A live vaccine rapidly protects against cholera in an infant rabbit model

INTRODUCTION
The massive and ongoing cholera epidemics in Yemen and Haiti illustrate that this ancient diarrheal disease remains a threat to public health (1, 2). Cholera results from ingesting water or food contaminated by *Vibrio cholerae*, a Gram-negative bacterial pathogen. *V. cholerae* colonizes the small intestine where it produces cholera toxin (CT), which induces profuse watery diarrhea and consequent dehydration that can be rapidly fatal in the absence of rehydration therapy (3). Public health interventions to limit cholera dissemination are critical because of the otherwise rampant spread of cholera epidemics, particularly in association with disruptions in sanitation infrastructure and water supplies. Oral cholera vaccines (OCVs) consisting of killed whole *V. cholerae* cells have modest protective efficacy in endemic regions (4). These vaccines were recently deployed during outbreaks in non-endemic areas as part of “reactive” vaccination programs aimed at blocking the spread of cholera (5). However, optimal efficacy of killed OCVs requires two refrigerated doses administered 14 days apart (6), and these features may limit killed OCVs’ capacity to rapidly constrain ongoing outbreaks in destabilized or resource-limited settings. Single-dose live attenuated OCVs showed efficacy in challenge studies (7) and early-phase clinical trials in endemic regions (8), and reactive vaccination with a live attenuated OCV may have contributed to a decrease in the incidence of cholera during an outbreak (9). However, no live OCVs are based on globally predominant “variant” El Tor strains, like the strain responsible for the 2010 Haitian cholera outbreak (10). Here, we created a live attenuated cholera vaccine based on the Haitian outbreak strain and found that it could protect infant rabbits against lethal cholera-like illness within 1 day of administration.

RESULTS
Design of HaitiV
We used nine different modifications to derive the new vaccine, HaitiV (Table 1), and whole-genome sequencing confirmed that all planned mutations were present. Mutations were engineered to ensure biosafety while maintaining HaitiV’s capacity for intestinal colonization so that, like wild-type (WT) *V. cholerae* and some previously tested live vaccine candidates (7, 11), it may impart long-term immunity after a single oral dose. To ensure the safety of HaitiV, we removed the bacteriophage (CTXΦ) encoding CT (Fig. 1A) (12), the pathogen’s principal virulence factor, and provided stringent impediments to toxigenic reversion. The boundaries of the CTXΦ deletion remove a sequence necessary for its chromosomal integration as well as the gene encoding a multifunctional toxin (MARTX) (13). In addition, HaitiV lacks *hupB*, a gene necessary for episomal maintenance of CTXΦ (14). Further vaccine engineering included steps to (i) reduce potential vaccine reactogenicity by deleting *V. cholerae*’s five flagellins (15); (ii) eliminate the vaccine’s capacity to transfer genes, conferring resistance to antibiotics, that lie within the SXT integrative conjugative element (ICE) (Fig. 1B); (iii) allow the vaccine to produce the nontoxic B subunit of CT (Fig. S1), an antigen that may elicit protection against diarrheal disease caused by enterotoxigenic *Escherichia coli* (ETEC) as well as *V. cholerae* (16); and (iv) minimize potential gene acquisition by deleting *recA*, thereby markedly reducing the strain’s capacity for DNA recombination. HaitiV also encodes a clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) system specifically targeting the toxin gene ctxA. The CTXΦ bacteriophage bearing intact ctxA was unable to infect the vaccine harboring the CRISPR/Cas9 system, whereas a CTXΦ variant lacking ctxA showed no such barrier to infection (Fig. 1C).

Intestinal colonization by HaitiV
Comparative studies of orogastrically inoculated HaitiV or the WT *V. cholerae* isolate from which it was derived (referred to as HaitiWT)
were performed in infant rabbits, a small-animal model that recapitulates many aspects of human cholera, including rapid mortality (17). All animals inoculated with HaitiWT progressed to a moribund state by 18 hours post inoculation (HPI). Upon necropsy, the ceca of these animals were filled with fluid (2A) previously found to resemble ctxAB-dependent choleric diarrhea (17). In marked contrast, minimal or no fluid had accumulated by 18 HPI in the ceca of littermates inoculated with HaitiV (Fig. 2A). Animals inoculated with HaitiV did not exhibit cholera-like illness during observation periods extending to 90 HPI, although in rare cases animals showed mild and self-limited noncholeric diarrhea. Animals inoculated with HaitiV continued to gain weight up to 90 HPI, providing further indication that HaitiV inoculation is not detrimental to overall health or development (Fig. 2B).

The distinct responses to HaitiWT or HaitiV inoculation were not associated with differences between intestinal colonization by the two strains. At 18 HPI, there was no statistically significant difference in *V. cholerae* colonization of the distal small intestine (dsII) between littermates inoculated with HaitiV or HaitiWT (Fig. 2C). HaitiV burden showed no reduction by 90 HPI (Fig. 2C), indicating that prolonged intestinal colonization by HaitiV does not cause disease. Although intestinal colonization by HaitiV and HaitiWT was not statistically distinguishable in single inoculation experiments, when animals were coinoculated with a 1:1 mixture of HaitiWT and HaitiV, the WT strain outcompeted the vaccine strain (Fig. 2D).

**HaitiV-mediated colonization resistance**

HaitiV’s robust occupancy of the intestine motivated us to test whether HaitiV-colonized animals might exhibit resistance to colonization by HaitiWT even before the development of an adaptive immune response due to, for example, alteration of the pathogen’s intestinal niche. To test this possibility, we inoculated animals with either HaitiV (live vaccine), formalin-killed HaitiV (killed vaccine), or a buffer control (mock), then challenged them 24 hours later with a lethal dose of HaitiWT. Animals in the buffer and formalin groups developed severe cholera-like illness after HaitiWT challenge, and intestinal burdens of HaitiWT in these animals resembled those without pretreatment (Fig. 3A). Conversely, no animals that received live vaccine exhibited signs of severe disease within 18 hours of HaitiWT challenge, and lower burdens of HaitiWT were recovered from the intestines of animals previously inoculated with live vaccine versus those inoculated with killed vaccine (Fig. 3B). The reduction in HaitiWT burden varied in magnitude across animals inoculated with live vaccine, with the burden falling below the limit of detection in two animals. The live vaccine’s antagonist of HaitiWT colonization (that is, colonization resistance) appears dependent on previous inoculation of HaitiV; normal burdens of HaitiWT were observed in animals inoculated with the two strains simultaneously rather than sequentially (compare Figs. 2E and 2B).

To assess the specificity of colonization resistance, we repeated the vaccine study, challenging with *V. cholerae* N16961, an early El Tor strain administered to human volunteers in studies of cholera vaccine efficacy (7). The Haitian and N16961 strains were isolated independently and are of distinct serotypes (10). Animals inoculated with live HaitiV, but not killed HaitiV, also exhibited colonization resistance against the N16961 WT challenge (Fig. 3C), demonstrating that HaitiV-mediated colonization resistance is neither strain- nor serotype-specific.

The colonization resistance evident in animals inoculated with live HaitiV led us to hypothesize that the vaccine’s occupancy of the intestine interfered with processes required for colonization by the challenge strain. We performed a forward genetic screen to identify mutations that allow HaitiWT to resist or evade vaccine-mediated antagonism. Such mutations could provide insight into the mechanism(s) by which

---

**Table 1. Genetic alterations in HaitiV, a live attenuated cholera vaccine.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔCTXΦ</td>
<td>Attenuates by removing the genes encoding CT and the multifunctional toxin MARTX (13); protects against toxigenic reversion by preventing chromosomal integration of CTXΦ (12)</td>
</tr>
<tr>
<td>ΔflaBDE/ΔflaAC</td>
<td>Attenuates and reduces potential reactivity (15)</td>
</tr>
<tr>
<td>ΔflaR-strAB-sul2ΔdfrA</td>
<td>Prevents the dispersal of antibiotic resistance genes</td>
</tr>
<tr>
<td>ΔupB</td>
<td>Protects from toxigenic reversion by inactivating the HU complex, which is necessary for extrachromosomal replication of CTXΦ (14)</td>
</tr>
<tr>
<td>lacZ:cas9-sgRNA_ctxA</td>
<td>Endonuclease targeting of ctxA prevents toxigenic reversion</td>
</tr>
<tr>
<td>ΔrecA</td>
<td>Prevents homologous recombination-dependent gene acquisition</td>
</tr>
</tbody>
</table>

---

**Fig. 1. Major genetic alterations in HaitiV.** (A) Deletion of the CTX prophage and adjacent sequences, including the satellite prophages TLC and RS1 and MARTX toxin genes (yellow area, deleted region). (B) Deletion of genes conferring resistance to trimethoprim (dfrA), sulfamethoxazole (sul2), streptomycin (strAB), and chloramphenicol (floR). (C) An anti-ctxA CRISPR system provides immunity to CTXΦ infection: Streptococcus pyogenes cas9 along with sequence encoding a single-guide RNA (sgRNA) targeting ctxA, integrated into the HaitiV lacZ locus; schematic showing targeting of the CTXΦ genome by the anti-ctxA Cas9-sgRNA complex; HaitiV with/without the CRISPR system (CRISPR+−) were infected with either CTXΦ-IgKn (Target+; integenic KanR cassette, intact ctxA) or CTX-Kn (Target−; ctxA replaced by KanR cassette), and the number of transductants was monitored. No detectable KanR transductants shown as ‘+’.
HaitiV mediates colonization resistance and were predicted to confer a fitness advantage to HaitiWT specifically in the HaitiV-colonized intestine; thus, animals were challenged with a pooled HaitiWT transposon-insertion library (HaitiTn) in the absence of pretreatment (single inoculation; Fig. 3, D and F) or 24 HPI with live vaccine (sequential inoculation; Fig. 3, E and G). HaitiTn colonization in the absence of pretreatment was indistinguishable from HaitiV inoculation of animals previously inoculated with a mock treatment or killed vaccine (Fig. 3). In addition, the range of HaitiTn colonization in vaccine-pretreated animals recapitulated the highly variable HaitiWT burden observed upon sequential inoculation of HaitiV and HaitiWT (Fig. 3).

To identify enriched mutants, we sequenced the transposon junctions from HaitiTn recovered from the dSL and performed a genome-wide comparison of mutant abundance in animals subjected to HaitiTn challenge without pretreatment (Fig. 3F and table S3) or after HaitiV inoculation (Fig. 3G and table S4). To ensure requisite statistical power, we restricted our analysis to animals colonized by diversely HaitiTn populations encompassing multiple independent disruptions per gene (rabbits r3 to r6 for single inoculation and rabbits r4 to r7 for sequential inoculation). Notably, insertions disrupting cqsS and hapR, components of a Vibrio-specific quorum sensing (QS) pathway, were enriched in multiple animals, independent of pretreatment (Fig. 3, F and G, fig. S2, and tables S3 and S4). QS down-regulates expression of virulence and colonization factors at high cell densities (18–21), and enrichment of cqsS and hapR mutants, which are blind to this inhibition, suggests that QS pathways constrain HaitiWT growth in the intestine. Corresponding enrichment of QS mutants was not identified in similar analyses of closely related V. cholerae isolates (22), suggesting that QS may play a distinct role in the pathogenesis of variant El Tor strains. Our genome-wide screen failed to identify any mutants that were consistently and specifically enriched in vaccine-colonized animals, indicating that single loss-of-function mutations are unlikely to enable HaitiWT to resist or evade vaccine-mediated antagonism.

The genetic diversity intrinsic to the HaitiTn library used above allowed us to assess whether HaitiV-mediated colonization resistance was associated with changes in the severity of the infection bottleneck that V. cholerae encounters in vivo (23, 24). V. cholerae recovered from the intestine arise from a founding population of organisms that persist after a stochastic constriction of the bacterial inoculum (23), and the severity of this infection bottleneck can be estimated from the number of unique transposon-insertion mutants recovered from the intestine (25). A subset of animals previously inoculated with live vaccine were colonized by relatively few unique insertion mutants and showed low HaitiTn burdens (Fig. 3E, rabbits r1 to r3), suggesting that HaitiV-mediated colonization resistance is, in some cases, associated with a highly restrictive infection bottleneck. There was no overlap in the mutants recovered from low-diversity animals, which indicates that the restrictive infection bottlenecks observed in some HaitiV-inoculated animals are stochastic and genotype-independent. We also observed reduced colonization in animals in which the vaccine did not appear to impose a bottleneck (Fig. 3D, rabbits r4 to r7). The variable bottlenecks observed in vaccine-colonized animals, along with our inability to identify mutants resistant to vaccine-mediated antagonism, highlights the possibility that the mechanism(s) underlying colonization resistance may be complex and/or multifactorial. However, the lower burdens of HaitiWT and the absence of severe disease after challenge in vaccine-colonized animals suggest that inoculation with HaitiV may be sufficient to protect against cholera-like illness.

**HaitiV-induced rapid protection against cholera-like illness**

To quantify HaitiV-dependent protection from cholera-like disease, we inoculated infant rabbits with live or killed vaccine, challenged them 24 hours later with HaitiWT, and conducted blinded hourly monitoring to assess their status. All animals inoculated with killed vaccine developed diarrhea (median onset, 15 hours) and progressed to a moribund state within 29 hours of HaitiWT inoculation (median, 18.8 hours) (Fig. 4A). In contrast, animals inoculated with live vaccine were significantly slower (P < 0.01, log-rank test) to develop diarrhea (median, 28.3 hours; Fig. 4A). To quantify HaitiV-induced protection against cholera-like illness, we quantified HaitiWT-dependent protection from cholera-like disease, we inoculated infant rabbits with live or killed vaccine, challenged them 24 hours later with HaitiWT, and conducted blinded hourly monitoring to assess their status.
41 hours after lethal challenge despite detectable HaitiWT colonization in all animals (Fig. 4, A and C). Thus, HaitiV may protect from disease even in the absence of absolute colonization resistance. The rapidity of HaitiV-induced colonization resistance and disease protection, and the observation of these phenotypes in a neonatal model of infection, are not consistent with vaccine-elicited adaptive immune protection. Instead, our data indicate that HaitiV colonizes the intestine and mediates viably-dependent protection against cholera, properties consistent with the definition of a probiotic agent (26).

To investigate how rapid protection, like that elicited by HaitiV, might affect reactive vaccination campaigns, we modified a previously published mathematical model of a cholera outbreak in a susceptible population (27), an epidemic context prioritized for reactive OCV interventions (28). Our modifications to the model (fig. S3A) allowed us to compute the effects of hypothetical vaccines that confer equal degrees of protection in 1 day [fast vaccine, based on observations in Figs. 3 and 4 (A and B)] or in 10 days [slow vaccine, when some recipients of killed OCV’s manifest vibriocidal antibody titers (29)]. The model does not account for transient protection and instead assumes that, once elicited, vaccine efficacy remains constant throughout the simulated epidemic. Varying different model parameters revealed that maximal benefit of a fast vaccine, relative to a slow vaccine, occurs under transmission dynamics consistent with recent outbreaks ($R_0 = 1.5$ to 3) and with rapid vaccine administration (figs. S3B and S4). These simulations revealed that, compared to a slow vaccine, an equally efficacious fast vaccine could avert an additional 20,000 infections in a population of 100,000 (Fig. 4D) by preventing infections that could be acquired in the window between administration of the slow vaccine and the emergence of protective immunity.

**DISCUSSION**

Here, we report the design and characterization of a new live attenuated cholera vaccine candidate, HaitiV. Our studies indicate that HaitiV is refractory to toxigenic reversion and that it colonizes an animal model of cholera without causing cholera-like disease or other untoward effects. The infant rabbit model is well suited for the intestinal colonization and disease progression studies reported above, but this model has limitations. Specifically, the poorly characterized intestinal microbiota and adaptive immune capacity of rabbit neonates restrict further investigation of HaitiV’s mechanism(s) of action and...
immunogenicity. There is no robust animal model to investigate adaptive immunity to cholera; as with previous cholera vaccines, evaluating the adaptive immune response elicited by HaitiV will require human volunteer studies. Encouragingly, HaitiV’s colonization was comparable to that of strains closely related to Peru-15 in the same model (15), an earlier live cholera vaccine candidate found to be safe in humans and to confer protective immunity with a single dose (11) even in children under 5 years old, who are not protected by killed OCVs (8).

Surprisingly, we found that HaitiV confers protection within 24 hours of administration, an interval that is not consistent with adaptive immunity. Notably, these effects required the use of viable HaitiV; formalin-killed HaitiV did not provide acute protection from disease, suggesting that rapid protection results from a probiotic effect that is unlikely to be elicited by killed OCVs. Typically, probiotics only temporarily occupy the intestine and require serial inoculation for sustained protection. Mao et al. (30) show that an existing probiotic, Lactococcus lactis, confers protection in an infant mouse cholera challenge model and can be engineered to detect Vibrio species. Unlike probiotics, HaitiV was engineered to retain the cholera pathogen’s remarkable capacity for colonizing the human small intestine.

Human challenge studies are a well-established system for assessing the adaptive immune protection elicited by OCVs (7, 11), but they have not evaluated the ability of probiotic strains to provide acute protection against cholera. Incorporating additional acute challenges (for example, within 24 hours of vaccine or probiotic administration) will illuminate the onset and duration of protection against cholera elicited by different interventions. In contrast to traditional probiotics and killed OCVs, our findings indicate that live OCVs, such as HaitiV, may elicit both the rapid benefits of probiotic strains and the prolonged protection of OCVs.

Although the mechanisms underlying HaitiV’s acute protection are likely complex and require further elucidation, our mathematical modeling indicates that the public health impacts of a hypothetical vaccine eliciting comparably rapid protection could be transformative in the context of reactive vaccination during cholera epidemics. Relative to controls, HaitiV-inoculated animals challenged with a lethal dose of HaitiWT survived longer after the onset of diarrhea, displayed lower HaitiWT colonization, and in some cases were completely protected from cholera. HaitiV’s slowing of disease progression suggests that individuals who are infected with pathogenic V. cholerae after being inoculated with HaitiV may have more time to access life-saving treatment after the onset of symptoms. The time that elapses between onset of symptoms and administration of treatment is often the determinant of case fatality rates during cholera outbreaks because rehydration therapy is sufficient to prevent death in virtually all symptomatic individuals (31). In addition, the colonization resistance mediated by HaitiV, but not formalin-killed HaitiV, suggests that inoculating with HaitiV may reduce shedding of toxigenic V. cholerae into the environment, the transmission route that perpetuates outbreaks. Although we did not incorporate HaitiV’s potential effects on transmission into our modeling studies, a reduction in transmission is likely to potentiate the effect that a rapidly protective vaccine could have on outbreak control. Overall, our findings suggest that probiotic vaccines, mediating rapid protection from disease while eliciting adaptive immunity, could constitute a new class of therapeutics with transformative impacts on outbreak control.

**MATERIALS AND METHODS**

**Study design**

The aim of this study was to design a new live attenuated cholera vaccine candidate, assess the strain’s capacity to safely colonize the intestine, determine whether the strain could protect animals from cholera-like illness shortly after its administration, and quantify the potential impact of observed protection parameters on the incidence of
cholera infection during a simulated epidemic. The vaccine candidate was derived from an isolate of the globally predominant V. cholerae strain via sequential allelic exchange steps (see the “Genetic manipulations” section), and mutations were verified by whole-genome sequencing (see the “Whole-genome sequencing” section). Studies of intestinal colonization and cholera-like illness were conducted, in compliance with federal and institutional guidelines regarding the use of animals in research, using the infant rabbit model of infection (see the “Infant rabbit infection studies” section). One- to 2-day-old animals were allocated to treatment groups randomly, and within-litter (that is, cohoused and age-matched) controls were used to minimize the impacts of litter-to-litter variation. For studies of disease progression, assessors were unaware of the treatment administered to each group, and animals found dead within 10 hours of challenge were excluded because of physical trauma consistent with maternal rejection. Transposon-insertion sequencing studies were conducted using the ARTIST pipeline, which models and compensates for experimental noise and offers recommendations for the imposition of effect size thresholds (see the “Transposon-insertion sequencing analysis” section). Lastly, our modeling incorporated a variable time to vaccine rejection. Transposon-insertion sequencing studies were conducted using the ARTIST pipeline, which models and compensates for experimental noise and offers recommendations for the imposition of effect size thresholds (see the “Transposon-insertion sequencing analysis” section).

Strains, media, and culture conditions
Table S1 contains a list of strains used in this work. Unless otherwise noted, V. cholerae and E. coli were grown in lysogeny broth [LB; tryptone (10 g/liter), yeast extract (5 g/liter), and NaCl (5 g/liter)] with shaking (250 rpm) at 37°C. Recipient strains in phage transduction assays were grown in AKI media [peptone (15 g/liter), yeast extract (10 g/liter), autoclaved, and then supplemented with freshly made, sterile-filtered NaHCO₃ to a final concentration of 0.3%]. Antibiotics and substrates were used in the following concentrations unless otherwise noted: streptomycin (Sm) (200 µg/ml), carbenicillin (50 µg/ml), chloramphenicol (20 µg/ml), SXT (sulfamethoxazole (160 µg/ml) and trimethoprim (32 µg/ml)), kanamycin (Kn) (50 µg/ml), and 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside [X-Gal (60 µg/ml)]

Genetic manipulations
All gene deletions and replacements were constructed via homologous recombination using the suicide vector pCD442, DH5α-λpir, and donor strain MFD-λpir (32) or SM10-λpir (table S1). For all deletions, ~500- to 700-base pair (bp) homologous regions upstream and downstream of the respective open reading frame were amplified using the primer combinations described below and cloned into XbaI-digested pCD442 using isothermal assembly. For derivation of HaitiV from the HaitiWT strain, first, the CTXΦ prophage and surrounding sequences were deleted using primers TDPsCTX1/TDPsCTX2 and TDPsCTX3/TDPsCTX4 to amplify homologous regions upstream of the rtx toxin transporter at the 5′ end and upstream of a putative dehydogenase on the 3′ end of this region. This resulted in a deletion of a 42,650-bp fragment that includes the entire CTX prophage, which includes ctxAB, the CTX attachment site, the RS1 and TLC satellite prophages, and the MARTX toxin genes rtxABCDE. The knockout was validated via polymerase chain reaction using primers TD1027/TDP1028.

Next, the flaBDE operon was deleted as previously described (33). The flaAC operon deletion plasmid was constructed using primers TDP1172/1174 (upstream homology) and TDP1173a/TDP1173 (downstream homology). The SXT ICE-encoded antibiotic resistance loci dfrA, sul2, strAB, and floR were then deleted using primers TDP1193/TDP1194 + TDP1195/1196 (dfrA, trimethoprim resistance) and TDP1287/TDP1288 + TDP1291/TDP1292 (sulfamethoxazole, streptomycin, and chloramphenicol resistance loci). Whole-genome sequencing revealed that the second crossover in the allele exchange process occurred not between the homologous regions included in the suicide plasmid, but rather between duplicate sequences flanking the flor sul region of the chromosome (N900_11210 and N900_11260). An SmR mutant of the vaccine precursor strain was isolated by plating on streptomycin (1000 µg/ml), and the rpsL K43R single nucleotide variant was confirmed by Sanger sequencing.

For CtxA overexpression, the htpG promoter was amplified from Peru-15 (34) using primers FD54/FD103 (adding the strong ribosome binding site AGGAGG), and ctxB was amplified from HaitiWT, which contains the ctxB7 allele, using primers FD33/FD34. Homologous regions flanking the intergenic region of the validated neutral locus vc0610/N900_11550 (23) were amplified with pairs FD30/FD31 and FD73/FD74. These fragments were then cloned into pCVD442 in a one-step isothermal assembly reaction. CtxB overexpression was confirmed by Western blot on cell-free supernatants from cultures grown in AKI conditions described above (Abcam, ab123129, anti-cholera toxin; fig. S1).

Next, the hupB deletion plasmid was constructed using primer pairs Vc-hupB5-F1/Vc-hupB5-R1 and Vc-hupB3-F1/Vc-hupB3-R1. The deletion was verified with primers VC-hupB-SF2/Bc-hupB-SR2. For the cas9-sgRNA module, cas9 was amplified from plasmid DS_SpCas9 (www.addgene.org/48645/), with primers TDP1747/TDP1748. The sgRNA region was amplified from gBlock “VC_3x_sgRNA_gBlock” (table S2), with primers TDP1761/TDP1762. Both fragments were combined and cloned in to the Stul site of pJL1 (35) via isothermal assembly. Sequencing revealed that a recombination event during assembly had removed two of three sgRNAs, leaving a single guide targeting ctxA. This suicide vector was introduced to the vaccine strain via triparental mating with the helper plasmid pRK600. Finally, a recA deletion plasmid was constructed using primer pairs Vc-recA5-F1/Vc-recA5-R1 and Vc-recA3-F1/Vc-recA3-R1; the deletion was verified with primers Vc-recA-SF2/Vc-recA-SR2.

Whole-genome sequencing
Genomic DNA from HaitiWT and HaitiV was prepared using the Nextera XT Library Prep Kit (Illumina) and sequenced on a MiSeq (reagent kit v2, 2×250). Each sample was mapped to its putative genome and variants were identified using GATK3.6.

CTXΦ transduction assay
Supernatant from V. cholerae O395 strains harboring CTXΦ-IGKn [a phage whose genome includes ctxA (36)] or CTX-KnΦ [a phage whose genome lacks ctxA (12)] [grown at 30°C in LB to an optical density at 600 nm (OD600) of 1.0] was concentrated (~50-fold; Ultragel 100K Centrifugal Filter, Millipore) and filtered (0.22-µm filter; Millipore) to get a cell-free phage supernatant. To induce expression of the toxin coregulated pilus (the phage receptor) in the strains being assayed for CTXΦ susceptibility, overnight LB cultures were back-diluted 1:100 into 10 ml of AKI in 16 × 150-mm glass culture tubes and incubated without shaking for 4 hours at 37°C. All but 1 ml of the culture was then discarded, and the culture was moved to a shaker (250 rpm) for aerobic culture at 37°C for an additional 2 hours. Recipient cultures were washed once by centrifugation, mixed 2:1 with phage supernatant.
and incubated at room temperature for 20 min. Serial dilutions were then plated on LB and LB + Kn (100 μg/ml) agar plates, and transduction efficiency was calculated as (CFU/ml)Kn100/(CFU/ml)LB.

Generation of HaitiWT-Tn library

*E. coli* SM10Δpir bearing the pir-dependent Himar transposon vector pSC189 (37) were conjugated with recipient HaitiWT to generate a transposon-insertion library. Overnight cultures of each strain were grown aerobically at 37°C and then diluted 1:100 in media at 37°C. After 4 hours of outgrowth, 4 ml of each culture was pelleted and washed once with LB. Cultures were then mixed in a 1:1 ratio, pelleted, and resuspended in 800 μl of LB. Fifty microliters of the mix was spotted onto 0.45-μm filters on LB agar plates for a total of 16 conjugation reactions. Reactions were incubated at 37°C for 4 hours, after which filters were vortexed in LB (1 ml per filter) to resuspend attached bacteria. Suspensions were plated onto 245 mm² LB + Sm/Kn agar plates to select for *V. cholerae* transconjugants (2 ml of suspension per plate). Plates were incubated at 30°C overnight to enumerate bacterial colonies. The library consisted of ~300,000 colonies and was scraped into LB + 25% glycerol. Incubated at 30°C overnight to enumerate bacterial colonies. The library

**Infant rabbit infection studies**

Infant rabbit studies were conducted according to protocols approved by the Brigham and Women’s Hospital Committee on Animals (Institutional Animal Care and Use Committee protocol number 2016N000334 and Animal Welfare Assurance of Compliance number A4752-01) and in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Welfare Act of the U.S. Department of Agriculture. To prepare bacteria for inoculation, overnight cultures were diluted 1:100 in 100 ml of LB and cultured with aeration at 37°C until late log phase (OD₆₀₀ of 0.5 to 0.9). About 2 × 10⁹ CFU were pelleted by centrifugation at 6000 rpm, the supernatant was removed, and cell pellets were resuspended in 10 ml of 2.5% sodium bicarbonate solution [2.5 g in 100 ml of water (pH 9.0)] to a final cell density of ~2 × 10⁹ CFU/ml. For coinfection studies, ~1 × 10¹⁰ CFU were pelleted by centrifugation at 6000 rpm, the supernatant was removed, cell pellets were resuspended in 5 ml of 2.5% sodium bicarbonate solution, and the resulting suspensions were combined to yield a 1:1 mixture at a cell density of ~2 × 10⁹ CFU/ml. For studies using the HaitiWT transposon library, a 1 ml frozen stock of the library (OD₆₀₀ = 10) was transferred to 100 ml of LB to an initial OD₆₀₀ of 0.1. The library was then cultured with aeration at 37°C to an OD₆₀₀ of 0.8 (~2 hours), and a cell suspension of ~2 × 10⁹ CFU/ml in 2.5% sodium bicarbonate solution was prepared as described above. Preparation of formalin-killed vaccine required the following additional steps: cell pellets were resuspended in 8 ml of 10% formalin, the formalin suspension was centrifuged at 6000 rpm, the supernatant was removed, cells were resuspended in five volumes of 1× phosphate-buffered saline (PBS) (40 ml of 1× PBS) to wash away excess formalin, the PBS suspension was centrifuged at 6000 rpm, the supernatant was removed, and cells were resuspended in 10 ml of 2.5% sodium bicarbonate solution. This procedure eliminated all viable *V. cholerae*. For all experiments, the final cell suspension was serially diluted in 1× PBS and plated in triplicate on LB + Sm/X-Gal, and incubated at 30°C overnight to enumerate the precise dose. For coinfection studies, the disruption of lacZ in HaitiV enabled enumeration of HaitiWT and HaitiV CFU, blue and white colonies, respectively. For studies using the HaitiWT transposon library, ~2 × 10¹⁰ CFU of the library inoculum were plated on LB + Sm/Kn and incubated at 30°C overnight to generate a representative sample of the library inoculum used for subsequent statistical comparisons.

Infant rabbit infections were performed as previously described (17), with minor modifications detailed below. All experiments were conducted using 1- to 4-day-old New Zealand White rabbits, and animals were cohoused with littermates and a lactating dam for the duration of all studies, which varied in length based on the phenotypes assessed. Animals were obtained from either Pine Acre Rabbitry or Charles River Canada, and phenotypes were consistent across animals from both vendors. Animal studies were always conducted using within-litter controls to minimize the impacts of litter-to-litter variation. Initial studies of HaitiWT and HaitiV colonization (Fig. 2, A to C) were conducted after intraperitoneal injection of ranitidine-hydrochloride (2 μg/g body weight) to reduce stomach acidity; however, this treatment was omitted from all subsequent studies because it had no discernible impact on HaitiWT or HaitiV colonization. Animals were orogastrically inoculated with ~10⁹ CFU (500 μl of a 2 × 10⁹ CFU/ml bacterial Suspension) using a size 5 French catheter. One-day-old animals were used for single-inoculation and coinoculation studies. These animals were typically euthanized ~18 HPI; however, longitudinal studies of HaitiV colonization were conducted by inoculating 1-day-old animals and monitoring their condition through ~90 HPI. For sequential inoculation studies, 1-day-old animals were inoculated with one of three treatments: “mock,” 500 μl of 2.5% sodium bicarbonate solution; “killed vaccine,” 500 μl of a 2 × 10⁹ CFU/ml suspension of formalin-killed HaitiV; or “live vaccine,” 500 μl of a 2 × 10⁹ CFU/ml suspension of HaitiV. Twenty-four hours later, the same animals were inoculated with ~10⁹ CFU (500 μl of a 2 × 10⁹ CFU/ml suspension of the challenge strain [HaitiWT, Figs. 3 (A and B) and 4; N16961, Fig. 3C; or HaitiTn, Fig. 3 (D and E)]. For sequential inoculation studies that report bacterial burden, animals were euthanized ~18 hours after challenge, with the exception of Fig. 4C. At necropsy, the entire intestinal tract was removed, cecal fluid was extracted using a 26½ gauge needle and transferred to a preweighed Eppendorf tube. Sections (2 to 3 cm) of the dSI, along with the entire cecum, were placed in preweighed homogenization tubes containing 1 ml of sterile PBS and two 3.2-mm stainless steel beads (BioSpec Products Inc.), and all filled tubes were weighed. The mass of fluid recovered from the cecum was divided by the mass of the cecum to obtain a FAR. The tubes containing tissue were homogenized for 2 min on a Mini-BeadBuster 16 (BioSpec Products Inc.) serially diluted in 1× PBS and plated. Plates were incubated at 30°C overnight, and the number of colonies was divided by the appropriate dilution ratio and the mass of the corresponding tissue/fluid sample to yield a measure of CFU/g of tissue. Homogenates were plated on LB + Sm/X-Gal to enumerate total burden for HaitiWT + HaitiV and/or on LB + SXT to enumerate HaitiWT burden alone. For coinoculation or sequential inoculation studies, the absence of a HaitiV-specific selectable marker prevented us from enumerating HaitiV CFU unless the burden of HaitiV was comparable to HaitiWT (that is, within 100-fold). Similarly, for studies using the N16961 WT strain, which is sensitive to SXT, the number of blue colonies on LB + Sm/X-Gal was used to enumerate WT burden. For coinoculation studies, a competitive index was calculated as

\[
\text{Competitive index} = \frac{(\text{HaitiV CFU} \div \text{HaitiWT CFU})_\text{dSI}}{(\text{HaitiV CFU} \div \text{HaitiWT CFU})_\text{inoculum}}
\]

For studies using the HaitiWT transposon library, the terminal 10 cm of the dSI was obtained at necropsy, weighed, and homogenized as described above. The homogenate was serially diluted in 1× PBS and plated.
on LB + Sm/X-Gal to enumerate total burden and LB + Sm/Kn to enumerate the burden of HaitiTn. The remaining 900 μl of undiluted tissue homogenate was plated on LB + Sm/Kn to recover representative samples of the in vivo passaged HaitiTn library that were used for subsequent analyses of sites of transposon insertion.

Colonization data were not reported for animals that reached a morbidity state of disease in studies of disease progression because the interval between inoculation and euthanasia, which varies substantially in these studies, is likely to affect bacterial burden. Instead, animals were euthanized upon assessment of moribund status characterized by a combination of visible diarrhea (staining of the ventral surface), dehydration (skin tenting), weight loss, lethargy (minimal movement), and decreased body temperature (cold to the touch). The individual who carried out these assessments was blinded to whether animals received killed vaccine or live vaccine. One animal progressed to moribund status without developing visible diarrhea, explaining the differences in sample sizes in Fig. 4 (A and B).

**Transposon-insertion sequencing analysis**

Transposon-insertion libraries were characterized by massively parallel sequencing; sequence data were processed and mapped to the *V. cholerae* H1 genome (38) as previously described (39). Higher complexity libraries (>30,000 unique genotypes) were compared to the input libraries using the ARTIST pipeline. Data were corrected for origin proximity using a LOESS correction of 100,000-bp windows. The inoculum data sets were independently normalized relative to intestinal data sets using Con-ARTIST’s multinomial distribution-based random samplings (n = 100). A modified version of Con-ARTIST’s Mann-Whitney U function was used to compare the intestinal data sets to their 100 simulated control data sets, and these data are reported in tables S3 and S4. Thresholds of mean informative sites > 5, $\log_2$ [mean fold change] > 1, and mean inverse $P > 100$ to $10^2$ were imposed to identify loci for which corresponding insertion mutants are enriched or depleted in the intestinal data sets relative to the inoculum.

**Modeling of cholera outbreaks**

Our model, adapted from a previous study (27), is depicted schematically in fig. S3A. We used previously published parameters for disease transmission (fig. S3B). The vaccine rollout was modeled as proceeding at a constant number of doses per day over the duration of the campaign (7 days in fig. S4, B and D; varied in fig. S4A) until 70% of the total population was vaccinated. The campaign was triggered when the number of symptomatic cases [estimated as 25% of total infections in a previously susceptible population (40)] exceeded a threshold (1000 people in Fig. 4D and fig. S4A; varied in fig. S4B). The transmission rate (β) used for simulations was calculated assuming a basic reproductive (R0) number in the range of 1 to 5, consistent with previous cholera outbreaks, with $R_0 = \beta / \gamma$. Consistent with previous modeling studies (41), we assumed the average duration of infectiousness (1/γ) estimated in a household transmission study (42). To compare the impact of using a fast vaccine over a slower-acting alternative with equal efficacy against infection, we assumed an average time to onset of protection of 1 day versus 10 days (1/γ) after receipt of a single dose. We modeled a “leaky” mode of vaccine action reducing the rate of acquisition by 70% (θ). Ordinary differential equations were solved in MATLAB R2016b (MathWorks) using the ode45 function, with initial conditions of a single exposed individual in an otherwise susceptible population. For our model, the system of differential equation is

$$\lambda = \beta(I_U + I_V + I_P)/N$$

$$\frac{dS_U}{dt} = -\lambda S_U - \rho(t)S_U/N_U$$

$$\frac{dE_U}{dt} = \lambda S_U - \sigma E_U - \rho(t)E_U/N_U$$

$$\frac{dI_U}{dt} = \sigma E_U - \gamma I_U - \rho(t)I_U/N_U$$

$$\frac{dR_U}{dt} = \gamma I_U - \rho(t)R_U/N_U$$

$$\frac{dS_V}{dt} = -\lambda S_V + \rho(t)S_U/N_U - \tau S_V$$

$$\frac{dE_V}{dt} = \lambda S_V - \sigma E_V + \rho(t)E_U/N_U - \tau E_V$$

$$\frac{dI_V}{dt} = \sigma E_V - \gamma I_V + \rho(t)I_U/N_U - \tau I_V$$

$$\frac{dR_V}{dt} = \gamma I_V + \rho(t)R_U/N_U - \tau R_V$$

$$\frac{dS_P}{dt} = -\lambda(1 - \theta)S_P + \tau S_V$$

$$\frac{dE_P}{dt} = \lambda(1 - \theta)S_P - \sigma E_P + \tau E_V$$

$$\frac{dI_P}{dt} = \sigma E_P - \lambda I_P + \tau E_V$$

$$\frac{dR_P}{dt} = \gamma I_P + \tau R_V$$

**Statistical analysis**

Comparisons of two samples were performed using two-sided testing (α = 0.05) for a t test (FARs), a Mann-Whitney U test for nonparametric data (bacterial burden), or a log-rank test (survival curve). Comparisons of three samples were performed using the Kruskall-Wallis test followed by Dunn’s multiple comparisons test (bacterial burden).

**SUPPLEMENTARY MATERIALS**

www.sciencetranslationalmedicine.org/cgi/content/full/10/445/eaap8423/DC1

Fig. S1. HaitIV produces only the B subunit of CT.

Fig. S2. Results of Con-ARTIST analysis.

Fig. S3. SEIR model and parameters.

Fig. S4. Impact of model parameters on relative protection.

Table S1. Strains and plasmids used in this study.

Table S2. Oligonucleotide sequences used in this study.

Table S3. Number of unique genotypes and the results of Con-ARTIST analysis of insertional sequencing (TIS) data from single-inoculation animals.

Table S4. Number of unique genotypes and the results of Con-ARTIST analysis of TIS data from sequential-inoculation animals.

Table S5. Primary data.
REFERENCES AND NOTES


Funding: This work was supported by NIH AI-042347 and Howard Hughes Medical Institute (M.K.W.), F31 AI-120665 to (T.P.H.), and NSERC PGS-D 487359 to (B.S.).


Data and materials availability: All data are available upon request from M.K.W.

A live vaccine rapidly protects against cholera in an infant rabbit model
Troy P. Hubbard, Gabriel Billings, Tobias Dörr, Brandon Sit, Alyson R. Warr, Carole J. Kuehl, Minsik Kim, Fernanda Delgado, John J. Mekalanos, Joseph A. Lewnard and Matthew K. Waldor

Sci Transl Med 10, eaap8423.
DOI: 10.1126/scitranslmed.aap8423

Designer bugs as drugs
The endemic persistence and outbreaks of *Vibrio cholerae* indicate a need for new methods of control; in this issue, two groups investigated the potential of engineered bacteria to mediate cholera resistance in animal models. Mao et al. discovered that lactic acid production by the probiotic *Lactococcus lactis* rendered the infant mouse gut hostile to *V. cholerae* and engineered *L. lactis* to detect breakthrough infection. Hubbard et al. extensively modified a contemporary *V. cholerae* strain for a live oral vaccine, which resulted in an attenuated strain that could protect infant rabbits from *V. cholerae* challenge within 24 hours of vaccine administration, indicating that the protective effects were not dependent on adaptive immunity. These papers showcase innovative approaches to tackling cholera.