Oral immunization with a probiotic cholera vaccine induces broad protective immunity against *Vibrio cholerae* colonization and disease in mice

Short title: Immunogenicity of HaitiV in germfree mice

Brandon Sit[1, 2], Ting Zhang[1, 2], Bolutife Fakoya[1, 2], Aklima Akter[3], Rajib Biswas[3], Edward T. Ryan[3, 4, 5], Matthew K. Waldor[1, 2, 4, 5, 6]*

[1]Division of Infectious Diseases, Brigham & Women's Hospital, Boston, Massachusetts, United States of America

[2]Department of Microbiology, Harvard Medical School, Boston, Massachusetts, United States of America

[3]Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts, United States of America

[4]Department of Medicine, Harvard Medical School, Boston, Massachusetts, United States of America

[5]Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, United States of America

[6]Howard Hughes Medical Institute, Bethesda, Maryland, USA

* Corresponding author

Email: mwaldor@research.bwh.harvard.edu (MKW)

Abstract

Oral cholera vaccines (OCVs) are being increasingly employed, but current killed formulations generally require multiple doses and lack efficacy in young children. We recently developed a new live-attenuated OCV candidate (HaitiV) derived from a *Vibrio cholerae* strain isolated during the 2010 Haiti cholera epidemic. HaitiV exhibited an unexpected probiotic-like activity in infant rabbits, preventing intestinal colonization and disease by wild-type *V. cholerae* before the onset of adaptive immunity. However, it remained unknown whether HaitiV would behave similarly to other OCVs to stimulate adaptive immunity against *V. cholerae.* Here, we orally immunized adult germ-free female mice to test HaitiV's immunogenicity. HaitiV safely and stably colonized vaccinated mice and induced known adaptive immune correlates of cholera protection within 14 days of administration. Pups born to immunized mice were protected against lethal challenges of both homologous and heterologous *V. cholerae* strains. Cross-fostering experiments revealed that protection was not dependent on vaccine colonization in or transmission to the pups. These findings demonstrate the protective immunogenicity of HaitiV and support its development as a new tool for limiting cholera.

Author summary

Vaccines for cholera are gaining acceptance as public health tools for prevention of cholera and curtailing the spread of outbreaks. However, current killed vaccines provide minimal protection in young children, who are especially susceptible to this diarrheal disease, and do not stimulate immunity against antigens that may only be expressed by live bacteria during infection. We recently developed HaitiV, an extensively engineered live-attenuated oral cholera vaccine candidate, derived from a clinical isolate from the Haiti cholera outbreak. Here, we found that the HaitiV induces immunological correlates of protection against cholera in germ free mice and leads

47 to protection against disease in their offspring. Protection in this model was dependent on
48 passively acquired factors in the milk of immunized dams and not transmission or colonization of
49 HaitiV. Coupling the immunogenicity data presented here with our previous observation that
50 HaitiV can protect from cholera prior to the induction of adaptive immunity, suggests that HaitiV
51 may provide both rapid-onset short-term protection from disease while eliciting stable and long-
52 lasting immunity against cholera.

53

54 Introduction

55 The bacterial pathogen *Vibrio cholerae* causes the severe human diarrheal disease cholera, a
56 potentially fatal illness characterized by rapid-onset of fluid loss and dehydration. Recent
57 estimates place the global burden of cholera at ~3 million cases per year, and over 1.3 billion
58 people are at risk of this disease [1]. *V. cholerae* proliferates in the small intestine and produces
59 cholera toxin (CT), which leads to water and electrolyte secretion into the intestinal lumen [2].

60 The O1 serogroup of *V. cholerae* causes virtually all epidemic cholera. This serogroup includes
61 two serotypes, Inaba and Ogawa, whose LPS structures differ by a single methyl group on the
62 terminal O-antigen sugar [3]. Serologic and epidemiologic studies have established the existence
63 of extensive serotype cross-reactivity and -protectivity, although immunogenicity and protection
64 is highest to the homologous serotype [4–7]. Toxigenic O1 strains are divided into two major
65 biotypes, classical and El Tor, but the former has not been isolated in over a decade and is thought
66 to be extinct [8]. Ongoing evolution of El Tor *V. cholerae* has given rise to “atypical El Tor” (AET)
67 strains, which are distinguishable from earlier strains by a variety of features, including the
68 expression of a non-canonical *ctxB* allele, that may impact disease severity in afflicted patients
69 [4,9,10]. AET strains, such as the strain responsible for the 2010 Haitian cholera epidemic, are
70 thought to be the globally dominant cause of cholera [10–12]. Currently, serogroup O139 isolates

71 only cause sporadic disease [13]. Notably, antibodies (or immune responses) targeting the O1 O-
72 antigen do not protect against O139 challenge and vice versa [14–16].

73 Oral cholera vaccines (OCVs) have recently become widely accepted as a tool for cholera control
74 [17]. Vaccines are a potent method to combat cholera due to their ability to both directly and
75 indirectly reduce disease and transmission [18]. Killed multivalent whole-cell OCVs, such as
76 Shancol, have shown promise both to prevent disease in endemic regions and as reactive agents
77 to limit cholera during epidemics [19]. However, killed OCVs tend to be less effective at eliciting
78 protective immunity in young children (<5 years old), who are most susceptible to cholera [20,21].
79 Additionally, these vaccines typically require two doses over the span of several weeks, although
80 recent studies suggest that a single dose may still lead to moderate protection [20,22,23].

81 There is no live-attenuated OCV licensed for use in cholera-endemic regions. The only clinically
82 available live-attenuated OCV is Vaxchora (CVD103-HgR), which is derived from a classical O1
83 Inaba *V. cholerae* strain and was approved by the US FDA in 2017 for use in travelers [24]. In
84 contrast to killed OCVs, live vaccines, such as CVD103-HgR and the El Tor-derived vaccine Peru-
85 15, elicit more potent immune responses in young children [25,26], potentially because they more
86 closely mimic natural infection than killed OCVs. In particular, live vaccines can produce antigens
87 in vivo that are not expressed in the in vitro growth conditions used to prepare killed vaccines;
88 furthermore, the inactivation processes used to formulate killed vaccines can destroy antigenic
89 epitopes [27].

90 In addition to the requirement for multiple doses of some OCVs for optimal protection, all current
91 live and killed OCVs are thought to be accompanied by a post-vaccination lag in protection during
92 induction of anti-*V. cholerae* adaptive immunity. The shortest reported time to protective efficacy
93 is 8 days post-vaccination, a delay that could hamper reactive vaccination campaigns designed
94 to limit the spread of cholera outbreaks [28]. We recently created HaitiV, a new live-attenuated
95 OCV candidate derived from an AET O1 Ogawa *V. cholerae* clinical isolate from the 2010 Haiti

cholera outbreak. HaitiV harbors many genetic alterations that render it avirulent and resistant to reversion while preserving its robust capacity for colonization of the small intestine [29]. In an infant rabbit model of cholera [30], intestinal colonization with HaitiV conferred protection against lethal wild-type (WT) *V. cholerae* challenge within 24 hours of vaccination, a timescale inconsistent with the development of adaptive immunity and suggestive of a "probiotic"-like mechanism of protection. Here, using a mouse model of *V. cholerae* intestinal colonization, we show that oral administration of HaitiV to female mice elicits serum vibriocidal antibodies and protects their pups from lethal challenge with virulent *V*. *cholerae*. Thus, HaitiV has the potential to provide rapid probiotic-like protection as well as to elicit long-lasting immune protection from cholera.

Methods

Bacterial strains and growth conditions

All bacteria were grown in Luria-Bertani (LB) broth supplemented with the relevant chemicals at the following concentrations: streptomycin (Sm, 200µg/mL), kanamycin (200µg/mL), carbenicillin (Cb, 50µg/mL), sulfamethoxazole/trimethoprim (SXT, 80 and 16µg/mL) and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal, 60µg/mL). For growth on plates, LB + 1.5% agar was used. All *V. cholerae* strains in this study were spontaneous Sm^R derivatives of the wild-type. Bacteria were stored as -80ºC stocks in LB with 35% glycerol. A list of strains and plasmids used in this study is listed in Supplementary Table 1.

Construction of *ΔctxAB* Haiti *V. cholerae*

The CT deletion strain in the H1 *V. cholerae* background (HaitiWT) was generated by allelic exchange as previously described, with an additional selection step to enhance the efficiency of obtaining a stable single crossover strain [29]. Briefly, HaitiWT was conjugated with SM10pir *E.*

coli bearing the suicide plasmid pCVD442-ctxAB-KnR, containing *sacB* as well as a kanamycin resistance cassette from pKD4 sandwiched by homology arms targeting the *ctxAB* operon (locus tags N900_RS07040 – N900_RS07045). Single crossovers were selected on LB+Sm/Cb/Kn agar plates. To select for a double crossover, verified single crossovers were grown in LB + Cb/Kn for 4 hours at 37ºC and then passaged in LB+10% sucrose overnight at room temperature. Sucrose-resistant (*sacB*-negative), Kn^R and Cb^S colonies were then conjugated with SM10pir *E. coli* bearing pCVD442-ctxAB (no Kn^R cassette) and clean Kn^S double crossovers generated via an identical protocol. The *ΔctxAB* deletion was verified by colony PCR with internal and flanking primers.

Oral immunization regimen

4-week old germ-free (GF) female C57BL/6 (Massachusetts Host-Microbiome Center) or Swiss-Webster (Taconic Farms) mice were housed in a BL-2 facility for the duration of the experiment. On Day 0, 2, 4, 6, 14, 28, 42 and 56, mice were anesthetized with isoflurane and orally gavaged with 10^9 CFU of an overnight culture of either HaitiV or CVD103-HgR in 100µL 2.5% Na_2CO_3. Mice were weighed at every immunization and once every 4-5 days between boosts. At each weighing, fresh fecal pellets were plated on LB + Sm to enumerate shed bacteria. At Day 7, 14, 28 and 42 post immunization, blood samples were obtained from each mouse by tail vein incision. A Day 1 blood sample was collected from the Swiss-Webster cohort and the single-dose C57BL/6 cohort. Blood was clotted at room temperature for 1 hour, centrifuged at 20000 x g for 5 minutes and the supernatant (serum) stored at -20ºC for analysis.

Quantification of vibriocidal responses

Vibriocidal antibody quantification was performed by complement-mediated cell lysis using PIC018 (Inaba) or PIC158 (Ogawa) *V. cholerae* as the target strain as previously described [31]. Seroconversion was defined as ≥4x increase in titer over the baseline measurement. The

144 characterized mouse monoclonal antibody 432A.1G8.G1.H12 targeting *V. cholerae* O1 OSP was
145 used as a positive control for the vibriocidal assay. Titers are reported as the dilution of serum
146 causing a 50% reduction in target optical density compared to no serum control wells.

147 **Quantification of anti-CtxB and anti-OSP responses**

148 Anti-CtxB and anti-OSP responses were measured by previously described isotype-specific
149 ELISAs [32,33]. Briefly, 96-well plates (Nunc) were coated with 1 µg/mL solution of bovine GM1
150 monosialoganglioside (Sigma) in 50mM carbonate buffer overnight. Next, 1µg/mL CtxB in 0.1%
151 BSA/PBS purified from the classical Inaba strain 569B (List Biological Laboratories) was layered
152 onto the GM1-coated wells. Wells were blocked with a 1% BSA/PBS mixture after which 1:50
153 dilutions of the mouse serum samples were loaded into each well. Goat anti-mouse IgA, IgG or
154 IgM secondaries conjugated to HRP (Southern Biotechnology) were then added at a
155 concentration of 1µg/mL in 0.1% BSA/0.05% Tween/PBS and incubated for 90 minutes. Detection
156 was performed by adding an ABTS/H_2O_2 mixture to the wells and taking an absorbance
157 measurement at 405nm with a Vmax microplate kinetic reader (Molecular Devices Corp.,
158 Sunnyvale, CA). Plates were read for 5 min at 30 s intervals, and the maximum slope for an optical
159 density change of 0.2 U was reported as millioptical density units per minute (mOD/min). Results
160 were normalized using pooled control serum from mice previously immunized against cholera and
161 reported as ELISA Units as previously described [32]. Anti-OSP responses were measured and
162 reported similarly to anti-CtxB responses, only instead of CtxB, purified OSP:BSA from either
163 PIC018 or PIC158 (1 µg/mL) was used to coat plates as previously described [34]. Additionally,
164 OSP ELISAs were carried out with 1:25 dilutions of the serum samples.

165 **Infant mouse challenge assay**

166 The infant mouse survival challenge was adapted from previous reports to optimize the dosage
167 for HaitiWT and to include more frequent monitoring intervals [31,32]. Pregnant dams were singly

168 housed at E18-19 for delivery. At P3 (third day of life), pups were orally inoculated with 10^7 CFU
169 *V. cholerae* in 50µL LB and returned to their dam. Infected pups were monitored every 4-6 hours
170 for onset of diarrhea and reduced body temperature. Once symptoms appeared, monitoring was
171 increased to every 30 minutes until moribundity was reached, at which point pups were removed
172 from the nest and euthanized by isoflurane inhalation followed by decapitation for dissection and
173 CFU plating of the small intestine on LB + Sm/X-gal. Pups that were alive at 48 hpi were deemed
174 protected from the challenge. Cross-fostering was performed by transferring up to half of a litter
175 between dams on the first day of life (P1). Fostering was maintained for at least 48 hours before
176 infection to fully replace the milk from the original dam. We excluded rejected pups from analyses
177 due to our inability to attribute mortality to infection alone.

178 **Statistical analysis**

179 Statistical analyses were performed with Prism 8 (Graphpad). Due to missing values from paired
180 measurements as a result of insufficient serum sampling, antibody titers could not be analyzed
181 by a typical one-way repeated measures ANOVA. Instead, we employed a mixed-effect model
182 ANOVA using the earliest sample (Day 1 or Day 7) as the control and performed post hoc tests
183 with Dunnett's multiple comparison test. The Geisser-Greenhouse correction was applied for the
184 ANOVA. Survival curves were analyzed with the log-rank test and CFU burdens were compared
185 with the Mann Whitney U test. A p-value <0.05 was considered statistically significant.

186 **Animal use statement**

187 This study was performed in accordance with the NIH Guide for Use and Care of Laboratory
188 animals and was approved by the Brigham and Women's Hospital IACUC (Protocol
189 #2016N000416). Infant (P14 or younger) mice were euthanized by isoflurane inhalation followed
190 by decapitation. At the end of the study, adult mice were euthanized by isoflurane inhalation
191 followed by cervical dislocation.

Results

Experimental design and vaccine colonization

While the infant rabbit model enables investigation of the progression of a *V. cholerae*-induced diarrheal disease that closely mimics human cholera [30], it is not appropriate to study vaccine immunogenicity because newborn animals lack a fully developed immune system. Instead, we used adult GF mice to study HaitiV immunogenicity. In contrast to normal adult mice, which are resistant to *V. cholerae* intestinal colonization, oral inoculation of GF mice with *V. cholerae* results in stable intestinal colonization without adverse effects [35–37]. In the GF model, serum markers of immunity, such as vibriocidal titers, can be measured, but challenge studies are not possible due to the persistent colonization of the vaccine strain and the resistance of adult mice to diarrheal disease. Here, we further developed a variation of the GF model [38]. Besides measuring serum markers in the orally vaccinated adult mice, neonatal pups (which are sensitive to *V. cholerae* induced diarrheal disease) born to these mice were subjected to challenge studies to evaluate vaccine protective efficacy.

We established two cohorts of orally immunized adult female GF mice. In the first cohort, a small pilot study was set up to compare the immunogenicity of HaitiV and a streptomycin-resistant derivative of CVD-103HgR. This cohort consisted of 4-week-old Swiss-Webster GF mice that were immunized with either vaccine strain (n=3 per group). Cohort 2 consisted of a set of seven 4-week-old C57BL/6 mice that were all immunized with HaitiV. We generally followed the multi-dose oral immunization scheme previously used in this model, which included eight doses of $1x10^9$ CFU vaccine over eight weeks [36,37]. After this vaccination regimen, the mice in cohort 2 were mated and vaccine-induced protective immunity was assessed in the progeny (Fig 1A).

Based on fecal CFU, all animals in both cohorts were stably colonized with high levels of either vaccine strain (Fig 1B). No adverse effects of long-term colonization with HaitiV or CVD103-HgR

were noted, and all mice gained weight over the course of the study (Fig 1B). Fecal shedding and presumably intestinal colonization of HaitiV in cohort 2 was eliminated after these dams were used to cross-foster pups born to specified-pathogen free (SPF) control mice (described below), suggesting that a normal microbiota can outcompete HaitiV.

HaitiV immunization elicits robust serum antibodies targeting *V. cholerae*

Serum samples from the immunized mice were used to quantify antibodies targeting several *V. cholerae* factors thought to play roles in protection from cholera. One of these metrics, the vibriocidal antibody titer, is a validated correlate of protection in vaccinated humans [39–42]. In cohort 1, all mice immunized with HaitiV or CVD-103HgR seroconverted within 2 weeks and developed vibriocidal titers consistent with those reported in human studies for live OCVs (Fig 2) [41,43]. Furthermore, HaitiV and CVD-103HgR elicited comparable vibriocidal titers.

In cohort 2, HaitiV immunization of C57BL/6 mice also induced high vibriocidal titers to Ogawa and Inaba target strains (Fig 2C). Isotype-specific levels of antibodies targeting Ogawa and Inaba O-antigen specific polysaccharide (OSP), and the B-subunit of CT (CtxB) were also measured since they also likely contribute to immunity to cholera [39]. Although we did not measure Day 1 titers in cohort 2, measurements from naïve GF C57BL/6 mice and baseline measurements from cohort 1, and Day 1 of HaitiV-inoculated C57BL/6 mice in a later cohort (Fig S2) showed undetectable levels of vibriocidal antibodies (Fig 2A, S2). The cohort 2 mice developed strong anti-Ogawa and anti-Inaba OSP responses (Fig 3, Table S2). The anti-Ogawa OSP titers were generally higher than those targeting Inaba OSP, likely reflecting the fact that HaitiV is an Ogawa strain. All mice in cohort 2 also developed high levels of anti-CtxB IgA, IgG and IgM antibodies (Fig 4, Table S1). The 100% seroconversion rate and general increase over time of all three humoral immune responses measured (vibriocidal, anti-CtxB and anti-OSP antibodies) reveals that orally delivered HaitiV can elicit *V. cholerae*-specific immune responses.

Pups born to HaitiV immunized dams are protected from lethal *V. cholerae* challenge

To assess the protective efficacy of HaitiV in this model, we challenged the neonatal progeny of HaitiV-immunized or control dams with lethal doses of different wild type *V. cholerae* strains. This assay has been used to study passive immunity elicited by cholera vaccines, but has not been characterized in vaccinated GF mice [31,32]. Initially, we optimized this assay with litters from SPF C57BL/6 control mice. Three or four-day old pups were inoculated with 10^7 or 10^8 CFU of HaitiWT, the virulent strain from which HaitiV was derived and returned to their dams for monitoring (Fig 5A). Infected pups from both groups rapidly developed signs of dehydrating diarrheal disease, including accumulation of nest material on their anogenital regions, lethargy, skin tenting and hypothermia. All infected pups died by 48 hours post inoculation (hpi), with a median time to moribundity of ~23-26 hpi (Fig 5B). At the time of death, all pups were heavily colonized, with >10^7 CFU/small intestine (Fig 5C), and had swollen ceca, another hallmark of productive cholera infection in mammalian models [30,44]. Since there were no significant differences in survival or bacterial loads in mice challenged with either 10^7 or 10^8 CFU, the smaller dose was used in subsequent experiments (Fig 5B). Diarrhea and death in this model were entirely dependent on CT; infant mice inoculated with HaitiWT Δ*ctxAB* or HaitiV were completely healthy at 48 hpi, despite sustained intestinal colonization (Fig 5C).

We next mated HaitiV-immunized animals from cohort 2 with age-matched GF male mice, thereby preserving their colonization with HaitiV. When challenged with HaitiWT, none of the 16 pups born to HaitiV-immunized dams developed signs of diarrhea or died by 48 hpi; in stark contrast, all pups born to non-immunized dams died within ~30 hpi (Fig 6A, left). There was a marked ~5,000-fold reduction in the intestinal load of HaitiWT in pups born to immunized versus control dams (Fig 6A, right). The pups of the immunized dams remained healthy for at least 2 weeks post-challenge, even though there were still detectable but very low levels of HaitiWT in their intestinal

265 homogenates (Fig S1). Thus, oral immunization with HaitiV elicits an immune response that
266 provides potent protection in nursing pups from diarrheal disease, death and *V. cholerae* intestinal
267 colonization.

268 Pups of HaitiV-immunized dams were similarly challenged with heterologous *V. cholerae* strains,
269 to test the serotype and serogroup specificity of protection engendered by oral immunization with
270 HaitiV. The additional challenge strains included an O1 Inaba strain (N16961) that has been used
271 as the challenge strain in several human volunteer cholera studies [43,45] and the serogroup
272 O139 strain MO10, which was isolated during the 1992 O139 outbreak in India. Most pups from
273 HaitiV-immunized dams were protected from N16961 *V. cholerae* challenge (7/10 survival at 48
274 hpi, Fig 6B). Despite the clinical protection, there was a much less dramatic reduction in the
275 intestinal burden of N16961 (~20-fold) compared to that observed with HaitiWT challenge,
276 indicating serotype-specific responses play an important role in limiting colonization. Surprisingly,
277 pups challenged with MO10 also exhibited some protection, but there was no concomitant
278 reduction in the intestinal burden of this O139 strain (Fig 6C). Together, these observations
279 demonstrate that animals can exhibit protection from death despite relatively robust colonization,
280 suggesting that protection from disease may result from immunity targeting factors such as CtxB,
281 in addition to those that impede colonization.

282

283 **Pups fostered by HaitiV-immunized dams are protected from lethal *V. cholerae***
284 **challenge**

285 Since our earlier studies indicated that HaitiV itself can mediate rapid protection against cholera
286 independent of an adaptive immune response, it was important to investigate whether pups
287 nursed by HaitiV immunized dams were colonized with the vaccine strain. Extensive plating of
288 intestinal samples from the >50 pups used for survival assays (limit of detection = 50 CFU/small

289 bowel) did not reveal any HaitiV CFU in the pups reared by HaitiV-shedding dams. Thus, vaccine
290 strain transmission and its probiotic effects are almost certainly not the explanation for the potent
291 protection observed in nursing pups.

292 Cross-fostering experiments were undertaken to investigate the likely passive nature of the
293 protection. P1 pups born to SPF dams were transferred to and reared by HaitiV-immunized dams
294 and then challenged 2 days later with HaitiWT (Fig 5A, between P1-P3). All pups crossed-fostered
295 by immunized dams were protected (100% survival at 48 hpi) and nearly all had marked
296 reductions (~1,000 fold) in their intestinal HaitiWT burdens (Fig 7A). These observations mirror
297 the challenge studies presented above (Fig 6A), indicating passive immunity from milk accounts
298 for the protection that HaitiV-immunized dams bestow to their progeny. Conversely, when pups
299 born to HaitiV-immunized dams were cross-fostered by SPF (non-vaccinated) dams, all
300 succumbed to HaitiWT challenge, albeit with an increase in median survival time (by ~6-hour) and
301 had high HaitiWT intestinal burdens (Fig 7B). The modest extension in survival time in these mice
302 may be due to trans-placentally derived immunity or residual milk from the HaitiV-immunized dam.

303

304 **A single oral dose of HaitiV is sufficient to elicit protective immunity**

305 Finally, to investigate whether the multiple dose regimen was necessary for stimulating protective
306 immunity, we tested whether a single oral dose of HaitiV stimulated protective immune responses
307 in female GF C57BL/6 mice (n=4). Like our studies with the multiple dose regimen, a single dose
308 of HaitiV led to sustained colonization in the mice (Fig S2). HaitiV induced vibriocidal antibody
309 titers comparable in magnitude to those from serially immunized mice (Fig 2C). Litters from singly-
310 immunized mice were also completely protected from disease resulting from HaitiWT challenge,
311 phenocopying pups from the first cohorts (Fig S2).

312

Discussion

Vaccines for cholera are being increasingly embraced as public health tools for prevention of endemic cholera and limiting the spread of cholera epidemics [17]. Killed OCVs are efficacious in endemic populations, but live OCVs promise to be more potent, particularly in young children [19]. Here, we showed that the live OCV candidate HaitiV induces vibriocidal antibodies and other immunological correlates of protection against cholera in GF mice and leads to protection against disease in their offspring. Protection in this model was dependent on passively acquired factors in the milk of immunized dams and not transmission or colonization of HaitiV. Although our relatively small cohort sizes precluded rigorous statistical comparisons of immune responses in the immunized mice, oral administration of even a single dose of HaitiV elicited detectable vibriocidal antibodies in all animals. These observations provide strong data establishing HaitiV's immunogenicity. Additionally, the comparable vibriocidal titers elicited by HaitiV and CVD-103HgR, a live OCV licensed by the FDA for travelers, bodes well for HaitiV's immunogenicity in humans. Combining the immunogenicity data presented here with our finding that HaitiV can protect from cholera prior to the induction of adaptive immunity [29], suggests that HaitiV may function as a dual-acting agent, providing both rapid-onset short-term protection from disease while eliciting stable and long-lasting immunity against cholera.

Data from the challenge experiments (Figure 6) are consistent with the prevailing notion that serogroup, and to a lesser extent serotype are major determinants of protection against *V. cholerae* challenge [39,46]. Although it is thought that exposure to Inaba strains is more cross-protective than exposure to Ogawa strains, the relative potency of Inaba versus Ogawa vaccines in eliciting dual protection against both O1 serotypes requires further definition, as it has been suggested that both Ogawa and Inaba vaccine strains are good candidates for development [4–7]. A mixture of Ogawa and Inaba serotypes either as distinct strains or one bivalent strain

337 (serotype Hikojima) may be beneficial in broadening the breadth of the immune response to HaitiV
338 [47,48].

339 The modest protection that HaitiV immunization provided against *V. cholerae* O139 was
340 unexpected. The epidemiology of the original O139 outbreak and experimental studies in rabbits
341 demonstrate a lack of cross-protection between the two serogroups [14,15,39]. Notably, although
342 pups born to HaitiV-immunized dams and challenged with MO10 survived longer than pups born
343 to non-immunized dams, there was little difference in the MO10 intestinal colonization between
344 these groups (Fig 6C). The discrepancy between clinical protection and relatively robust
345 colonization suggests that HaitiV stimulates immune responses to *V. cholerae* factors, like CT,
346 that may contribute to disease but not directly to colonization. The capacity of live OCVs to induce
347 immune responses to in vivo-expressed antigens, including CtxB, is a property that heightens the
348 appeal of live vs killed OCVs [27,49].

349 Although GF mice enabled us to test the protective efficacy of a candidate live OCV, the absence
350 of the microbiota and resulting improper immune development in these mice, are important
351 caveats to consider. The GF model does not recapitulate the competitive microbial environment
352 that live OCVs will encounter in the human host. We observed similar prolonged shedding
353 patterns for both CVD-103HgR and HaitiV in the GF mice (Fig 1), yet CVD-103HgR is known to
354 be shed by human volunteers at a low frequency for a short period [25,50]. Thus, our findings
355 likely overestimate HaitiV's capacity to colonize the human intestine. The observation that
356 exposure of HaitiV-immunized dams to SPF-derived pups during the cross-fostering experiments
357 led to the elimination of detectable HaitiV in feces supports the prediction that this vaccine will not
358 stably colonize humans. It is an open question whether transient exposure of naïve mice to HaitiV
359 will also stimulate protective immunity, as has been shown in the context of vaccination with *V.*
360 *cholerae* outer membrane vesicles [51,52]. The streptomycin-treated mouse model of *V. cholerae*
361 colonization, which allows for temporary intestinal colonization, may also be useful to investigate

362 the duration of colonization required for immunity [53]. Ultimately, the capacity of HaitiV to
363 colonize the intestine and the relationship between colonization and protective immunity will need
364 to be defined in human volunteers.

365 The immunogenicity of live OCVs in mice has only been investigated in GF animals because adult
366 mice with intact microbiota are refractory to intestinal *V. cholerae* replication and colonization.
367 However, previous studies of live OCVs in GF mice only analyzed immune correlates of protection
368 and not protection against challenge [36,37]. The combination of the neonatal survival assay with
369 the oral GF vaccination model builds on existing knowledge of these mice to assay both the
370 immunogenicity and protective efficacy of live OCV candidates [38]. This model may be a useful
371 addition to existing approaches that probe the molecular bases of vaccine-mediated mucosal
372 protection against pathogens, a topic with significant translational potential that remains poorly
373 understood [52,54]. A recent report employing a similar maternal-infant transmission model in the
374 context of intraperitoneally-delivered heat-killed *Citrobacter rodentium* highlights the versatility of
375 assessing vaccine protective efficacy using the infant progeny of immunized animals as readouts
376 [55]. The broad availability of genetically engineered mice and the relative ease of GF-derivation
377 provides a powerful opportunity to leverage both host and bacterial genetics to explore how live-
378 OCVs can be optimized to better defend against this ancient pathogen.

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Author Contributions

Conceptualization: BS, TZ, ETR, MKW. Methodology: BS, TZ, ETR, MKW. Investigation: BS, TZ, BF, AA, RB. Supervision: ETR, MKW. Visualization: BS. Writing (Original Draft Preparation): BS and MKW. Writing (Review and Editing): BS, TZ, BF, AA, RB, ETR, MKW.

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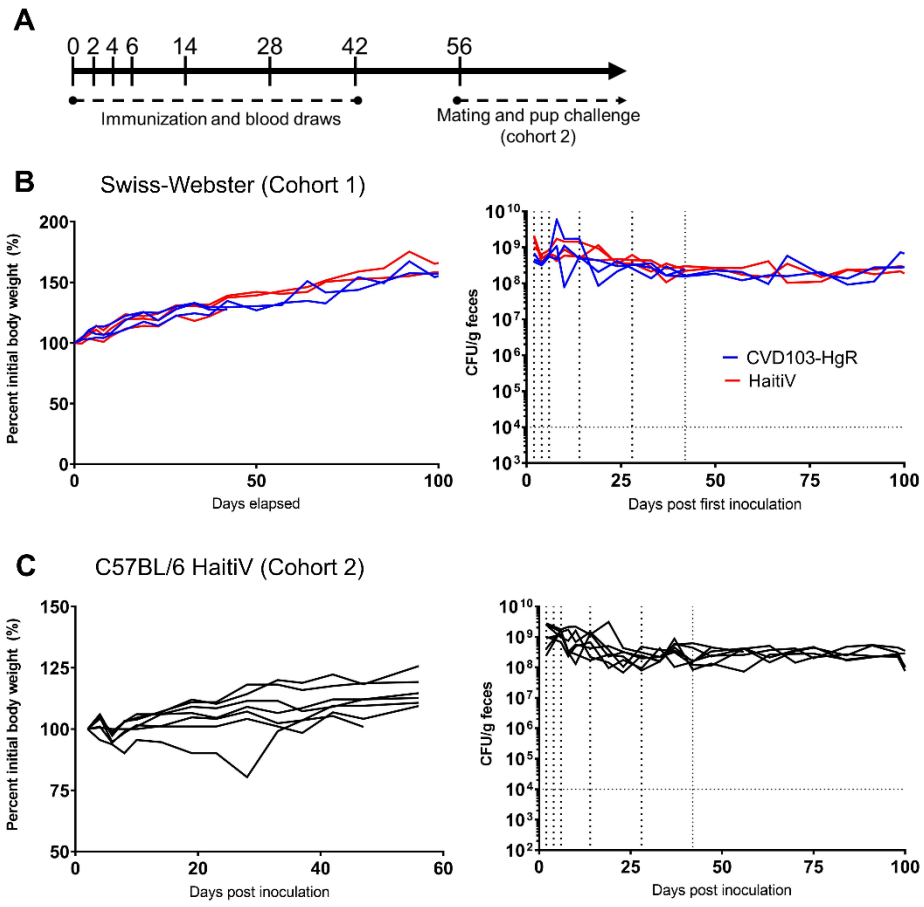


Fig 1. Fecal vaccine shedding and weight of adult GF mice after oral administration of live attenuated cholera vaccines. (A): Schematic of vaccination regime – note cohort 1 does not extend past Day 42. (B and C): Bodyweight (left) and fecal CFU shedding of HaitiV (right) for cohort 1 and 2 respectively.

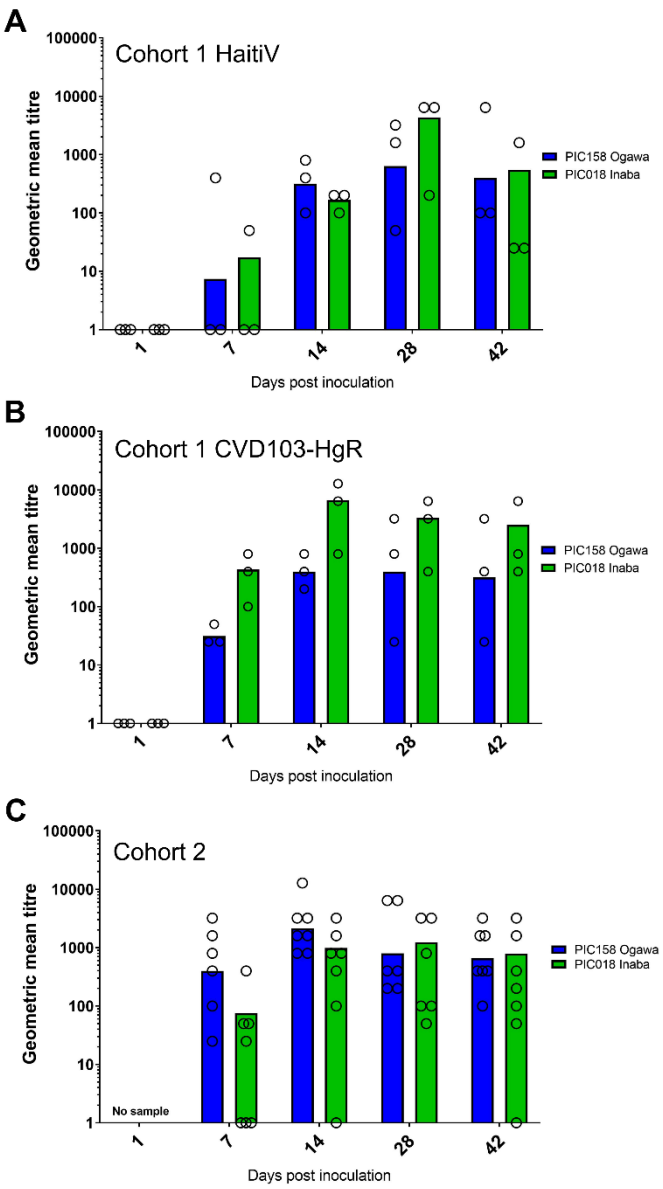
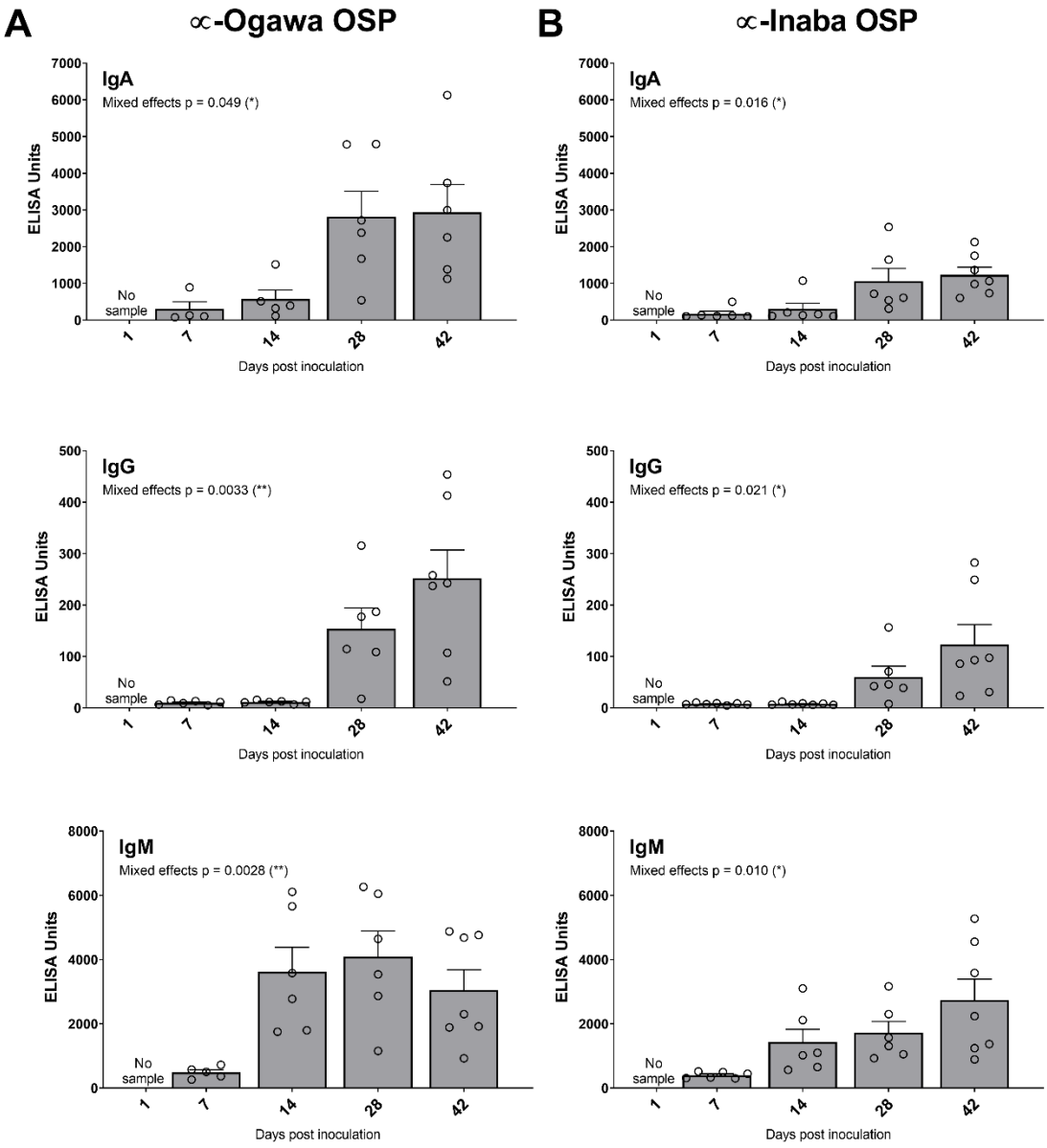


Fig 2. Vibriocidal titers in immunized mice. Vibriocidal titers measured in cohort 1 mice immunized with (A): HaitiV or (B): CVD103-HgR, and in (C): cohort 2 mice immunized with HaitiV are shown. Each circle corresponds to the lowest dilution at which vibriocidal activity was observed and the height of the bars represent geometric mean titers in each group. Ogawa-specific responses, measured using strain PIC158, are shown in blue and Inaba-specific responses, measured using strain PIC018, are shown in green. Serum samples with undetectable vibriocidal activity were assigned a titer of 1



627 **Fig 3. Ogawa- and Inaba-specific anti-OSP titers in C57BL/6 mice immunized with HaitiV.**

628 (A) Anti-Ogawa OSP titers with specific isotype data for IgA (top), IgG (middle) and IgM (bottom).

629 (B) Anti-Ogawa OSP titers with specific isotype data for IgA (top), IgG (middle) and IgM (bottom).

630 The mean ± SEM is indicated. Mixed-effect p-values were determined by one-way ANOVA

631 analysis. Multiple comparison p-values for this data are reported in Table S1. Although there were

632 7 mice per group, some samples were of insufficient volume for analysis.

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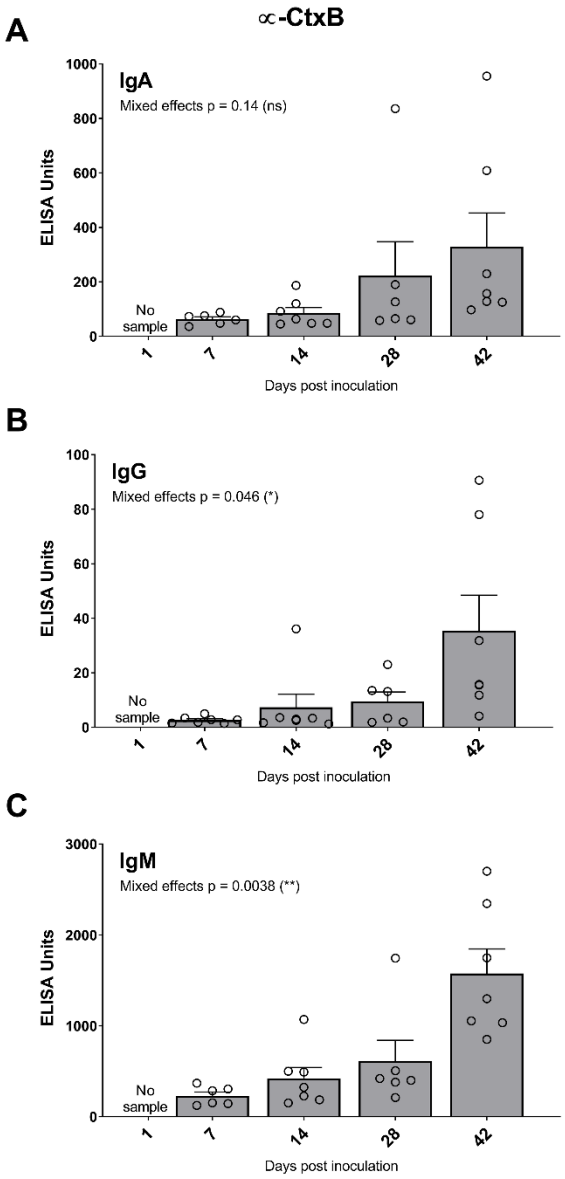
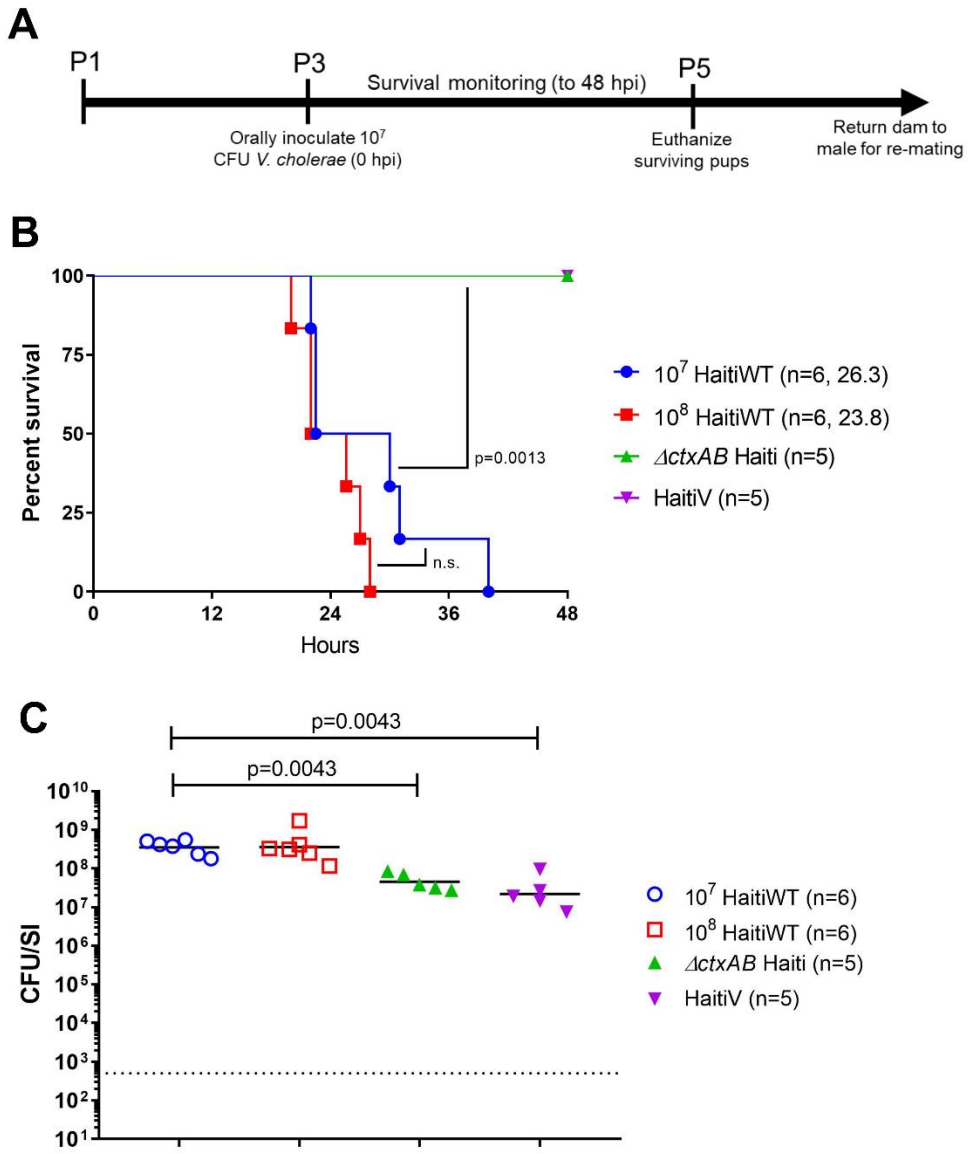


Fig 4. Isotype specific anti-CtxB titers in C57BL/6 mice immunized with HaitiV. Anti-CtxB titers with specific isotype analysis for IgA (A), IgG (B) and IgM (C) were performed for each serum sample. The mean ± SEM is indicated. Mixed-effect p-values were determined by one-way ANOVA analysis. Multiple comparison p-values for this data are reported in Table S1. Although there were 7 mice per group, some samples were of insufficient volume for analysis.



668 **Fig 5. Survival of P3 C57BL/6 pups challenged with Haiti *V. cholerae***. (A): Timeline of oral
669 challenge assays. (B) Survival curves for 48 hours post inoculation with the indicated strains; the
670 number of pups challenged and the median survival time in hours is shown. P-values were
671 determined by the Mantel-Cox method. (C) Intestinal *V. cholerae* burden at the time of death
672 (open shapes) or at 48 hours (filled triangles). Burden is plotted as colony-forming units per small
673 intestine (CFU/SI). The dotted line marks the limit of detection (50 CFU/SI). P-values were
674 determined by the Mann-Whitney U test.

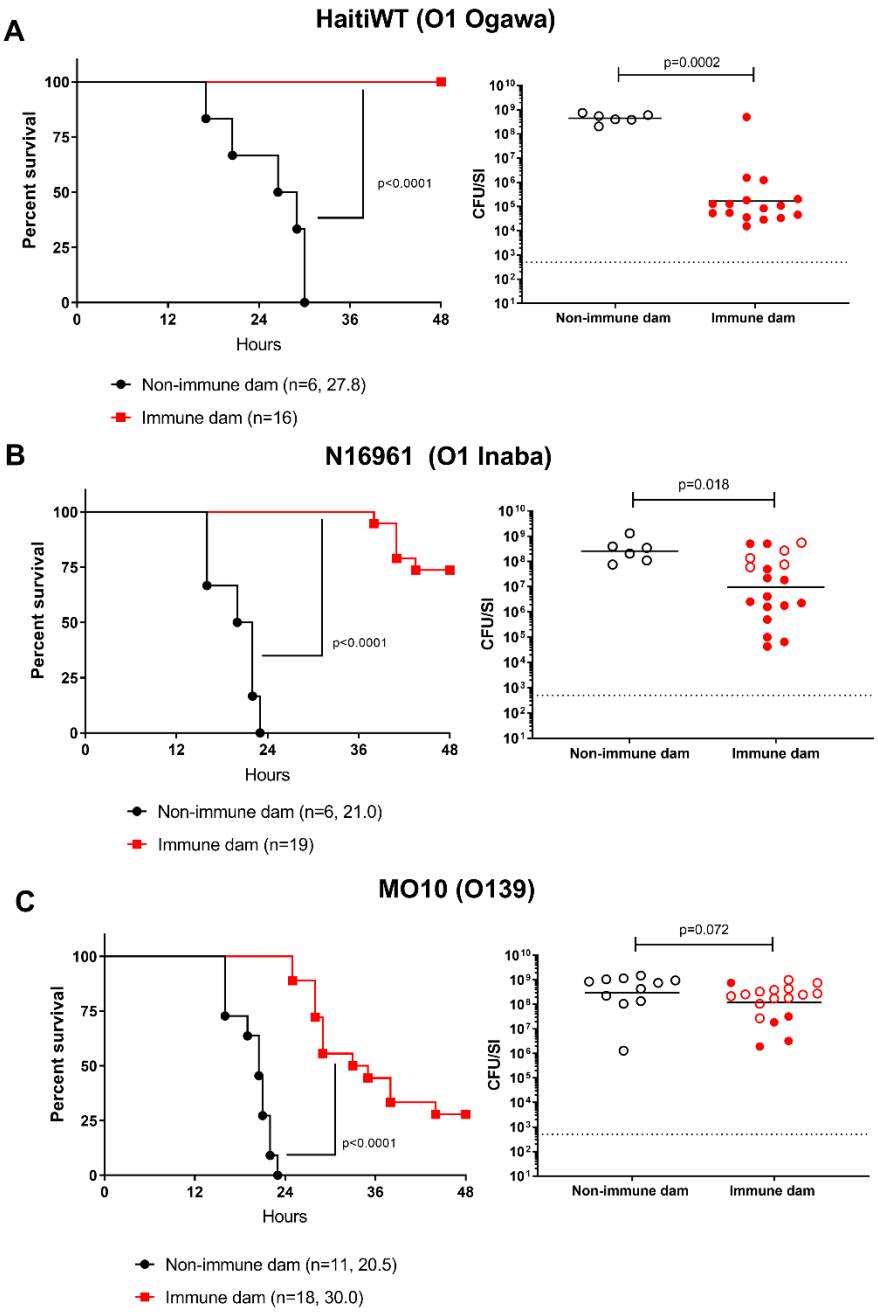


Fig 6. Survival of P3 C57BL/6 pups born to HaitiV-immunized or non-immunized dams after challenge with different serotype and serogroup *V. cholerae* strains. Pups born to either HaitiV-immunized dams (red) or non-immunized SPF dams (black) were challenged with either the O1 Ogawa strain HaitiWT (A), the O1 Inaba strain N16961 (B) or the O139 strain MO10 (C). Panels on the left show survival curves for infected pups up to 48 hours post inoculation with the

696 indicated strains; the number of pups challenged and the median survival time in hours is shown.
697 P-values were determined by the Mantel-Cox method. Panels on the right show the intestinal *V.*
698 *cholerae* burden in pups at the time of death (open circles) or at 48 hpi (closed circles). Burden is
699 plotted as CFU/SI. The dotted line marks the limit of detection (50 CFU/SI) and grey open circles
700 indicate pups with burdens below this limit. P-values were determined by the Mann-Whitney U
701 test.

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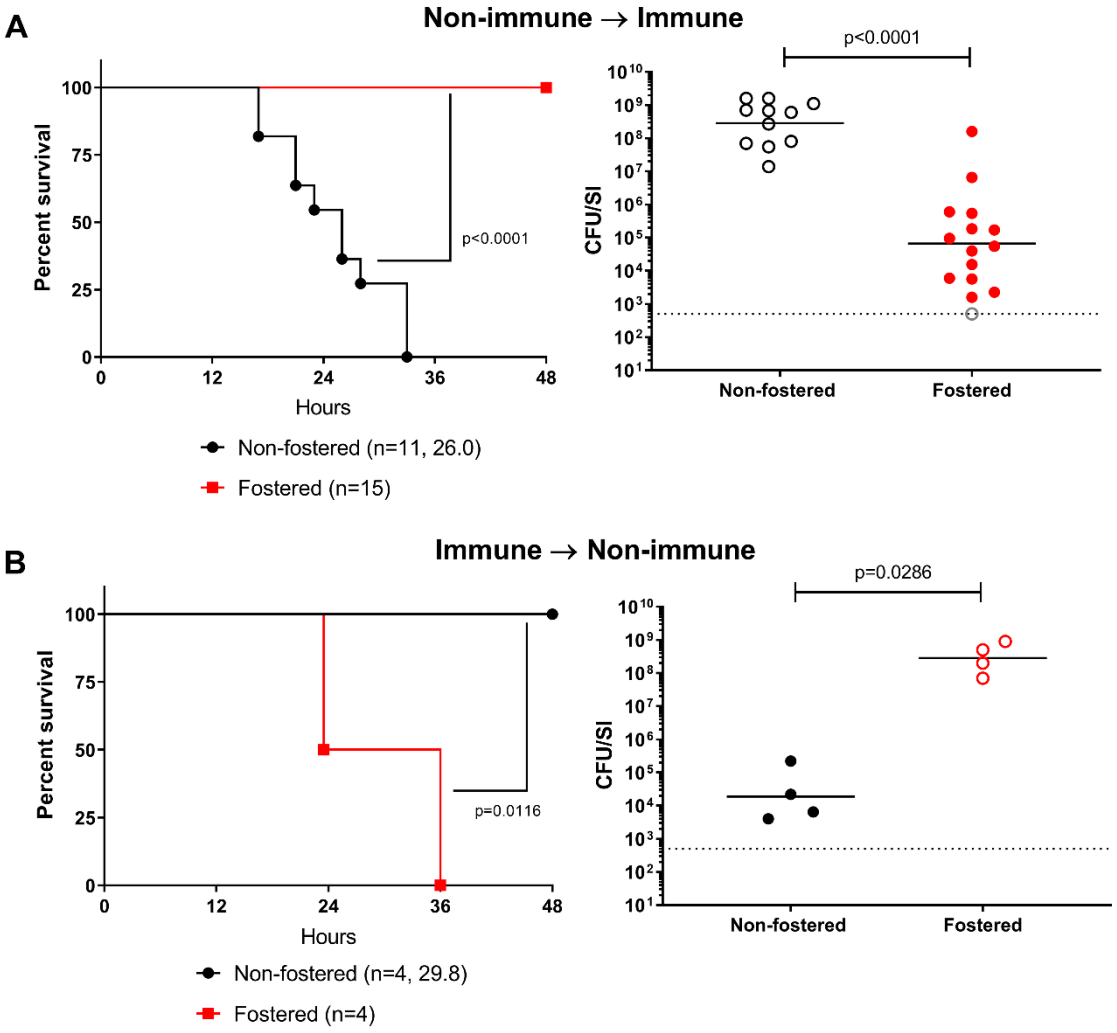

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706 **Fig 7. Survival of pups cross-fostered by HaitiV-immunized or non-immunized dams after**
707 **challenge with HaitiWT.** (A) Survival and *V. cholerae* intestinal colonization in pups born to non-
708 immunized dams fostered by HaitiV-immunized dams. (B) Survival and *V. cholerae* intestinal
709 colonization in pups born to HaitiV-immunized dams and fostered by non-immunized dams. Panel
710 labeling, and statistics are as described in Figure 6.

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Supporting information

S1 Fig. Duration of protection from HaitiWT in progeny from HaitiV-immunized dams. Litters were inoculated identically to initial challenge studies and allowed to age normally for up to 14 days (336 hours) before enumeration of intestinal burdens.

S2 Fig. Effectiveness of a single-dose HaitiV vaccination regimen in C57BL/6 mice. (A): Fecal shedding of HaitiV from mice given a single dose of HaitiV at Day 0. (B): Vibriocidal antibody titers from singly-immunized mice against either Ogawa (blue) or Inaba (green) target strains. (C): Survival (left) and intestinal colonization (right) of pups from singly-immunized dams challenged with a lethal dose of HaitiWT. The dotted line marks the limit of detection.

734 **S1 Table. Bacterial strains used in this study.** ET = El Tor, AET = Altered El Tor, Sm =
735 streptomycin, SXT = sulfamethoxazole/trimethoprim, R = resistant, S = sensitive.

Strain	Notes	Source
E. coli SM10pir	Donor strain for *ctxAB* deletion	[29]
V. cholerae HaitiWT	O1 Ogawa AET, SmR SXTR, *lac*+	[10]
V. cholerae Haiti *ΔctxAB*	O1 Ogawa AET, SmR SXTR, *lac*+	This study
V. cholerae HaitiV	O1 Ogawa AET, SmR SXTS, *lac- recA*+	[29]
V. cholerae CVD103-HgR	O1 Inaba Classical, SmR, *lac*+	This study
V. cholerae N16961	O1 Inaba ET, SmR, *lac*+	[56]
V. cholerae MO10	O139, SXTR, *lac*+	[57]
V. cholerae PIC018	O1 Inaba ET	[34]
V. cholerae PIC158	O1 Ogawa ET	[34]

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738 **S2 Table. Multiple comparisons testing of antibody titers in cohort 2.** P-values shown were
739 calculated from a Dunnett's multiple-comparison test comparing Day 14, 28 or 42 mean titers to
740 the mean titer at Day 7. P-values are shown to three significant figures and values < 0.05 are
741 bolded.

Sample	Day 14 vs. Day 7	Day 28 vs. Day 7	Day 42 vs. Day 7
Inaba OSP IgA	0.560	0.112	**0.0197**
Inaba OSP IgG	0.999	0.117	0.0579
Inaba OSP IgM	**0.0157**	**0.0428**	**0.0357**
Ogawa OSP IgA	0.531	0.0828	0.146
Ogawa OSP IgG	0.550	**0.0205**	**0.0172**
Ogawa OSP IgM	0.0630	**0.0253**	**0.0343**
CtxB IgA	0.750	0.571	0.207
CtxB IgG	0.694	0.263	0.112
CtxB IgM	0.267	**0.0498**	**0.013**

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