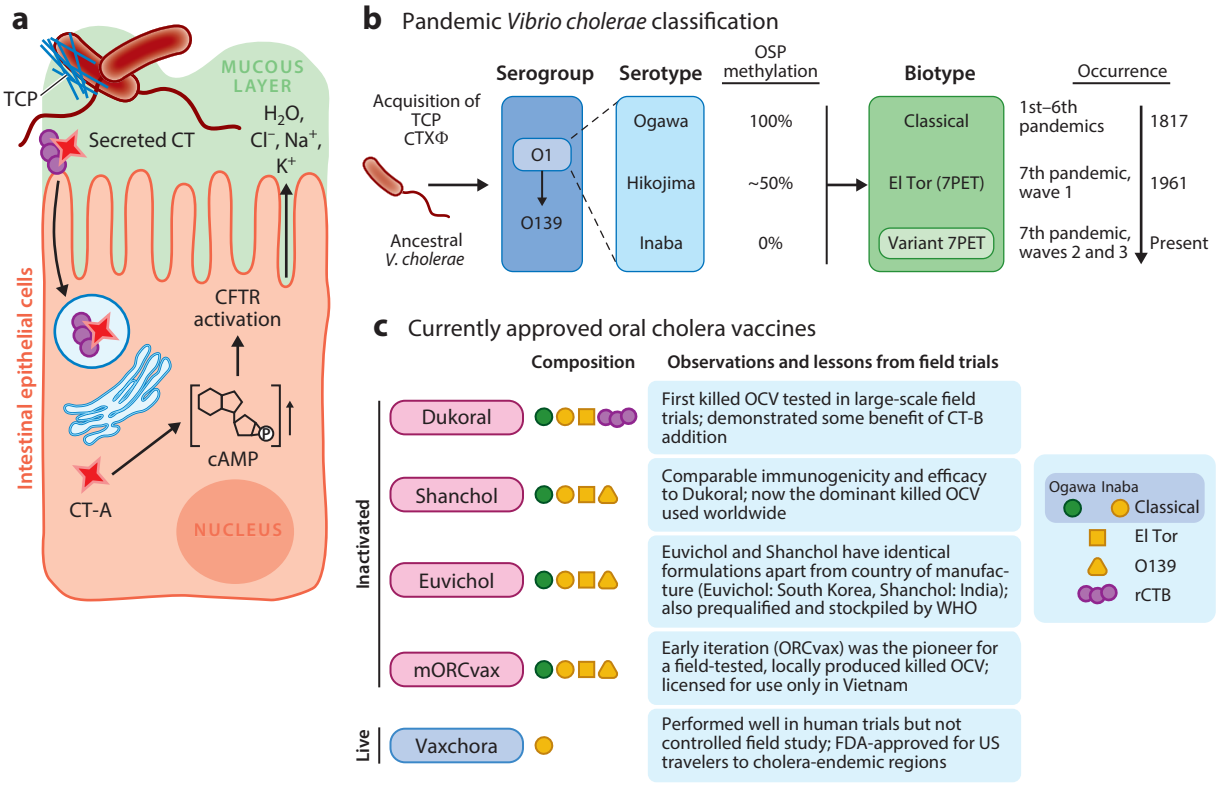


**Figure 2**

Pandemic *Vibrio cholerae* infection and classification and currently approved vaccine formulations. (*a*) Pandemic *V. cholerae*, once ingested, colonizes the human small intestine in a TCP-dependent manner, penetrating the mucous layer (*green*) and releasing CT. CT intoxicates host cells by leading to elevations in intracellular cAMP concentrations and a massive efflux of water and ions into the intestinal lumen. (*b*) Evolution and classification of pandemic *V. cholerae*. Serotype differences are important for cholera vaccine development, as immunity against *V. cholerae* is known to be serotype-biased. (*c*) Formulations of selected oral cholera vaccines and key findings from field trials. Shanchol and Euvichol are currently stockpiled by the WHO. Abbreviations: 7PET, seventh-pandemic El Tor; CT, cholera toxin; FDA, US Food and Drug Administration; OCV, oral cholera vaccine; OSP, O-specific polysaccharide; rCTB, recombinant B subunit of CT; TCP, toxin coregulated pilus; WHO, World Health Organization. Figure adapted from images created with BioRender.com.

A biotyping scheme has also been used to classify *V. cholerae* O1 strains based on several phenotypic traits (**Figure 2*b***). O1 *V. cholerae* strains belonging to the classical biotype caused the first six cholera pandemics, but they were ultimately replaced by strains of the El Tor biotype; classical *V. cholerae* is now considered extinct (37). With the emergence of next-generation DNA sequencing, whole-genome sequencing has become a new standard for classifying seventh-pandemic El Tor (7PET) isolates. Comparative genomics studies suggest that three genetically distinct waves of El Tor strains have successively emerged from the Ganges Delta since the onset of the seventh pandemic in 1961 (100). Wave 3 7PET strains emerged during 1990–2000 and are now the dominant cause of cholera globally (46, 148). Importantly, wave 3 strains have acquired distinguishable genetic features such as the *ctxB7* and $tcpA^{N89S}$ alleles of their virulence genes, with implications for the clinical presentation of cholera and the pathogen's antigenic profile (77).

In 2017, the World Health Organization (WHO) created the Global Task Force on Cholera Control and outlined a roadmap to eliminate endemic cholera in 20 countries and reduce cholera

deaths by 90% by 2030 (55) (**Figure 1**). This approach includes steps to augment public health systems and infrastructures as well as deployment of oral cholera vaccines (OCVs). Here, we focus on OCVs, the only clinical intervention called for by the roadmap.

## Protective Immunity to Cholera

Studies with convalescent cholera patients in both natural infection and volunteer challenge settings have yielded important insights about human immunity to cholera (113). Repeated observations that infection with *V. cholerae* confers strong immunity to disease from future exposure underscore the feasibility of vaccines as an approach to combat this pathogen (86). As the exact mechanism(s) of immune-mediated protection against cholera remains unknown, correlates of protection are often invoked to describe the immune response to cholera. Correlates of protection are immune metrics that correlate with, but do not necessarily account for, protection against disease (66). The best-characterized cholera correlate of protection is vibriocidal antibody titer (VAT), a measure of serum antibodies that inhibit *V. cholerae* growth in vitro. VATs can be easily measured from blood samples and are well-suited for large-scale clinical trials. High VATs correlate well with short-term protection in adults, but they are less useful in children and wane after infection and immunization by 6–12 months, rendering them inappropriate as a long-term surrogate measure of vaccine efficacy (1, 66, 117). There is also no known VAT threshold at which protection is absolute—populations in endemic regions often have higher VAT baselines, likely due to repeated subclinical exposures to *V. cholerae* that boost immunity (19, 124).

Early epidemiological and clinical studies suggested that immune responses to the *V. cholerae* O-antigen ("antibacterial") play a dominant role compared to those against CT ("antitoxic") in protective immunity against cholera (88, 101, 128). This was further demonstrated by observations that O1 and O139 *V. cholerae* infections were not cross-protective, strongly suggesting that immune responses to the O-antigen are protective (5–7). This concept has been experimentally tested by recent studies of one correlate of protection, antibodies against the O-specific polysaccharide (OSP, essentially the O-antigen). Several studies have now shown that anti-OSP antibodies may function in protection by blocking *V. cholerae* motility in vivo, potentially inhibiting the pathogen from accessing its preferred niche in the intestine (25, 32, 145). It remains an open question how serum-derived antibodies that target OSP access the intestinal lumen, although it seems likely that mucosal anti-OSP secretory IgA also contributes to protection. As additional correlates of protection are found and investigated in human settings, a more complete model of immunity to cholera, as well as methods to more effectively track cholera incidence in endemic regions and outbreaks, is likely to emerge (20, 121).

## Landscape and Limitations of Current Cholera Vaccines

The long arc of cholera vaccine development (reviewed extensively in References 63 and 93) has involved dozens of vaccine candidates and widespread clinical studies. The first recognized cholera vaccination campaign was conducted in 1885 by Jaime Ferrán y Clúa, just two years after the isolation of *V. cholerae* by Robert Koch (26). Ferrán's vaccine consisted of intramuscularly injected live virulent *V. cholerae*. It resulted in an apparent protective efficacy (PE) of more than 80% but was accompanied by high frequencies of systemic adverse effects (26). Like this vaccine, the first widely used cholera vaccines were injected (parenteral) formulations that consisted of killed or live whole-cell *V. cholerae*. These vaccines conferred some protection against cholera but were thought to have modest and short-lived PE and unfavorable safety profiles. Although retrospective meta-analysis has suggested their drawbacks may have been overestimated, by the 1980s parenteral cholera vaccines had been superseded by killed OCVs (56). Compared to injected vaccines, OCVs

have the key advantage of being mucosal vaccines. They are able to induce immune responses at the intestinal surface, the primary colonization site of *V. cholerae*.

The first major killed OCV to be developed was Dukoral, a mixture of three heat- or formalin-inactivated O1 pre–wave 3 7PET *V. cholerae* strains (classical Inaba Cairo 48, classical Ogawa Cairo 50, and El Tor Inaba Phil 6973) and recombinantly produced CT-B. Large, placebo-controlled field trials of Dukoral in Bangladesh beginning in 1985 were instrumental in demonstrating the utility of OCVs in an endemic region (38) (**Figure 1**). At six months after immunization, the PEs of triple doses of Dukoral with and without CT-B were 85% and 53%, respectively, indicating that adding CT-B to a killed OCV provides a protective benefit (38). However at one year and at three years, although moderate PE (~40–50%) was maintained, the benefit of CT-B addition was lost (38, 39). A five-year follow-up study revealed that PE of both formulations was lost after three years (142).

CT-B was removed from subsequently developed OCVs to simplify manufacturing and improve vaccine stability, but the Dukoral strain composition was retained. Addition of a killed O139 *V. cholerae* strain after the 1992–1996 O139 epidemic led to the current versions of these vaccines: Shanchol, Euvichol, and mORCvax in India, South Korea, and Vietnam, respectively (**Figure 2*c***). These vaccines, which are delivered as two doses spaced 14 days apart, were first tested in Vietnam and exhibited a PE of 66% at one year, comparing favorably to Dukoral (139). Similar PE results were observed in a five-year study of Shanchol in India (23, 136). These findings were largely replicated in a recently completed five-year urban feasibility trial of Shanchol in Bangladesh (11, 109). Reanalysis of data from the Dukoral trial in 2005 and subsequent studies of Shanchol have revealed strong signals of herd immunity, further buttressing the case for use of OCVs in endemic regions (8, 9, 12). The 2010 cholera outbreak in Haiti provided an example of the need for readily accessible OCV inventories. The WHO established a global cholera vaccine stockpile in 2013 and now distributes approximately 20 million doses of OCVs each year (24, 104, 143). Substantially more doses are requested than the WHO distributes, suggesting that increased manufacturing and storage of killed OCVs is necessary.

Despite their demonstrated efficacy in the field, killed OCVs have important shortcomings (**Table 1**). Field trials of both Shanchol and Dukoral have consistently reported low immunogenicity and minimal PE in children younger than five years, the age group most susceptible to death and long-term effects from cholera (18, 23, 85, 142). Killed OCVs also require multiple doses to induce high-level protection, may not provide sufficient protection beyond five years, and have a characteristic lag between dosing and onset of immunity (typically one to two weeks); these limitations may be inherent to killed whole-cell OCVs. The formalin and heat used to kill the *V. cholerae* strains in these vaccines may destroy or alter protein epitopes that could contribute to PE (45); in some sense, these vaccines likely function primarily as oral Inaba and Ogawa LPS doses. The conditions used to manufacture these vaccines also do not mimic those encountered by *V. cholerae* in vivo, and as a result these vaccines do not express known in vivo–induced antigens such as TcpA, potentially restricting killed OCV immunogenicity and efficacy (16, 60, 118).

Live attenuated OCVs, which mimic natural infection with *V. cholerae*, have the potential to circumvent many of the limitations of killed OCVs. Attempts to create live attenuated vaccines began in the late 1970s and were propelled forward with the advent of genetic engineering technologies that enabled site-directed alterations to the *V. cholerae* genome, particularly deletion of *ctxA* (72, 98). In contrast to killed OCVs, live attenuated OCVs express TCP and transiently colonize the small intestine, where they express the suite of in vivo–expressed *V. cholerae* antigens and therefore provoke immune responses to a wide range of antigens (120, 147). Only one live OCV, Vaxchora, a Δ*ctxA* derivative of a classical O1 Inaba *V. cholerae* strain, has been approved in the United States for travelers to cholera-endemic regions (87) (**Figure 2*c***). Vaxchora had a single-dose PE of more

## Table 1 Approved and candidate cholera vaccine classes

| | Vaccine type | | | | |
|---|---|---|---|---|---|
| | **Killed** | **Live** | **Conjugate** | **Outer membrane vesicle** | **Protein antigen** |
| **Composition** | Heat/formalin-inactivated, whole-cell *V. cholerae* with or without recombinant CT-B | Live attenuated, whole-cell *V. cholerae* | *V. cholerae* O-antigen linked to carrier protein | *V. cholerae* outer membrane vesicles | Protein antigens (TcpA, CT-B, chimeras) |
| **Developmental stage** | Several approved and WHO-prequalified; some preclinical and phase 1 candidates | One approved (Vaxchora) but none WHO prequalified; many candidates at varying stages | Not available; current candidates have not advanced to human testing | Not available; no candidate ever tested in humans | Not available; no candidates other than CT-B tested in humans |
| **Key advantages** | ■ Scalable and inexpensive manufacture<br>■ Tested in millions of humans in field trials<br>■ Well tolerated | ■ Single-dose regimen<br>■ Can produce in vivo antigens<br>■ May provide rapid protection against disease<br>■ Scalable, inexpensive manufacture | ■ Delivers a purified and likely major protective *V. cholerae* antigen<br>■ Tunability with carrier protein<br>■ Should function well to boost OCV regimens | ■ Potential stability benefits<br>■ Fewer biosafety concerns than live vaccines<br>■ Present antigens in their native conformation and context | ■ Do not require large-scale *V. cholerae* culture<br>■ Direct administration of protective antigens to immune system |
| **Key limitations** | ■ Require two doses and >1 week for maximum protection<br>■ Less protective in children <5 years<br>■ Unknown how inactivation alters antigens<br>■ Lack in vivo antigens<br>■ Current formulations require production of 4 *V. cholerae* strains | ■ Potential biosafety concerns<br>■ More reactogenic than killed OCVs<br>■ Have yet to demonstrate strong protection in controlled field trials | ■ Requires isolation of O-antigen and conjugation to carrier<br>■ Less easily administered than OCVs<br>■ Expensive manufacture related to whole-cell OCVs<br>■ Untested in humans | ■ Manufacturing potentially expensive and more involved than whole-cell preparations<br>■ Potential LPS toxicity concerns<br>■ Untested in humans | ■ Individual protein antigens may not be sufficient for protection and weaker than OSP<br>■ Early human trials for CT-B showed no protection<br>■ Require industrial protein purification |
| **Recent candidates near or in human testing** | ■ Hillchol (Hikojima killed OCV); phase 1 trial reported in 2020 | ■ *V. cholerae* 638 (Vax-COLER); phase 1/2 field trial last reported in 2011<br>■ VA1.4; phase 2 field trial last reported in 2014<br>■ Peru-15 (CholeraGarde); phase 1/2 field trial last reported in 2015<br>■ HaitiV (Panchol); phase 1 trial planned for 2022 | ■ OSP-rTTHc conjugate; scalable manufacturing reported in 2021 | None | ■ MucoRice-CTB; phase 1 reported in 2021 |

Abbreviations: CT, cholera toxin; LPS, lipopolysaccharide; OCV, oral cholera vaccine; OSP, O-specific polysaccharide; rTTHc, recombinant tetanus toxin heavy chain; *V. cholerae*, *Vibrio cholerae*; WHO, World Health Organization.

than 90% at six months in human studies and provoked promising immune responses in the field, and a phase 2 trial comparing it to Shanchol suggested it exhibited higher immunogenicity (33, 134, 135). However, the vaccine is not WHO prequalified, due in part to its poor performance in a large-scale field efficacy study in Indonesia, a trial likely confounded by a low incidence of cholera (116). Wide usage of Vaxchora may be precluded by other limitations, including potential adverse consequences of reintroducing classical *V. cholerae* genetic content to the environment and possible toxigenic reversion of the vaccine by CTXΦ infection (73).

Despite the utility of currently available OCVs, their biological and practical limitations suggest that improved cholera vaccines could play a role in global cholera control and potentially as vaccine platforms. In the next section, we discuss emerging themes in OCV and nonoral cholera vaccine research, outlining recent progress and highlighting areas for future investigation.

## CHOLERA VACCINE CANDIDATES AND CONCEPTS

### Honing Deployment and Usage of Killed Oral Cholera Vaccines

As killed OCVs are already embedded within many cholera prevention programs, most work on killed OCVs has focused on increasing the efficiency of vaccine deployment and investigating how alternative dosing methods modify vaccine potency. Significant effort has been invested in studying the efficacy of a single dose of killed OCV versus the currently used two-dose regimen. A large clinical trial in Bangladesh showed that while a single dose appears to induce strong short-term immune responses and protection against cholera, protection is relatively short-lived and wanes by two to three years after vaccination (112). Data from this trial and others indicate that single-dose regimens, similarly to the current two-dose format, provide comparatively weak immunogenicity and protection in young children, reinforcing this deficiency as a key limitation of killed OCVs (92). Recent work has also shown that the interval between the first and second dose of killed OCVs can be as long as 28 days and that the cold-chain requirements of killed OCVs may not be as strict as previously thought, allowing for more flexible vaccination campaigns (70, 123). Aided by the global OCV stockpile, field investigations in multiple countries have revealed the utility of reactive immunization campaigns in response to developing cholera outbreaks (i.e., beyond the effort to curtail endemic cholera) (115). Initial two-dose studies in Vietnam and Guinea during emergent cholera outbreaks revealed a more than 70% PE of rapidly administered killed OCVs (15, 94). Successful single-dose reactive campaigns in at least three countries have been reported, suggesting that even incomplete vaccination regimens can buffer rapidly spreading outbreaks (14, 49, 76).

A new killed OCV candidate is Hillchol, which uses a single Hikojima serotype O1 *V. cholerae* strain instead of the four strains that are mixed to create Shanchol. Hillchol is a genetically engineered derivative of *V. cholerae* Phil 6973 (a component of Shanchol) that contains a hypomorphic allele of *wbeT*, which encodes the methyltransferase that determines O1 serotype (74, 130). The premise of Hillchol is that a single-strain manufacturing process will be less expensive than current OCVs while maintaining the mixed O1 serotype antigen composition. Hillchol has shown promise in both animal models and a recent phase 1 clinical trial in which it was shown to be not inferior to Shanchol (130). However, as Shanchol and Euvichol have achieved economies of scale with manufacturing costs of $1–2 per dose, without a clear advantage in immunogenicity or protection it is unclear whether newer killed OCVs will establish themselves in the cholera vaccine space (129). Additional new killed OCV candidates that are produced in virulence-inducing conditions and thus contain TCP have been described but have not progressed to human trials (60).

## Live Oral Cholera Vaccines for Single-Dose, Long-Lasting Immunity

Compared to killed OCVs, development of live OCV candidates and formulations has been more wide-ranging and focused on preclinical and basic studies. Given that initial cholera infection is associated with long-lasting protection, live OCVs are also likely to be more conducive to single-dose immunization schema. Underlying live OCV development is the idea that *V. cholerae* can be sufficiently attenuated without substantial loss of immunogenicity. In contrast to the case of killed OCVs, which have proven safe, attenuation of *V. cholerae* through deletion of *ctxA* does not guarantee sufficient reduction of reactogenicity. Furthermore, live OCVs have biosafety issues beyond safety for individual vaccinees. As living organisms, these vaccines have the potential for toxigenic reversion and for exchange of genetic material with other organisms (**Table 1**).

Many live OCV candidates have been generated, and several of them have advanced to phase 1/2 clinical testing, but none apart from Vaxchora has been tested in large-scale field studies and attained approval. The earliest live OCV candidates, some of which were produced by spontaneous mutagenesis and not targeted genetic manipulation, stalled due to high rates of reactogenic diarrhea in controlled human infection studies (120). However, their strong immunogenicity in humans propelled the development of newer rationally engineered candidates that stimulate immune responses while minimizing adverse effects. These strains are all nontoxigenic owing to deletion or insertional inactivation of *ctxA* or removal of the entire CTXΦ prophage region. Some of them also include *ctxB* expressed heterogeneously from another genomic site, as well as other modifications that potentially increase antigenicity and safety (reviewed in 120).

Besides Vaxchora, only two live O1 OCV candidates have advanced to human challenge studies within the last three decades. Peru-15 (CholeraGarde), reported in 1995, is an El Tor Inaba–derived live OCV strain that, besides deletion of the entire CTXΦ prophage and its chromosomal attachment site, contains a spontaneous mutation rendering it nonmotile (further attenuating the strain) and an insertion of constitutively expressed *ctxB* in the *recA* gene required for homologous recombination (thus markedly reducing its capacity to engage in horizontal genetic exchange) (35). Peru-15 was well-tolerated, immunogenic, and protective in phase 2 trials in the United States, Bangladesh, and Thailand, but it did not progress to field efficacy trials (40, 110, 111, 114, 122). Another candidate, *V. cholerae* 638 (Vax-COLER), contains a deletion of the CTXΦ prophage region but retains the CTXΦ attachment site and has an insertion of selection marker *celA* in the *hapA* locus, which encodes a virulence-associated protease (22, 141). *V. cholerae* 638 was immunogenic and protective in volunteer studies in Cuba and Mozambique (53, 54, 140). A third OCV candidate, VA1.3/1.4, has been recently reported as safe and immunogenic in a trial in India, but it has not been tested in protection studies (71, 96). Notably, many live OCV candidate trials have involved single-dose regimens, reinforcing the idea that these vaccines can be effective following a single oral dose. However, besides Vaxchora, no live OCV has been approved for any indication.

The most recently described live OCV candidate is HaitiV, an attenuated vaccine derived from the wave 3 El Tor *V. cholerae* strain responsible for the 2010 Haiti cholera epidemic. HaitiV contains a suite of targeted mutations that attenuate its pathogenicity, retain its capacity to colonize the small intestine, and provide additional biosafety safeguards (65). Like Peru-15, HaitiV contains a deletion of the CTXΦ prophage, including its chromosomal attachment site and surrounding virulence-associated sequences such as the accessory toxin *rtxA*; in addition, the vaccine contains a heterologously expressed *ctxB* cassette and a deletion in *recA* (65). HaitiV also carries several key alterations that distinguish it from other live OCVs. These include an active Cas9 along with a *ctxA*-targeting single-guide RNA, to prevent toxigenic reversion by any mechanism of horizontal gene transfer, deletion of antibiotic resistance loci in the SXT integrative conjugative element,

and deletion of genes encoding all five *V. cholerae* flagellins to minimize reactogenicity (65). It is also the only live vaccine candidate that is derived from a virulent wave 3 7PET strain and thus is antigenically matched to the currently circulating pandemic *V. cholerae* clone. Later iterations of HaitiV, now known as Panchol, include deletion of *hlyA* and its conversion to a Hikojima strain with the same mutation used in Hillchol (132).

Although HaitiV has not yet advanced to human studies, extensive testing in animal models of *V. cholerae* infection and immunity has proven useful for characterizing this vaccine. Investigations using infant and adult mouse models of OCV administration demonstrated that HaitiV induces robust and cross-protective adaptive immune responses (48, 132, 133). The most recent of these studies demonstrated serotype-specific contributions to live OCV efficacy, and that the Hikojima version of HaitiV provided strong cross-serotype protection and immunogenicity (132). However, the most remarkable finding came from the infant rabbit model of severe cholera-like illness. In this model, administration of HaitiV one day before challenge with a lethal dose of wild-type *V. cholerae* protected animals from both development of diarrhea and death (65). The kinetics of this protection, which was dependent on the ability of HaitiV to precolonize the small intestine, are inconsistent with adaptive immune responses. This rapid protection is particularly relevant to a disease such as cholera that manifests within hours and can be fatal within the span of a day.

## Rapid Vaccine-Mediated Protection

The observation that administration of HaitiV to infant rabbits one day prior to infection provides protection from cholera-like disease brings into question the traditional notion that vaccines work solely by triggering adaptive immune responses. Rapid protection by HaitiV was dependent on the vaccine's capacity to colonize the intestine prior to wild-type challenge and was active against both wave 1 and wave 3 7PET strain challenges; a killed version of HaitiV did not provide rapid protection. Interestingly, although rapid protection was observed in virtually every immunized animal, the level of colonization of the wild-type *V. cholerae* challenge strain varied from undetectable to $\sim 10^9$ CFU/g, the level observed in nonimmunized animals. Thus, the mechanisms underlying rapid protection in experimental cholera are not solely attributable to colonization resistance and may be manifold (**Figure 3**).

A large body of work on *V. cholerae* community behaviors suggests cholera-specific pathways may contribute to rapid protection. In *V. cholerae*, the interbacterial communication system of quorum sensing is an important axis of virulence regulation, including CT production (152). *V. cholerae* quorum-sensing pathways are complex and involve multiple secreted quorum-sensing ligands (103). It has been proposed that *V. cholerae* intestinal density and resulting activation of quorum-sensing signaling pathways can impact the course of infection by differentially regulating genes involved in colonization and pathogen egress/transmission (17, 91, 150, 151). Production of quorum-sensing mediators by precolonized HaitiV could thus impede the establishment and normal kinetics of infection by the wild-type pathogen. This concept was demonstrated using a probiotic *E. coli* Nissle 1917 strain engineered to overproduce *V. cholerae* quorum-sensing molecules, which provided protection against subsequent challenge with virulent *V. cholerae* (47).

Rapid vaccine-mediated protection could also be explained at least in part through prechallenge exposure of the mucosal innate immune system to the vaccine. For example, a recent report of rapid protection by a killed intranasal *Acinetobacter baumannii* vaccine attributed this effect to general innate immune priming defending against not only *A. baumannii* but also *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* challenge (57). Additional findings of adaptive immune-independent vaccine or attenuated strain–mediated protection include both bacterial and viral contexts. Rapid protection conferred by a nontoxigenic *Clostridioides difficile* strain against virulent *C. difficile* was found to be due to intraspecies competition for glycine, a key germination signal (84). Studies of

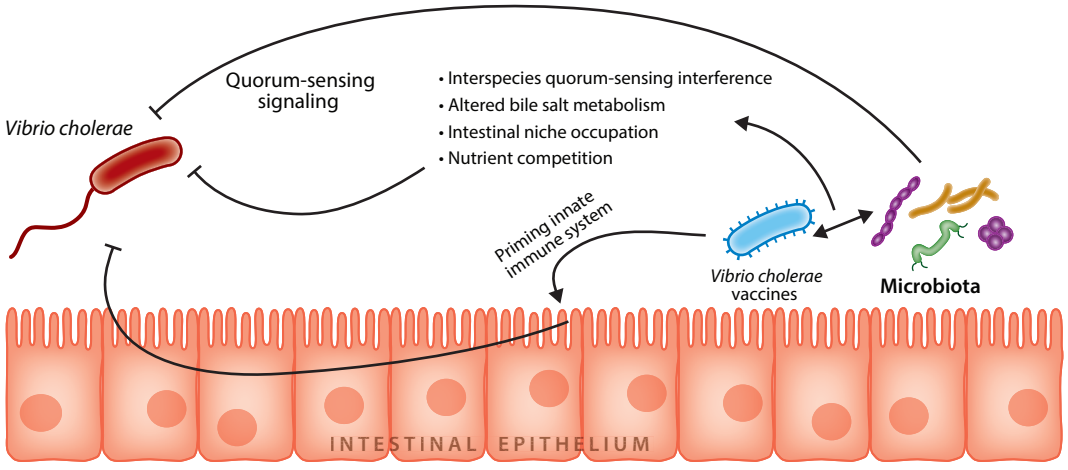


**Figure 3**

Potential mechanisms of rapid protection from vaccines and engineered bacteria. Rapid protection in a live attenuated oral cholera vaccine could occur through direct antagonism of wild-type *Vibrio cholerae* or indirectly through effects on the host innate immune system or modulation of microbiota function to resist infection. Figure adapted from images created with BioRender.com.

influenza vaccine candidates that display rapid protection have implicated innate immune priming, although the exact mechanisms remain unknown and may be adjuvant dependent (36, 52). The full scope and specificity of rapid protection by vaccines remains to be seen, and the mechanisms responsible likely vary depending on the identity of the vaccine, pathogen, and route of immunization. Regardless of the mechanisms by which HaitiV elicits rapid protection, outbreak modeling revealed that a rapid-acting live vaccine such as HaitiV could be transformative for reactive vaccine campaigns against cholera (65). Thus, it will be important to test whether rapid protection is recapitulated in human challenge studies. Rapid protection is an emerging concept in vaccinology that could become a critical aspect of next-generation vaccine design, especially against explosively spreading pathogens such as *V. cholerae*.

We anticipate that increasing the potency of the rapid protection mediated by HaitiV and other vaccines will become possible when our understanding of the mechanisms that underlie this intriguing phenotype deepens. For example, it may be possible to harness *V. cholerae*'s type VI secretion system (T6SS), a multicomponent secretion apparatus that targets other bacteria and injects effector proteins that kill the target cell or inhibit its growth (41), to enhance a vaccine's capacity to provide rapid protection from cholera. T6SS effectors have a wide variety of toxic activities and are almost always accompanied by a cognate immunity protein that protects the primary cell and its kin. Effector-immunity pairing is species specific but modular, meaning that pairs from a given species can be expressed in heterologous systems. Thus, T6SS activity represents an axis by which a live OCV strain could be engineered to specifically target and antagonize the wild-type pathogen. Engineering of heterologous T6SS activity has been demonstrated in *V. cholerae*, and the *V. cholerae* T6SS is thought to be active in vivo (62, 150), but this system has not yet been exploited to enhance rapid action of vaccines.

## Impacts of Commensal Microbes on Rapid and Long-Term Oral Cholera Vaccine Performance

It is important to consider how the rapid and long-term effects of cholera vaccines are influenced by the gut microbiome. An emerging body of evidence with wild-type *V. cholerae* and new studies

with OCV strains in animal and human investigations is revealing that the microbiome can shape outcomes of both cholera infection and immunity (146). Several axes of interactions of virulent *V. cholerae* with gut commensal bacteria have been proposed (recently reviewed in 34). These include interspecies quorum-sensing interference and altered bile salt metabolism, both of which are key regulatory factors of the *V. cholerae* virulence program (4, 64). Many of the hypotheses for these mechanistic microbiome studies and investigations into potential *V. cholerae*–commensal interactions have been informed by observations from human trials. Leveraging these pathways will be valuable for OCV engineering to enhance rapid protection in two ways: first, to facilitate optimal (yet ultimately transient) colonization for immune induction, and second, to maximize rapid antagonism of wild-type *V. cholerae* elicited by the vaccine strain itself (e.g., by OCV strains or formulations that overproduce quorum-sensing factors) (**Figure 3**).

Beyond rapid protection, intestinal commensal organisms are now recognized as critical players in adaptive immunity. The gut microbiome may alter not only immune responses and host outcomes to initial infection but also vaccine-induced immunity and protection against subsequent or future infections (50, 95). Studies have attempted to relate the gut microbiome to variable human immune responses to two OCV formulations (31, 149). However, given the small sizes of the study populations and the scope of these studies, at this time it is difficult to draw links between microbial clades and the potency of responses to vaccination against cholera. A recent study of CT vaccination in a gnotobiotic mouse model of undernourishment suggested that specific commensal species can determine the immune response to this important *V. cholerae* antigen (44). This is particularly relevant to cholera, since the incidence of this disease and other diarrheal illnesses is markedly higher in areas that also have higher rates of undernourishment. More research is needed in this area to fully understand how microbiome composition, including the presence of gut-resident fungi and bacteriophages, may be exploited to provide enhanced adaptive immune responses from OCVs.

## *V. cholerae* as a Heterologous Mucosal Antigen Delivery Platform

Genetic engineering in *V. cholerae* is straightforward, suggesting that this bacterium could be a useful platform for presentation of heterologous antigens from other pathogens. The appeal of this approach is heightened by the fact that live attenuated *V. cholerae* specifically targets the small-intestine surface. Mucosal presentation of heterologous antigens is likely to stimulate immunity at other mucosal sites as well, suggesting that using *V. cholerae* to deliver antigens from pathogens that target other mucosal sites (e.g., the lungs) in addition to the intestine could be effective (80). Heterologous antigen systems have been established in other attenuated bacterial vectors, such as *Salmonella typhi* and *Listeria monocytogenes*. Inactivated and live-attenuated *V. cholerae* vaccine candidates have been reported that express diverse bacterial and viral heterologous sequences, including entire proteins and chimeric epitopes inserted into CT-B (21, 28, 29, 131). One potential limitation of this approach is that the major protective antigens of many pathogens are surface-exposed polysaccharides synthesized by multiple genes. Mobilizing these operons for heterologous expression can be complex, but it has been shown to be feasible (97). These obstacles may be overcome with the application of multiplex gene-editing systems that have been developed in *V. cholerae*, although their effectiveness is strain dependent (42, 43). There has also been work to engineer new antigen-delivery platforms in *V. cholerae*. One recent example is a *V. cholerae*–based platform that uses antigens fused to the biofilm structural protein RbmA to enable extracellular antigen display and adjuvant delivery through the biofilm matrix (89, 90). It has been proposed that biofilms are created by *V. cholerae* along mucosal surfaces, and concentrating heterologous antigens in an extracellular space such as a biofilm could potentiate immune responses. Application

of emerging techniques in genome engineering holds great promise for the development of next-generation live attenuated OCVs for delivery of diverse heterologous antigens and potentially additional cargo types to the mucosal surface of the small intestine.

## Outer Membrane Vesicle Vaccines—A Cell-Free Vaccination Approach

A cell-free vaccine formulation that has been recently advanced uses *V. cholerae* outer membrane vesicles (OMVs). OMVs are outer membrane blebs of gram-negative bacteria that can introduce antigens to the immune system. Such vaccines have potential storage and delivery advantages over live-cell formulations. An approved OMV vaccine against *Neisseria meningitidis* (BEXSERO) is available and has excellent PE (99). Proteomic profiling of *V. cholerae* OMVs derived from infected rabbits suggests that they can present infection-relevant antigens such as TCP and CT-B (13). However, manufacture of OMVs in virulence-inducing conditions in culture at scale could prove costly and challenging. OMVs from a variety of toxin-replete and -deficient *V. cholerae* serotypes and serogroups have been tested across a range of administration routes in mouse models. Results from these studies have shown that mucosal (in particular, intranasal) OMV administration provides the highest levels of protection against subsequent infection (25, 127). Like OCVs, OMV-induced immunity primarily targets LPS-associated serotype and serogroup antigens. Mechanistic experiments in OMV-immunized animals were critical for establishing O-antigen-targeted antibodies as a likely critical protective factor in *V. cholerae*–induced immunity (25, 82, 145). OMVs can also be combined, akin to components of OCVs. A recent report suggests that mixing OMVs derived from *V. cholerae* and enterotoxigenic *E. coli* (ETEC) can confer protection against both pathogens (83). Although *V. cholerae* OMVs represent a potential alternative to OCVs, they have yet to be tested in human trials and face a long path to the clinic.

## Conjugate Vaccines for Complementing Oral Cholera Vaccines

The knowledge that *V. cholerae* LPS (in particular, the O-antigen) is likely the principal protective antigen bodes well for the development of conjugate vaccines for cholera prevention. These vaccines use different forms of the *V. cholerae* O-antigen coupled to various protein carriers to induce T cell–dependent immune responses following parenteral inoculation. The first generation of O1 conjugate vaccines used detoxified LPS purified from *V. cholerae* conjugated to CT or tetanus toxin and demonstrated immunogenicity and protection in animals (27, 58). These vaccines were tested in a phase 1 human trial and proved immunogenic but were not evaluated further (59). Subsequent iterations used a synthetic hexasaccharide representative of *V. cholerae* OSP in the place of whole LPS. Interestingly, these vaccines could be administered transcutaneously, potentially obviating the requirement for needles. These vaccines provoked strong immune responses in mice but did not independently induce high levels of protection (119). The newest generation of conjugate vaccines use whole Inaba or Ogawa OSP moieties purified from *V. cholerae* conjugated to recombinant tetanus toxin heavy chain (rTTHc) (3, 126).

Recent studies of conjugate vaccines have addressed the likely circumstances in the field in which, if approved, these vaccines would be used alongside OCVs. Both the hexasaccharide and full OSP glycoconjugates have shown promise in mice as a booster following initial OCV administration with a single dose of a live attenuated *V. cholerae* strain or after initial exposure to *V. cholerae* (2, 137). The OSP:rTTHc conjugate candidate has now been produced in a scalable manner, priming it for testing in humans (67). As conjugate vaccines are available for other bacterial pathogens such as *S. typhi* that cocirculate with *V. cholerae* in some regions, combined conjugate immunization approaches could be an efficient means to combat multiple pathogens at once.

## Protein Antigen–Based Vaccines and Discovery of New Protective Antigens

Although OSP is considered the major *V. cholerae*–protective antigen, other known *V. cholerae* antigens have been investigated as vaccine components. The best-characterized of these is CT. CT is a potent mucosal adjuvant, but the holotoxin cannot be used because of its diarrhea-inducing effects (125). A variety of approaches have been used to engineer inactive forms of the toxin (i.e., toxoids) that preserve its structure and immunogenicity. Inactive forms of CT were first identified by isolation of nontoxic *V. cholerae* strains, and they were subsequently designed by structure-guided mutagenesis. The most recently reported version is called multiple-mutated CT (mmCT) (81). However, to circumvent the need for mutagenesis or toxoid assembly, most vaccines that include CT antigens, such as Dukoral, only use CT-B, the nontoxic receptor-binding subunit of CT.

In humans, immunization with CT-B alone apparently does not yield protection against challenge (88). However, the Dukoral trials revealed at least some protective benefit to adding CT-B to a killed OCV, and mouse studies have demonstrated that CT-B can be a protective antigen, suggesting there may be some benefit of antitoxic immunity (38, 107). An interesting new oral vaccine delivery mechanism for CT-B, MucoRice-CTB, employs a genetically modified rice strain that constitutively expresses CT-B. Transgenic CT-B-expressing rice seeds are directly crushed into a powder and taken orally in a suspension. MucoRice-CTB was immunogenic and protective in several animal models of enteropathogen-induced diarrhea (102, 138). In a recently reported phase 1 study, MucoRice-CTB induced dose-dependent CTB-specific IgA and IgG responses (149). These antibodies also cross-reacted with ETEC heat-labile toxin (LT), suggesting that MucoRice-CTB could serve as a multipurpose vaccine. However, MucoRice's applicability to cholera appears limited, given that immunity to CT-B is likely not sufficient to prevent cholera.

As TCP is essential for *V. cholerae* small-intestine colonization, there have been some efforts to develop TCP-based vaccines. Administration of TcpF or TcpA induces protective adaptive immunity against *V. cholerae* in mice, and merging of these efforts with those targeting CT as an antigen led to the creation of a TcpF-CTA2-CT-B chimeric fusion antigen (78, 118). This antigen was immunogenic and protective in mice and demonstrated additive effects between the TCP- and CT-derived components of the chimeric antigen (106). Newer antigen discovery efforts involving single-cell analysis of memory cells from convalescent cholera patients have reidentified OSP as the dominant antigen and CT and TCP as secondary antigens, validating their status as prominent research targets (75). These efforts have also uncovered additional surface-exposed/secreted antigen candidates, such as the secreted sialidase NanH. Antibody responses to NanH were recently demonstrated to correlate strongly with protection against cholera, suggesting that this protein is a bona fide protective antigen and warrants further investigation as a vaccine target (68). Despite these preclinical data, it is unclear whether any of these individual candidate protective antigens are sufficient for protection against cholera in humans. Given the emergence of single-cell sequencing and technologies for profiling immune responses, it seems likely that additional antigen candidates will be identified and that future investigations will turn to multi-antigen combinatorial or chimeric antigen subunit-based vaccines for *V. cholerae*; alternatively, such antigens could be overexpressed and delivered in whole-cell formats such as a live OCV.

# CONCLUSIONS AND OPPORTUNITIES

The development and use of cholera vaccines has been instructive for creation of other similar prophylactic interventions, and these vaccines are reducing the burden of cholera. However, there are clear limitations to current killed OCVs, and there is a need to develop new cholera vaccine candidates. Next-generation cholera vaccines will benefit from emerging knowledge about *V. cholerae* infection and immunity to specifically address shortcomings of previous vaccines, such

as their weak immunogenicity in young children. The development of new candidates will be aided by advances in both single-cell and genome-scale technologies. Studies of *V. cholerae* pathogenesis using cutting-edge approaches have allowed high-throughput dissection of pathogen intestinal colonization at genomic and proteomic levels, offering new insights into vulnerabilities that could be targeted by vaccines. Expanded longitudinal studies of the human immune response to cholera will be impactful for our understanding of the immune landscape during different stages of the disease. The establishment of *V. cholerae* genome-editing technologies, including multiplex engineering and CRISPR interference systems (30), offers expanded capabilities for investigators to engineer the pathogen and maximize its potential as a vaccine. It is exciting to consider next-generation vaccine designs that incorporate the roles and consequences of microbiome variation in their design. Insights into rapid protection afforded by live OCVs may inform not only the augmentation of this property but also the translation of this capability to other live attenuated vaccines. *V. cholerae*'s versatility as an antigen-delivery platform mean that highly complex, engineered cholera vaccine candidates may arise in the future that combat both cholera and other infectious ailments.

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