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

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Abstract

Outbreaks of cholera, a rapidly fatal diarrheal disease, often spread explosively. The efficacy of reactive vaccination campaigns - deploying *Vibrio cholerae* vaccines during epidemics - is partially limited by the time required for vaccine recipients to develop adaptive immunity. We created HaitiV, a live attenuated cholera vaccine candidate, by deleting diarrheagenic factors from a recent clinical isolate of *V. cholerae* and incorporating safeguards against vaccine reversion. We demonstrate that administration of HaitiV 24hrs prior to lethal challenge with wild-type *V. cholerae* reduced intestinal colonization by the wild-type strain, slowed disease progression, and reduced mortality in an infant rabbit model of cholera. HaitiV-mediated protection required viable vaccine, and rapid protection kinetics are not consistent with development of adaptive immunity. These features suggest that HaitiV mediates probiotic-like protection from cholera, a mechanism that is not known to be elicited by traditional vaccines. Mathematical modeling indicates that an intervention that works at the speed of HaitiV-mediated protection could improve the public health impact of reactive vaccination.

One Sentence Summary:

An engineered cholera vaccine candidate mediates colonization resistance and disease protection within one day in an infant rabbit model.

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Author Contributions:

TPH, GB, JJM, and MKW designed experiments conducted by TPH, GB, TD, BS, ARW, CJK, MK, FD, and JAL. TPH, GB, and MKW wrote the manuscript. TPH and MKW provided funding.

Competing Interests:

The authors have no competing interests to declare. TPH, GB, and MKW have filed a provisional patent on HaitiV (62/531,551).

Overline: Infectious disease

Data and materials availability:

All data are available upon request from MKW.

Editor's summary: 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 lactate 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 twenty-four hours of vaccine administration, indicating the protective effects were not dependent on adaptive immunity. These papers showcase innovative approaches to tackling cholera.

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, 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 2 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 one day of administration.

Results

Design of HaitiV

We employed 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 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 cholera toxin (CT) (12)

(Fig. 1A), 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). Additionally, HaitiV lacks hupB, a gene necessary for episomal maintenance of CTXΦ (14). Further vaccine engineering included steps to 1) reduce potential vaccine reactogenicity by deleting V. cholerae's 5 flagellins (15); 2) eliminate the vaccine's capacity to transfer genes, conferring resistance to antibiotics, that lie within the SXT Integrative Conjugative Element (ICE) (Fig. 1B); 3) allow the vaccine to produce the non-toxic B subunit of CT (Fig. S1), an antigen which may elicit protection against diarrheal disease caused by enterotoxigenic *E. coli* as well as *V. cholerae* (16); and 4) minimize potential gene acquisition by deleting recA, thereby markedly reducing the strain's capacity for DNA recombination. HaitiV also encodes a CRISPR/Cas9 system specifically targeting the toxin gene ctxA. CTXΦ bacteriophage bearing intact ctxA were 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 oro-gastrically inoculated HaitiV or the wild-type *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 (Fig. 2A) previously found to resemble *ctxAB*-dependent choleric diarrhea (17). In marked contrast, minimal or no fluid had accumulated by 18HPI 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 90HPI, although in rare cases animals showed mild and self-limited non-choleric diarrhea. Animals inoculated with HaitiV continued to gain weight up to 90HPI, 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 18HPI, there was no statistically significant difference in *V. cholerae* colonization of the distal small intestine between littermates inoculated with HaitiV or HaitiWT (Fig. 2C). HaitiV burden showed no reduction by 90HPI (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 co-inoculated with a 1:1 mixture of HaitiWT and HaitiV, the wild-type strain outcompeted the vaccine strain (Fig. 2D).

HaitiV mediates 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 prior to the development of an adaptive immune response due, for example, to alteration of the pathogen's intestinal niche. To test this possibility, animals were inoculated either with HaitiV (live vaccine),

formalin-killed HaitiV (killed vaccine), or a buffer control (mock), then challenged 24 hours later with a lethal dose of HaitiWT. Animals in the buffer and formalin groups developed severe cholera-like illness following 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 antagonism of HaitiWT colonization (i.e., colonization resistance) appears dependent on prior inoculation of HaitiV; normal burdens of HaitiWT were observed in animals inoculated with the two strains simultaneously rather than sequentially (Fig. 3B).

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). Importantly, 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 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. 3DF) or 24 hours post-inoculation with live vaccine (sequential inoculation, Fig. 3EG). HaitiTn colonization in the absence of pretreatment was indistinguishable from HaitiWT colonization of animals previously inoculated with a mock treatment or killed vaccine (Fig. 3). Additionally, 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 distal small intestine and performed a genome-wide comparison of mutant abundance in animals subjected to HaitiTn challenge without pretreatment (Fig. 3F, Table S3) or following HaitiV inoculation (Fig. 3G, Table S4). To ensure requisite statistical power, our analysis was restricted to animals colonized by sufficiently diverse HaitiTn populations encompassing multiple independent disruptions per gene (rabbits r3–r6 for single inoculation, rabbits r4–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. 3FG, Fig. S2 and Table S3,4). 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 utilized 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 following 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–r3), suggesting that HaitiV-mediated colonization resistance is, in some cases, associated with a highly restrictive infection bottleneck. Importantly, 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 r4–7). 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 multi-factorial. However, the lower burdens of HaitiWT and absence of severe disease following challenge in vaccine-colonized animals suggests that inoculation with HaitiV may be sufficient to protect against cholera-like illness.

HaitiV rapidly protects 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; one animal did not develop diarrhea) and showed a marked increase in survival time post lethal challenge (median > 41.3 hours; Fig. 4A) and in survival time post onset of diarrhea (>13 hours vs. 5 hours in control animals; Fig. 4B). Additionally, 4 animals inoculated with live vaccine had not reached a moribund state when the study was concluded 40 hours post lethal challenge despite detectable HaitiWT colonization in all animals (Fig. 4A,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 viability-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 impact 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 4AB) or in 10 days (slow vaccine – when some recipients of killed OCVs manifest vibriocidal antibody titers (29)). Importantly, 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 (Fig. S3B, 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. Specifically, our study is limited by the poorly characterized intestinal microbiota and adaptive immune capacity of rabbit neonates, which 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, 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 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.* show that an existing probiotic, *Lactococcus lactis*, confers protection in an infant mouse cholera challenge model and can be engineered to detect Vibrio species (30). 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 (e.g., 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 following 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 following 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 re-hydration therapy is sufficient to prevent death in virtually all symptomatic individuals (31). Additionally, 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 Genetic manipulations), and mutations were verified by whole-genome sequencing (see Whole genome sequencing). 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 Infant rabbit infection studies). 1–2 day old animals were allocated to treatment groups randomly, and within-litter (i.e., co-housed 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 due to 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 Transposon-insertion sequencing analysis). Lastly, our modeling incorporated a variable time to vaccine protection into a set of previously published parameters for disease transmission (see Modeling of cholera outbreaks). Primary data are reported in table S5.

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: 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) with shaking (250 RPM) at 37°C. Recipient strains in phage transduction assays were grown in AKI media (15g/L peptone, 4g/L yeast extract, 5g/L NaCl, autoclaved, then supplemented with freshly made, sterile-filtered 0.3% $NaHCO_3$). Antibiotics and substrates were used in the following concentrations unless otherwise noted: streptomycin (Sm) (200μg/ml), carbenicillin (50ug/ml), chloramphenicol (20ug/ml), SXT (160ug/ml sulfamethoxazole, 32μg/ml trimethoprim), kanamycin (Kn) (50ug/ml) and 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (X-Gal 60mg/mL).

Genetic manipulations

All gene deletions and replacements were constructed via homologous recombination using the suicide vector pCVD442, DH5α-λpir and donor strains MFD-λpir (32) or SM10-λpir (Table S1). For all deletions, ~500–700 bp homologous regions upstream and downstream of the respective ORF were amplified using the primer combinations described below and cloned into XbaI-digested pCVD442 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 dehydrogenase on the 3’ end of this region. This results in a deletion of a 42650 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 PCR 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 Sm^R mutant of the vaccine precursor strain was isolated by plating on streptomycin (1000ug/ml), and the $rpsL^{K43R}$ SNV was confirmed by Sanger sequencing.

For CtxB 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 (https://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 StuI site of pJL1 (35) via isothermal assembly. Sequencing revealed that a recombination event during assembly had removed 2 of 3 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 preparation kit (Illumina) and sequenced on a MiSeq (Reagent kit v2, 2×250). Each sample was mapped to its putative genome and variants identified using GATK3.6.

CTXΦ transduction assay

Supernatant from *Vibrio 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 OD_{600} of 1.0) was concentrated (~50 fold; Ultracel-100K centrifugal filter, Millipore) and filtered (0.22-um filter, Millipore) to get a cell-free phage supernatant. In order to induce expression of TCP (the phage receptor) in the strains being assayed for CTXΦ susceptibility, overnight LB cultures were back-diluted 1:100 into 10ml AKI in 16×150mm glass culture tubes and incubated without shaking for 4hr at 37°C. All but 1ml of the culture was then discarded, and the culture was moved to a shaker (250rpm) for aerobic culture at 37°C for an additional 2hr. 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+Kanamycin (100ug/ml) agar plates, and transduction efficiency was calculated as $(CFU/ml)_{Kan100}/(CFU/ml)_{LB}$.

Generation of HaitiWT-Tn library

E. coli SM10λpir 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, 4mL of each culture was pelleted and washed once with LB. Cultures were then mixed in a 1:1 ratio, pelleted and re-suspended in 800μL LB. 50μL of the mix was spotted onto 0.45μm 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 (1mL/filter) to re-suspend attached bacteria. Suspensions were plated onto 245mm^2 LB +Sm/Kan agar plates to select for *V. cholerae* trans-conjugants (2mL suspension/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. The OD_{600} was adjusted to ~10 and aliquots were stored at −80°C for downstream use.

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, 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 United States Department of Agriculture. To prepare bacteria for inoculation, overnight cultures were diluted 1:100 in 100mL LB and cultured with aeration at 37°C until late-log phase (OD_{600} 0.5 to 0.9). ~2×10^{10} CFU were pelleted by centrifugation at 6000rpm, the supernatant was removed, and cell pellets were re-suspended in 10mL of 2.5% sodium bicarbonate solution (2.5g in 100mL water; pH 9.0) to a final cell density of ~2×10^9 CFU/mL. For co-infection studies, ~1×10^{10} CFU were pelleted by centrifugation at 6000rpm, the supernatant was removed, cell pellets were re-suspended in 5mL 2.5% sodium bicarbonate solution, and the resulting suspensions were combined to yield a 1:1 mixture at a cell density of ~2×10^9 CFU/mL. For studies using the HaitiWT transposon library, a 1mL frozen stock of the library (OD_{600} = 10) was transferred to 100mL LB to an initial OD_{600} of 0.1. The library was then cultured with aeration at 37°C to OD_{600} 0.8 (~2 hours) and a ~2×10^9 CFU/mL cell suspension in 2.5% sodium bicarbonate solution was prepared as described above. Preparation of formalin-killed vaccine required the following additional steps: cell pellets were re-suspended in 8mL of 10% formalin, the formalin suspension was centrifuged at 6000rpm, the supernatant was removed, cells were re-suspended in 5 volumes of 1× phosphate buffered saline (40mL 1× PBS) to wash away excess formalin, the PBS suspension was centrifuged at 6000rpm, the supernatant was removed, and cells were re-suspended in 10mL 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 co-infection 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, ~2×10^{10} CFU of the library inoculum were plated on LB+Sm200/Kan50 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 1–4 day old New Zealand White Rabbits, and animals were co-housed 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. 2A–C) were conducted following 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 oro-gastrically inoculated with ~10^9 CFU (500μL of a 2×10^9 CFU/mL bacterial suspension) using a size 5 French catheter. One-day-old animals were used for single-inoculation and co-inoculation studies. These animals were typically euthanized ~18hrs post-inoculation; however, longitudinal studies of HaitiV colonization were conducted by inoculating 1 day old animals and monitoring their condition through ~90hrs post-inoculation. For sequential inoculation studies, 1 day old animals were inoculated with one of 3 treatments: “mock” – 500μL 2.5% sodium bicarbonate solution, “killed vaccine” – 500μL of a 2×10^9 CFU/mL suspension of formalin-killed HaitiV, or “live vaccine” - 500μL of a 2×10^9 CFU/mL suspension of HaitiV. 24 hours later, the same animals were inoculated with ~10^9 CFU (500μL of a 2×10^9 CFU/mL suspension of the challenge strain (HaitiWT – Fig. 3AB and Fig. 4; N16961 – Fig. 3C; or HaitiTn – Fig 3DE). For sequential inoculation studies that report bacterial burden, animals were euthanized ~18hrs after challenge, with the exception of Fig. 4C.

At necropsy, the entire intestinal tract was removed, cecal fluid was extracted using a 26-½ gauge needle and transferred to a pre-weighed Eppendorf tube. 2–3cm sections of the distal small intestine, along with the entire cecum, were placed in pre-weighed homogenization tubes containing 1mL 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 fluid accumulation ratio (FAR). The tubes containing tissue were homogenized for 2 minutes on a mini-beadbeater-16 (BioSpec Products Inc.) serially diluted in 1× PBS, and plated. Plates were incubated at 30°C overnight and the number of observed colonies was divided by the appropriate dilution ratio and the mass of the corresponding tissue/fluid sample to yield a measure of CFU/g tissue. Homogenates were plated on LB+Sm200/X-Gal60 to enumerate total burden for HaitiWT + HaitiV and/or on LB+SXT to enumerate HaitiWT burden alone. For co-inoculation 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 (i.e., within 100-fold). Similarly, for studies utilizing the N16961 WT strain, which is sensitive to SXT, the number of blue colonies on LB+Sm200/X-Gal60 was used to enumerate WT burden. For co-inoculation studies, a competitive index was calculated as: $Competitive\ Index = \frac{(HaitiV\ CFU \div HaitiWT\ CFU)_{distal\ small\ intestine}}{(HaitiV\ CFU \div HaitiWT\ CFU)_{inoculum}}$. For studies using the HaitiWT transposon library, the terminal 10cm of the distal small intestine were obtained at necropsy, weighed, and homogenized as described above. The homogenate was serially

diluted in 1× PBS and plated on LB+Sm200/X-Gal60 to enumerate total burden and LB +Sm200/Kan50 to enumerate the burden of HaitiTn. The remaining 900μL of undiluted tissue homogenate were plated on LB+Sm200/Kan50 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 moribund state of disease in studies of disease progression because the interval between inoculation and euthanasia, which varies substantially in these studies, is likely to impact 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 between Fig. 4A and Fig 4B).

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 100,000bp 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 Table S3 and S4. Thresholds of mean informative sites > 5, $|\mathrm{Log}_2(\text{mean fold change})| > 1$, mean inverse P-value > 100 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 Figure 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. S4D, S4B; 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, S4A; varied in Fig. S4B). The transmission rate (β) used for simulations was calculated assuming a basic reproductive (R_0) 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 ($1/\gamma$) 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 ($1/\tau$) 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:

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

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

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

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

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

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

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

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

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

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

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

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

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

Statistical Analysis

Comparisons of two samples were performed using two-sided testing (α=0.05) for a t test (fluid accumulation ratios), 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 Material

Refer to Web version on PubMed Central for supplementary material.

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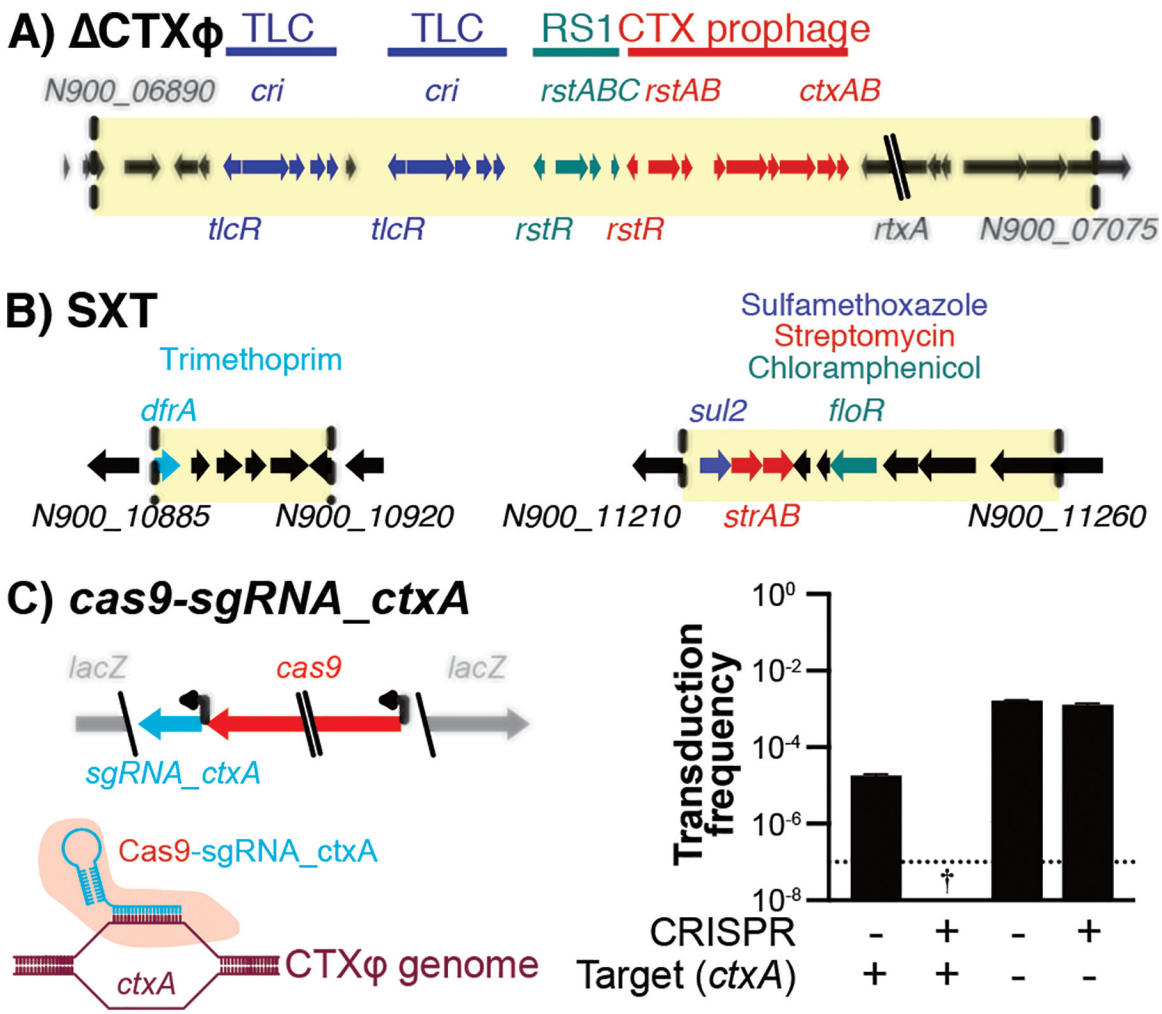

Fig. 1:

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: *S. 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+; intergenic Kan^{R} cassette, intact *ctxA*) or CTX-KnΦ (Target-; *ctxA* replaced by Kan^{R} cassette), and the number of transductants was monitored. No detectable Kan^{R} transductants shown as (†).

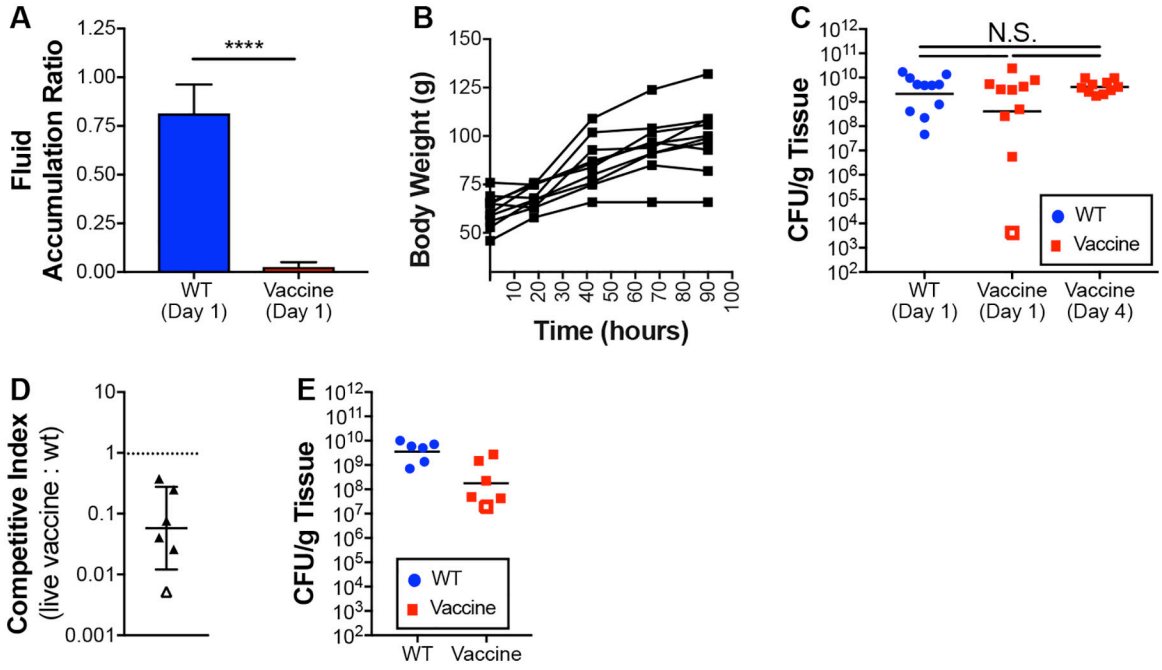

Fig. 2:

HaitiV colonizes the infant rabbit intestine without causing cholera-like illness. **A**) Fluid accumulation ratios after littermates were pre-treated with ranitidine-hydrochloride, to reduce stomach acidity, and inoculated with either WT (n=11) or HaitiV (vaccine; n=10). Plots show mean and standard deviation derived from 2 litters. **** P<0.001, unpaired t-test. **B**) Successive daily weights of animals inoculated with 10^9 CFU HaitiV (n=10). **C**) WT (blue circles) or HaitiV (red squares) CFU recovered from rabbit distal small intestines (dSI) at Day 1 or 4 post-inoculation (each of the 3 groups consists of animals from at least 2 litters). Lines indicate geometric means, and the open symbol indicates limit of detection for the single animal from which no CFU were recovered. NS: P≥0.05, Kruskall-Wallis test followed by Dunn's multiple comparisons test. **D**) Competitive indices (CI) of dSI bacteria 1 day post-inoculation with a 1:1 mixture of WT and HaitiV. The open symbol indicates limit of detection for the single animal from which no vaccine CFU were recovered; lines and bars indicate geometric means and geometric standard deviation of CIs across 2 litters, (n=6). **E**) WT (blue) and HaitiV (red) CFU recovered from co-inoculated animals. The open symbol indicates limit of detection the single animal, from which no vaccine CFU were recovered; lines indicate geometric means.

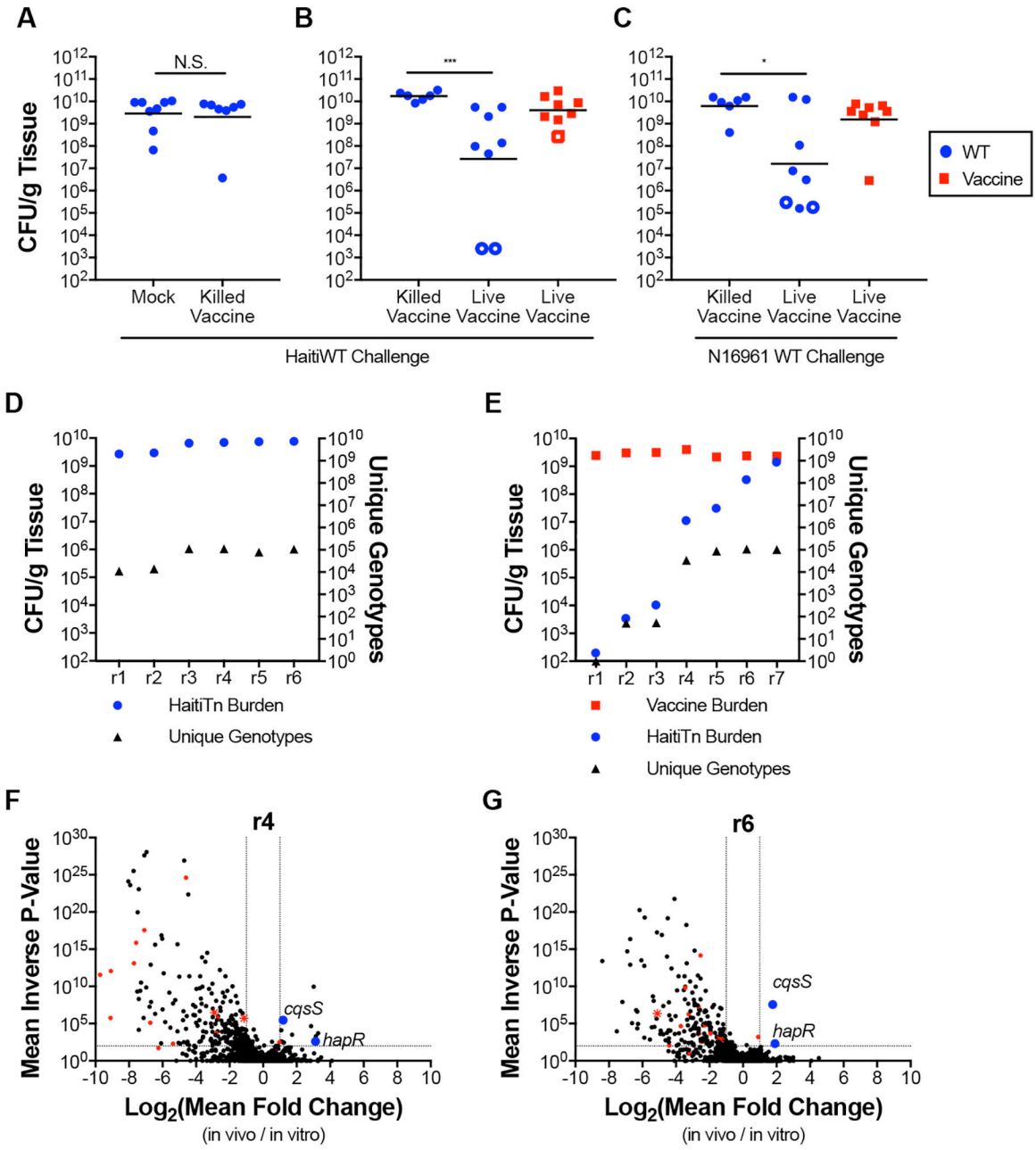

Fig. 3:

HaitiV mediates colonization resistance associated with variably-sized infection bottlenecks. **A**) WT CFU (blue circles) recovered from dSI of animals 18 hours after inoculation with WT. Littermates were pretreated with sodium bicarbonate buffer (mock, n=8) or formalin-killed HaitiV (killed vaccine, n=7) 24 hours prior to WT challenge; geometric means of each group across 3 litters are shown. NS: P>0.05, Mann-Whitney test. HaitiV and HaitiWT (**B**) or N16961 WT (**C**) CFU recovered from the dSI of animals 18 hours post-challenge. Animals were pretreated with killed (n=6) or live (n=8) vaccine 24 hours prior to challenge. Open symbols indicate limit of detection for 5 animals in which no CFU were recovered, and lines indicate the geometric mean of each group across 2 litters. ***P<0.001, *P<0.05,

Mann-Whitney test. **D**) WT CFU (blue circles), and unique transposon mutants (black triangles) recovered from the dSI of individual animals (rabbits r1 to r6) one day after inoculation of the transposon mutant library without pretreatment. **E**) HaitiTn CFU (blue circles), HaitiV CFU (red squares), and unique transposon mutants (black triangles) recovered from the dSI of individual animals (rabbits r1 to r7) 18hrs after inoculation of the transposon mutant library. Animals were pretreated with HaitiV 24 hours prior to challenge with the transposon mutant library. **F)** and **G)** Results of Con-ARTIST (39) analysis for single inoculation (rabbit r4) and sequential inoculation (rabbit r6) samples with the largest number of unique genotypes. The x-axis indicates the change in relative abundance of insertion mutants per gene in vivo, and the y-axis indicates the concordance of independent insertion mutants within each gene. Genes exhibiting a greater than 2-fold change (Log_2(mean fold change) <−1 or > 1) across multiple mutants (mean inverse P-value > 10^2) are considered depleted/enriched. Enriched mutants *cqsS* and *hapR* are indicated in blue. Mutations in known colonization factors, including toxin co-regulated pilus biogenesis (red circles), and the associated transcriptional regulators *toxR* and *toxS* (red asterisks), were depleted.

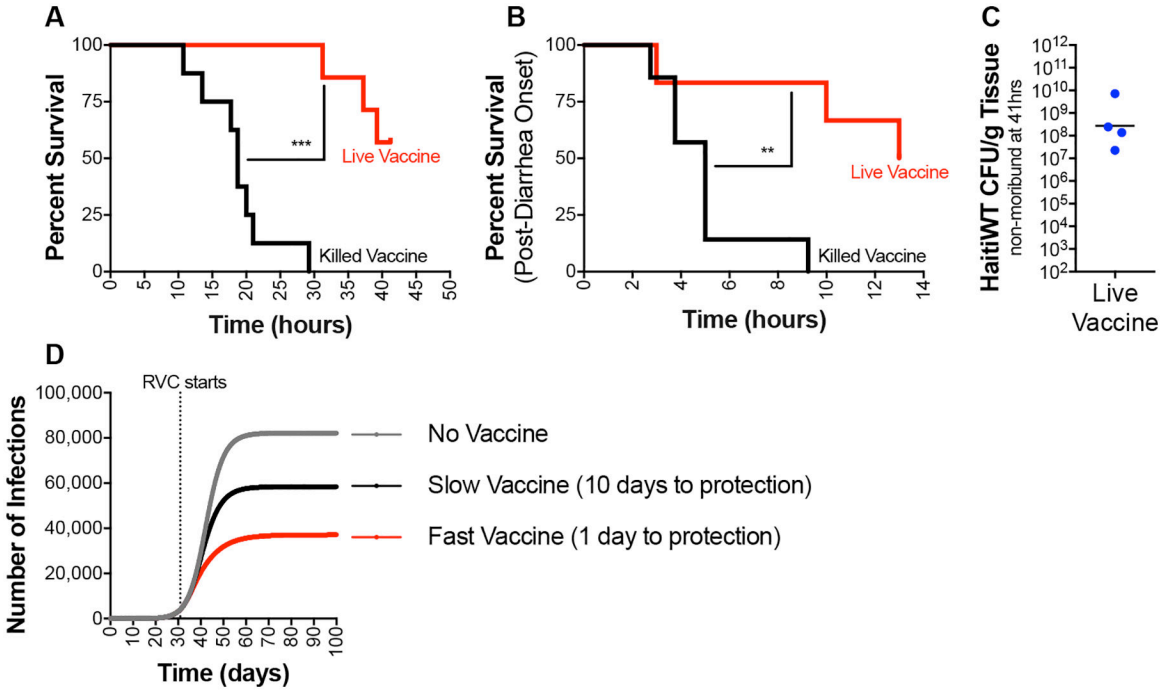

Fig. 4:

HaitiV colonization protects from disease following HaitiWT challenge, and modeling demonstrates the benefit of rapid protection during a cholera outbreak. **A**) Survival curves tracking progression to moribund disease status in animals inoculated with WT at 0 hours after pretreatment (at t = −24 hours) with killed (black: n=8) or live vaccine (red: n=7). ***P<0.001, Log-rank test. **B**) Disease progression from the onset of diarrhea to moribund status in animals, pretreated with killed (black: n=7) or live vaccine (red: n=6), that developed visible diarrhea. ***P<0.001, Log-rank test. **C**) WT CFU (blue circles) recovered from dSI of animals 41 h post-challenge (from A) that did not progress to moribund disease status. **D**) Effect of reactive vaccination on the number of cholera infections in a simulated outbreak (R_0=2.1) starting with a single infection in a population of 100,000 susceptible individuals where the reactive vaccination campaign (RVC) is triggered once the number of symptomatic individuals reaches 1000 (1% of the total population), indicated by the dashed line. Rollout of doses are modeled with a constant rate over 7 days until 70% of the population is vaccinated, as achieved by recent reactive vaccination campaigns. Modeling parameters are described in Fig. S3B.

Table 1:

genetic alterations in HaitiV, a live attenuated cholera vaccine

Mutation	Rationale
ΔCTXΦ	Attenuates by removing the genes encoding cholera toxin and the multifunctional toxin MARTX (13); protects against toxigenic reversion by preventing chromosomal integration of CTXΦ (12)
Δ*flaBDE* / Δ*flaAC*	Attenuates and reduces potential reactogenicity (15)
Δ*floR-strAB-sul2* / Δ*dfrA*	Prevents the dispersal of antibiotic resistance genes
N900_11550::P*htpg-ctxB*	Constitutive expression of CtxB (Fig. S1) promotes anti-CtxB immune responses that may protect against diarrheal disease caused by *V. cholerae* and ETEC (16)
Δ*hupB*	Protects from toxigenic reversion by inactivating the HU complex, which is necessary for extra-chromosomal replication of CTXΦ (14)
lacZ::cas9-sgRNA_ctxA	Endonuclease targeting of *ctxA* prevents toxigenic reversion
Δ*recA*	Prevents homologous recombination-dependent gene acquisition