

1 **Dissecting serotype-specific contributions to live oral cholera vaccine**
2 **efficacy**

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20 **Abstract**

21 The O1 serogroup of *Vibrio cholerae* causes pandemic cholera and is divided into Ogawa and
22 Inaba serotypes. The O-antigen is *V. cholerae*'s immunodominant antigen, and the two serotypes,
23 which differ by the presence or absence of a terminally methylated O-antigen, likely influence
24 development of immunity to cholera and oral cholera vaccines (OCVs). However, there is no
25 consensus regarding the relative immunological potency of each serotype, in part because
26 previous studies relied on genetically heterogenous strains. Here, we engineered matched
27 serotype variants of a live OCV candidate, HaitiV, and used a germ-free mouse model to evaluate
28 the immunogenicity and protective efficacy of each vaccine serotype. By combining vibriocidal
29 antibody quantification with single and mixed strain infection assays, we found that all three HaitiV
30 variants - Inaba^V, Ogawa^V, and Hiko^V (bivalent Inaba/Ogawa) - were immunogenic and protective,
31 suggesting the impact of O1 serotype variation on OCV function may be minimal. The potency of
32 OCVs was found to be challenge strain-dependent, emphasizing the importance of appropriate
33 strain selection for cholera challenge studies. Our findings and experimental approaches will be
34 valuable for guiding the development of live OCVs and oral vaccines for additional pathogens.

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42 **Introduction**

43 The human bacterial pathogen *Vibrio cholerae* causes cholera, a severe and potentially fatal
44 diarrheal disease. In the small intestine, *V. cholerae* produces cholera toxin (Ctx), an AB₅ toxin
45 that induces ion imbalances and a secretory response that largely accounts for the massive fluid
46 loss associated with cholera¹. Although effectively treated with rehydration therapy, cholera
47 remains a threat to public health. The disease is endemic in over 50 countries, and is especially
48 dangerous where access to clean water and sanitation remains limited¹. There are an estimated
49 3,000,000 cases and ~100,000 deaths due to cholera worldwide each year². The magnitude of
50 this threat has propelled interest in understanding *V. cholerae*-host immune system interactions
51 for the refinement of oral cholera vaccines (OCVs), an important frontline intervention to reduce
52 both cholera incidence and transmission³.

53 Both killed (inactivated) and live OCV formulations have been developed. Killed whole-cell OCVs
54 (e.g. Shanchol), which consist of a mixture of heat or formalin-inactivated *V. cholerae* strains,
55 have been generally efficacious in both endemic and epidemic settings⁴. However, inactivated
56 vaccines have limited efficacy in young children (<5 years old), who are most susceptible to
57 severe cholera, and require multi-dose immunization regimens for long-lived immunity, although
58 recent studies suggest that single or higher dose schedules may still offer shorter term protection⁵⁻
59 ⁸. In contrast to killed OCVs, live OCVs are likely to be more effective after a single dose and in
60 young children, since such vaccines more closely mimic authentic infection; during their *in vivo*
61 replication in the intestine, live OCVs produce intact antigens, including infection-induced
62 colonization factors, such as the toxin co-regulated pilus, that are targets for protective immunity⁹⁻
63 ¹¹. However, to date, no live OCV is approved for use in cholera endemic countries, highlighting
64 the need for development of new live OCVs for global public health. There is a live OCV
65 (Vaxchora) that is commercially available in the USA, but its indication is limited to travel-related
66 use¹².

67 Although there are >200 known *V. cholerae* serogroups, all pandemic cholera has been caused
68 by O1 serogroup *V. cholerae*¹³. Continued evolution of this dominant *V. cholerae* pandemic
69 serogroup has given rise to several genetically and phenotypically distinct *V. cholerae* lineages
70 (i.e. biotypes). Classical biotype *V. cholerae*, now thought to be extinct, likely caused the 1st-6th
71 cholera pandemics. The ongoing 7th cholera pandemic, which began in 1961, is caused by the 7th
72 pandemic El Tor (7PET) biotype of O1 *V. cholerae*. Extensive genomic analyses of 7PET *V.*
73 *cholerae* have demonstrated additional changes acquired by more recent clinical isolates,
74 including the *ctxB7* allele of Ctx and the SXT antibiotic resistance element^{14–16}. These “Wave 3”
75 7PET strains are now the primary cause of cholera worldwide, and have caused dramatic
76 outbreaks in Haiti (2010-2019) and Yemen (2017-present)^{17,18}.

77 The O-antigen moiety of lipopolysaccharide (LPS) is thought to be the primary antigenic
78 determinant of protective immunity resulting either from O1 *V. cholerae* infection or vaccination¹⁹.
79 The O1 serogroup includes two serotypes, Ogawa and Inaba, which differ by the presence or
80 absence, respectively, of a methyl group on the terminal sugar of the LPS O-antigen (Figure
81 1A)^{1,20–22}. Both Ogawa and Inaba strains cause epidemic cholera and circulate globally, often
82 replacing each other in cyclical outbreaks in the same region^{23–25}. Studies of natural infection
83 indicate that immune responses and subsequent protection against future infection are strongest
84 against the homologous (initial) serotype²⁶. However, the relative potency of the cross-
85 protectiveness of these responses, particularly whether one serotype confers greater cross-
86 protectivity, remains unclear. Studies on this topic have not used matched isogenic strains to
87 investigate the impact of variation in this immunodominant antigen in isolation from the complex
88 suite of *V. cholerae* virulence and colonization factors. Similarly, while the serotype/biotype
89 landscape of current OCVs is varied, ranging from monovalent (Vaxchora, classical Inaba) to
90 trivalent (Shanchol, classical and El Tor Ogawa/Inaba/O139) formulations, evidence for their
91 cross-serotype protective capacity from controlled comparisons of vaccines of varying serotypes

92 is lacking. Understanding these concepts could guide design of a more potent O1 OCV against
93 both serotypes.

94 O1 serotypes are determined by the activity of the O-antigen methyltransferase WbeT (previously
95 known as RfbT), and loss-of-function mutations in this enzyme produce Inaba strains²⁷. In rare
96 cases where WbeT function is impaired, but not eliminated, Hikojima strains, which
97 simultaneously produce methylated (Ogawa) and un-methylated (Inaba) LPS, can arise^{27,28}.
98 Although Hikojima is thought to be an unstable phenotype, Hikojima strains have historically and
99 recently been isolated in the clinic²⁹⁻³¹, and stable Hikojima-generating point mutations in WbeT
100 have recently been identified^{32,33}, raising the idea that an OCV bearing this bivalent O1 serotype
101 could elicit superior cross-serotype protection while retaining the manufacturing advantages of a
102 single strain vaccine. To this end, an inactivated Hikojima vaccine (Hillchol) has been produced,
103 with promising early clinical results suggesting non-inferior immunogenicity compared to
104 Shanchol³⁴.

105 We recently described a new live-attenuated OCV candidate, HaitiV, an engineered derivative of
106 a toxigenic Wave 3 7PET O1 Ogawa *V. cholerae* clinical isolate (HaitiWT) from the 2010 Haiti
107 cholera epidemic. HaitiV contains a set of genetic modifications that reduce its potential
108 reactogenicity and enhance its biosafety, and allow it to over-produce the non-toxic B subunit of
109 Ctx to boost immunogenicity³⁵. In two mouse models, we showed that HaitiV is immunogenic and
110 elicits robust protective adaptive immune responses^{36,37}. In addition to its function as a
111 conventional live OCV, HaitiV also has an unprecedented, rapid-acting function that protects
112 animals against lethal *V. cholerae* challenge within 24 hours post-immunization. Thus, HaitiV
113 could be an OCV with both short- and long-term protective functions³⁵. Here, to understand how
114 vaccine serotype influences the generation of serotype-specific vibriocidal antibodies and
115 protective immunity, we engineered isogenic Inaba, Ogawa and Hikojima variants of HaitiV. The
116 protective capacities of these vaccines were tested in a germ-free (GF) mouse OCV immunization

117 model using isogenic Inaba and Ogawa challenge strains. All three serotype vaccines functioned
118 as excellent OCVs, with subtle but detectable differences in immunogenicity and protective
119 efficacy. Our study provides insight into the *in vivo* biology of *V. cholerae* serotypes, demonstrates
120 the utility of the GF mouse platform for rapidly assessing the OCV candidates, and offers guidance
121 for design of future OCV trials.

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123 **Results**

124 **Generation of genetically matched HaitiV serotype variant strains**

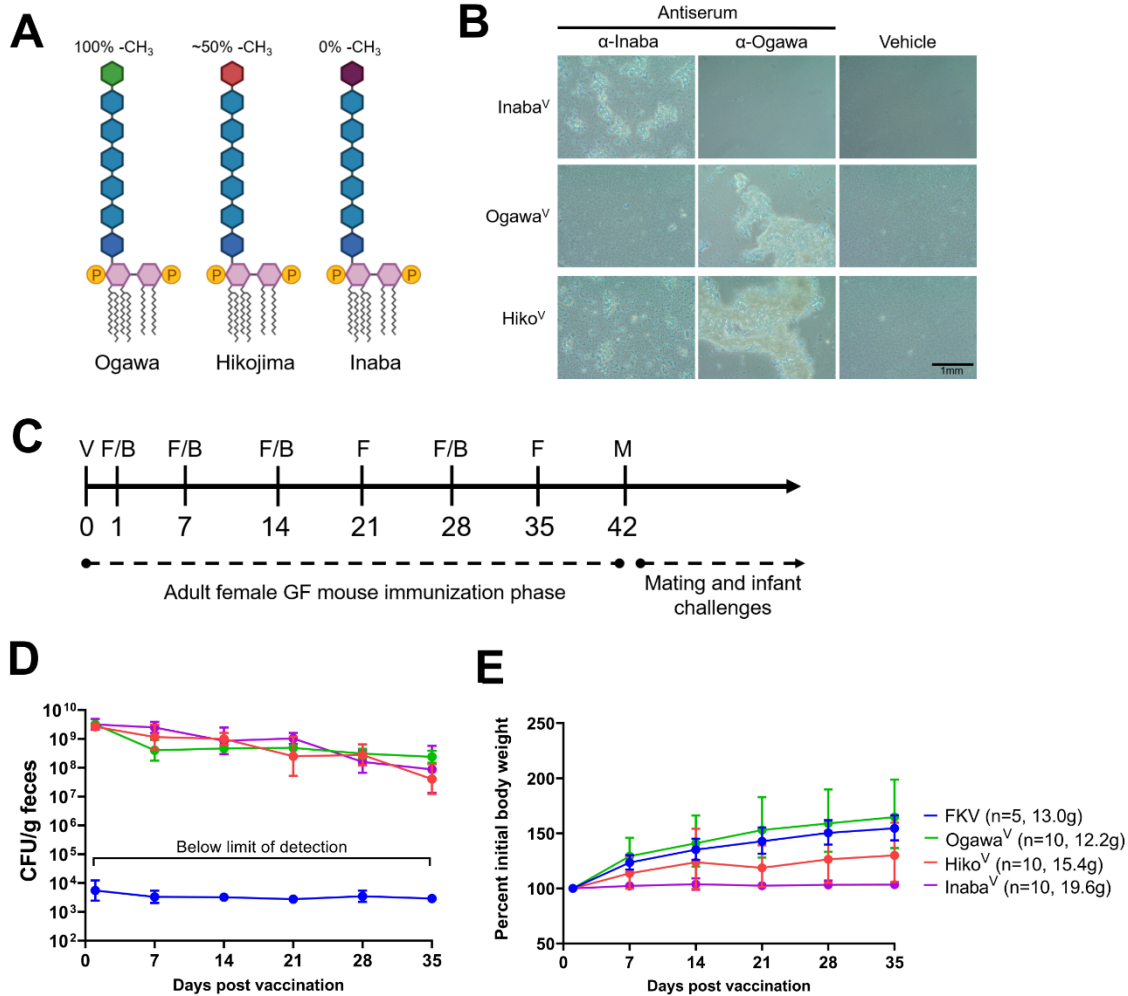
125 Among the suite of genetic modifications in HaitiV is a deletion of the recombinase *recA* (VC0543),
126 which limits the vaccine's capacity to acquire new genetic material, but is required for engineering
127 mutations by homologous recombination. To further modify HaitiV, we restored a precursor of
128 HaitiV to *recA*⁺. Subsequently, the hemolysin *hlyA* (VCA0219) was deleted, since HlyA is a
129 suspected *V. cholerae* virulence factor³⁸. We then introduced the following reported point mutants
130 of WbeT (VC0255) at Ser158 in the original Ogawa variant of HaitiV: S158F (Hikojima), and
131 S158P (Inaba)³². Finally, *recA* was deleted from each strain to yield three HaitiV-derived isogenic
132 $\Delta hlyA/\Delta recA$ strains: Ogawa^V (WbeT^{S158}), Hiko^V (WbeT^{S158F}), and Inaba^V (WbeT^{S158P}) (Figure 1A).
133 Each vaccine strain's serotype was confirmed by slide agglutination (Figure 1B).

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135 **Vaccine serotype influences the specificity of vibriocidal responses**

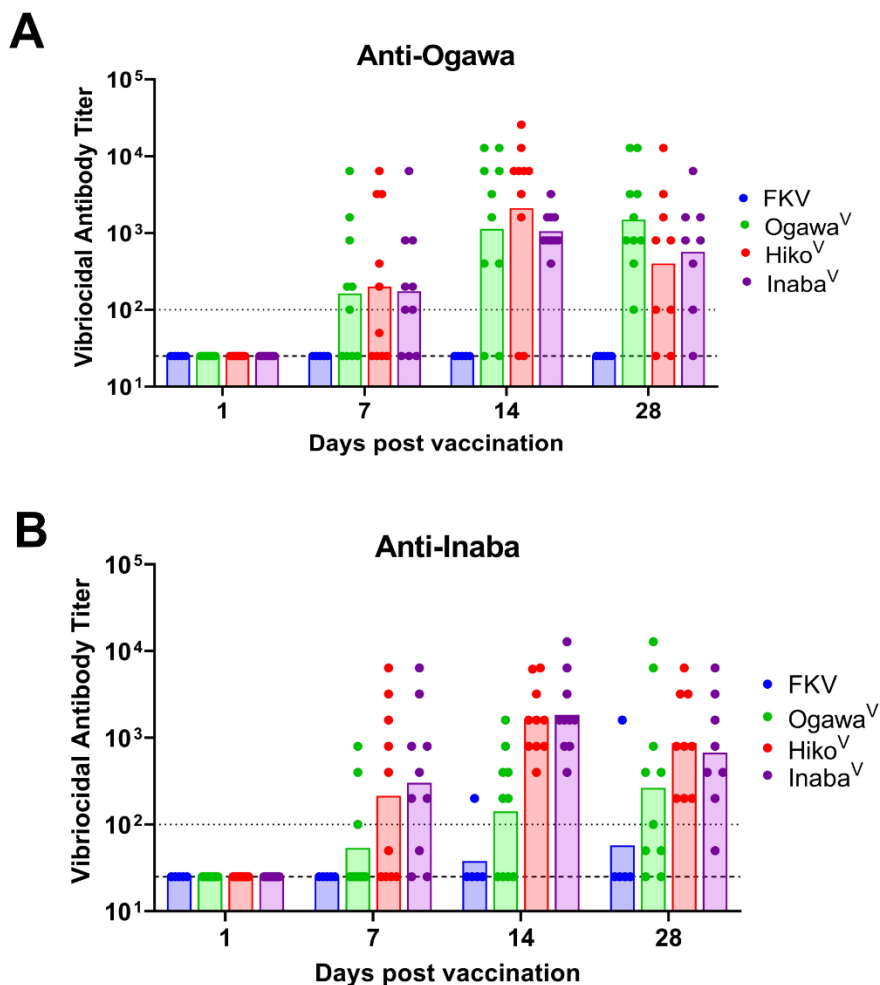
136 We used the GF adult mouse oral immunization model to compare the relative potency of the
137 immune responses elicited by these three HaitiV serotype variants in adult female mice^{36,39,40}. 3-
138 6-week-old female GF mice (n=5/group) were immunized with a single oral dose of 10⁹ CFU of
139 live Ogawa^V, Inaba^V, Hiko^V or formalin-inactivated Hiko^V (FKV) (Figure 1C). All three live variants

140 were shed in feces (i.e. colonized) at equivalent levels with comparable kinetics over the course
 141 of the study (Figure 1D). Although there was some variance in mean initial weights of each group
 142 due to mouse availability, mice weights remained stable or increased over the course of the study,
 143 indicating that prolonged colonization by all 3 vaccine serotypes is safe (Figure 1E).



144 **Figure 1. Vaccine shedding and bodyweights of adult germ-free mice orally immunized**
 145 **with isogenic vaccine serotype variants.** (A): Schematic of the three known O1 *V. cholerae*
 146 serotypes. Internal and terminal perosamine residues are indicated by blue or otherwise colored
 147 diamonds, respectively, with the approximate degree of methylation of the terminal perosamine
 148 shown. (B): Representative slide agglutination of the three HaitiV vaccine serotypes. (C): Oral
 149 immunization and sampling regime for adult mouse phase of this study. V – oral vaccination, F –
 150 fecal pellet collection, B – blood sample collection, M – mating. (D) Fecal shedding and (E)
 151 bodyweight of mice orally immunized with a single dose of the indicated inactivated or live vaccine
 152 strain at Day 1.

153 We next gauged immune responses to the vaccine variants by measuring vibriocidal antibody
154 titers (VATs) in serum samples from the mice. VATs are a strong clinical correlate of protection in
155 human *V. cholerae* infections and report on serotype-specific antibody responses against Ogawa
156 and Inaba target strains¹⁹. All but two (28/30) mice immunized with live vaccines seroconverted
157 (>4x from baseline VAT) against at least one O1 serotype within 14 days post-immunization, with
158 the remaining two seroconverting by Day 28 (Figure 2). Anti-Ogawa responses were comparable
159 between the three vaccine variants and $\geq 80\%$ of animals responded to vaccination (Fig 2A). In
160 contrast, anti-Inaba responses in Ogawa^V-immunized mice were of lower titer than those in
161 Inaba^V- or Hiko^V-immunized mice and were not detected in some animals 14 and 28 days post-
162 vaccination (Fig 2B), suggesting that vaccine strain serotype biases the potency of serotype-
163 specific vibriocidal immune responses. Only one FKV-immunized mouse seroconverted,
164 demonstrating that the inactivated vaccine is markedly less immunogenic in this model. In a
165 separate GF mouse cohort, we observed similar responses to *hlyA*⁺ Ogawa^V as the *hlyA* mutant,
166 indicating that deletion of this locus did not broadly impact immunogenicity (Supplementary Figure
167 1).



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169 **Figure 2. Vibriocidal antibody titers in adult germ-free mice orally immunized with isogenic**
170 **vaccine serotype variants.** Anti-Ogawa (A) and -Inaba (B) titers are plotted as geometric means
171 of each group with individual values for each mouse shown. Individual values correspond to the
172 highest dilution at which vibriocidal activity was observed. The lower dotted line represents the
173 lower limit of detection (1:25 serum dilution), with the upper dotted line indicating the
174 seroconversion threshold (4-fold increase over the baseline limit of detection).

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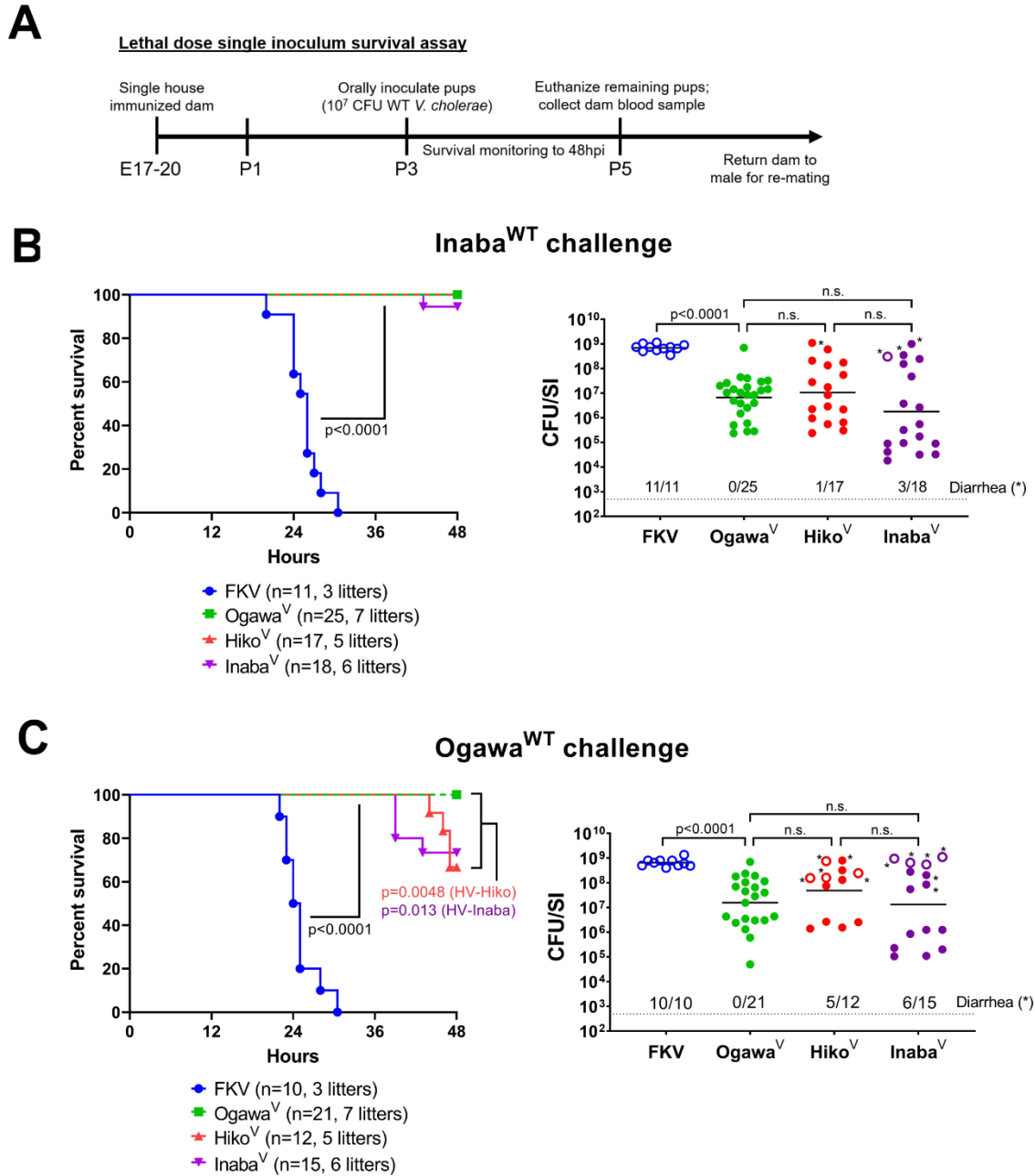
180 **Vaccine serotype influences vaccine protective efficacy**

181 VATs are not a direct measure of vaccine protective efficacy, and adult mice are refractory to
182 cholera-like illness. To assess whether the immune responses in the vaccinated female adults
183 were protective, and whether protection was biased by the serotype of the vaccine strain, we
184 tested the susceptibility of their neonatal progeny to lethal challenge with either Haiti^{WT} Ogawa
185 (Ogawa^{WT}) or Inaba (Inaba^{WT}) isogenic challenge strains (Supplementary Figure 2). To control for
186 litter-to-litter variations in maternal care and immune responses, which could affect comparisons,
187 each litter was randomly split into two groups of pups, which received either a lethal Ogawa^{WT} or
188 Inaba^{WT} challenge (Figure 3A). Pups in all three live vaccine groups were significantly protected
189 from both death and diarrhea caused by challenge with either serotype compared with pups in
190 the FKV group that exhibited similar kinetics of mortality as pups of unvaccinated dams, indicating
191 that the presence of VATs (i.e. seroconversion) is tightly correlated with protection in this model
192 (Figure 3BC)^{36,37}. However, the protective efficacy of the three vaccine serotypes differed
193 depending on the serotype of the challenge strain. All three live vaccines provided similar
194 protection against Inaba^{WT} challenge (Figure 3B), but pups from Ogawa^V-immunized dams were
195 protected from death and diarrhea to a greater extent than pups from the other two groups against
196 Ogawa^{WT} challenge (Figure 3C, $p = 0.0033$ and 0.0026 for diarrhea incidence in pups from
197 Ogawa^V dams versus Hiko^V and Inaba^V dams, respectively). These findings suggest that Ogawa^V
198 elicits more potent protective immune responses to homologous challenge and that vaccine
199 serotype modifies the protective capacity of OCVs. In contrast to their differential protective
200 capacities, all three vaccines conferred similar levels of colonization suppression (Fig 3BC),
201 illustrating that suppression of colonization is not strictly equivalent to protection against disease.

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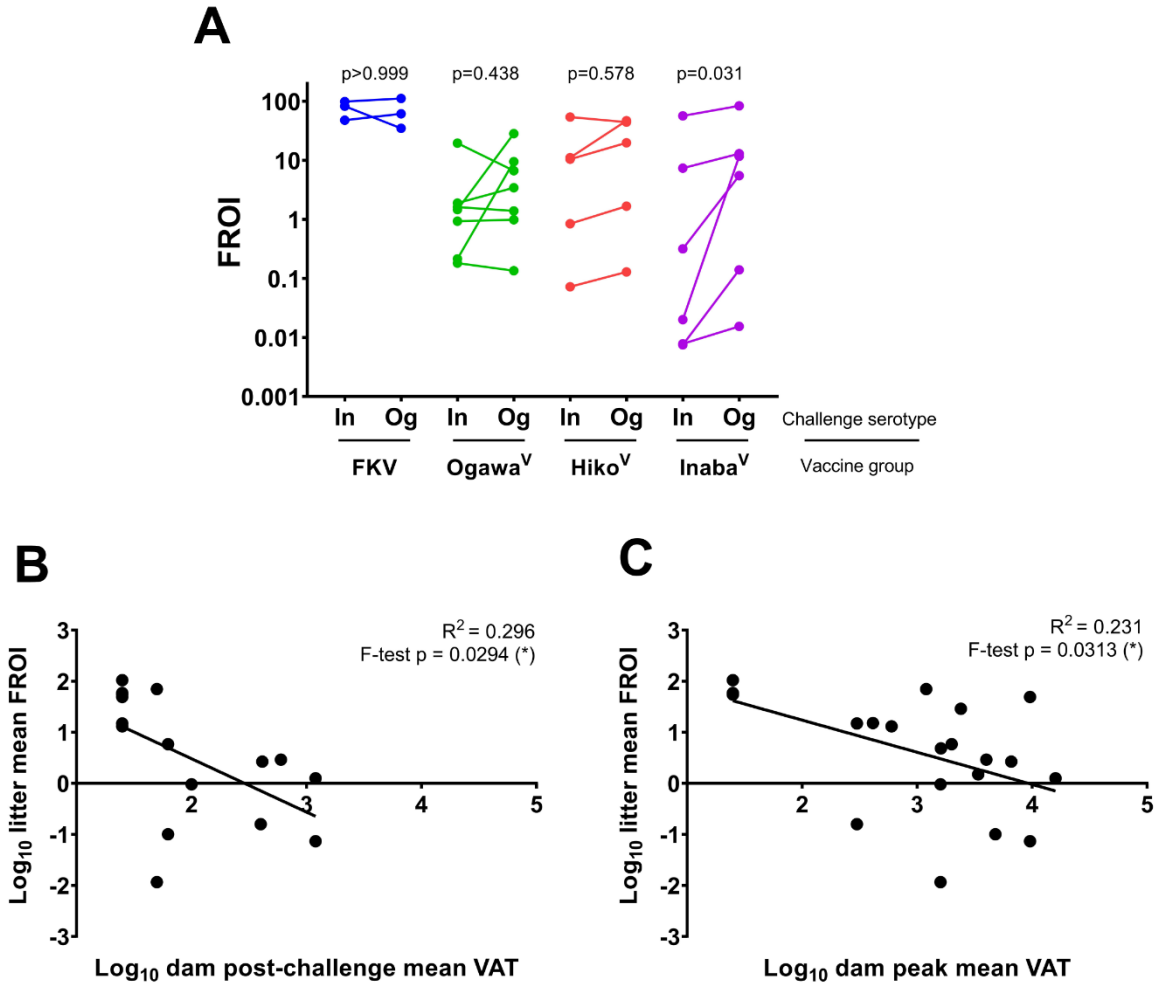
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205 **Figure 3. Protective efficacy of isogenic vaccine serotype variants using isogenic single**
 206 **strain challenges in pups from immunized dams.** (A) Timeline of challenge experiments
 207 depicted in Figures 3, 4 and 6. For Figure 3 and 4, P3-4 pups in each litter were randomly assigned
 208 to receive a lethal dose of either Inaba^{WT} (B) or Ogawa^{WT} (C). Panels on the left depict survival
 209 kinetics for challenged pups from the indicated vaccine groups, with associated median survival
 210 times and sample sizes. P-values were determined by the Mantel-Cox test. Panels on the right
 211 show small intestinal (SI) *V. cholerae* burden in the same pups at the time of death (open circles)
 212 or at assay endpoint (48 hpi, closed circles). Burden is plotted as CFU/SI and pups with visible
 213 signs of diarrhea at the time of sacrifice are marked with an asterisk. P-values were determined
 214 by the Mann-Whitney U test.

215 We also analyzed the expansion of Inaba^{WT} or Ogawa^{WT} within each litter to counter potential
216 confounding effects of variations in maternal care between litters. The *in vivo* expansion of the
217 challenge strains was estimated by dividing the number of CFU recovered from the intestine by
218 the CFU in the inocula, yielding a fold replication over inoculum (FROI) index. FROI comparisons
219 revealed that litters from FKV, Ogawa^V and Hiko^V immunized dams did not display differential
220 expansion of either the Inaba or Ogawa challenge strain (Figure 4A); in each group, litters showed
221 comparable replication of either serotype. In contrast, in pups from all six litters from Inaba^V
222 immunized dams, Ogawa^{WT} expanded to a greater extent than Inaba^{WT}, suggesting that Inaba^V-
223 induced immune responses have diminished capacity to suppress the expansion of virulent
224 Ogawa versus Inaba *V. cholerae* in the intestine. There was a statistically significant, but modest
225 negative correlation between both peak or post-challenge mean dam VAT and FROI in the
226 associated litters (Figure 4BC), suggesting that these metrics of vaccine potency are related in
227 GF mice. Significant correlations between serotype-specific VAT and serotype-specific expansion
228 were not detected in all groups, possibly due to insufficient statistical power (Supplementary
229 Figure 3A-D). Importantly, other metrics such as dam bodyweight were not correlated to FROI,
230 indicating the specificity of VAT correlations with pathogen replication in the GF mouse OCV
231 model (Supplementary Figure 3EF).



232

233 **Figure 4. Replication of WT challenge strains in pups from dams immunized with isogenic**
 234 **serotype variants of OCV.** (A): Fold replication over inoculum (FROI) for the indicated challenge

235 strain (In: Inaba^{WT}, Og: Ogawa^{WT}) using colonization data from right panels in Figure 3. Solid lines
 236 connect FROI values for In- or Og-challenged pups in the same litter. P-values were calculated
 237 by the Wilcoxon matched-pairs signed rank test. (B): Correlation of litter-specific FROI with the
 238 post-challenge mean vibriocidal antibody titer (VAT) in the dam. Mean post-challenge VAT was
 239 determined by averaging the anti-Inaba and -Ogawa VAT values from the blood sample taken at
 240 the time the dam's litter was euthanized. (C): Correlation of litter-specific FROI with the peak
 241 mean vibriocidal antibody titer (VAT) in the dam. Mean peak VAT was determined by averaging
 242 the highest measured anti-Inaba and -Ogawa VAT values from the mouse at any point during the
 243 study. The p-value of the fitted linear regression was calculated with the F-test.

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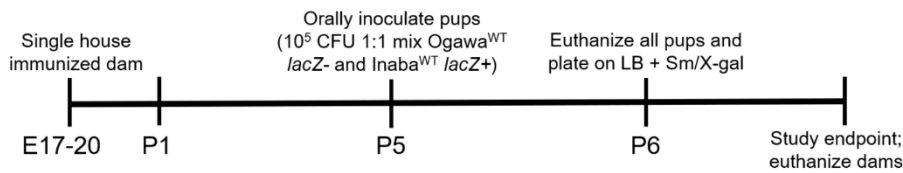
246 To further probe the serotype-specific protective efficacy of a bivalent vaccine strain such as
247 Hiko^V, we carried out within-pup comparisons using competitive infections, thus controlling for
248 pup-to-pup variations in maternal care. These experiments used a lower challenge inoculum (10⁵
249 CFU) composed of a 1:1 mixture of Ogawa^{WT} and Inaba^{WT} to robustly assay colonization in the
250 absence of signs of disease (Figure 5A). Consistent with the single infection data, the total CFU
251 burden of WT *V. cholerae* in pups from Hiko^V dams was significantly reduced (~100x) compared
252 to pups from unimmunized dams (Figure 5B). Unexpectedly, in pups from unimmunized dams,
253 the baseline competitive index (CI) was not 1, suggesting that Ogawa^{WT} has a modest competitive
254 advantage in the infant mouse SI relative to Inaba^{WT} (Figure 5C). CIs in pups from Hiko^V-
255 immunized dams were significantly higher than in the controls, suggesting that Hiko^V vaccination
256 elicits immune responses that are less potent at impeding Ogawa^{WT} expansion and/or more
257 potent at impairing Inaba^{WT} replication (Figure 5C). These observations provide additional
258 evidence that OCV serotype exerts serotype-specific impacts on *V. cholerae* fitness, even within
259 a single animal.

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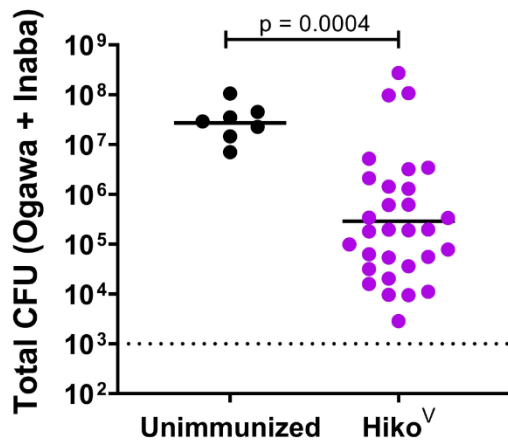
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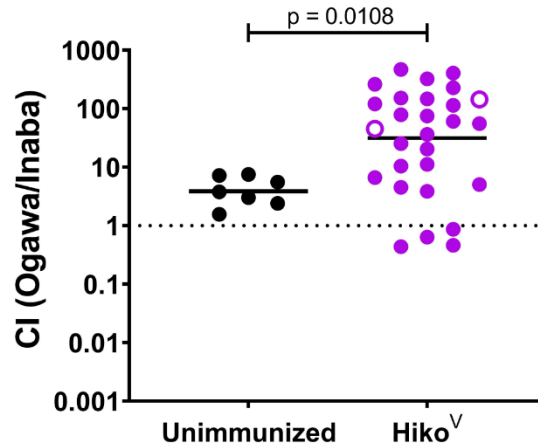
Non-lethal mixed inoculum competitive index assay



B



C



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263 **Figure 5. Mixed-strain challenges in pups from dams immunized with *Hiko*^V.** (A): Timeline
264 of mixed strain challenge experiments. (B): Colonization burdens in P6 pups from unimmunized
265 and *Hiko*^V vaccinated adults challenged with a 1:1 mixture of *Inaba*^{WT} and *Ogawa*^{WT}. The dotted
266 line indicates the limit of detection. (C): Competitive index (CI) values from pups in Panel B. Open
267 circles denote pups where the denominator was 0 in the CI calculation, forcing an imputed 1 and
268 hence representing the upper limit of detection. The dotted line indicates the line of parity (CI =
269 1). Pups with a total colonization burden of less than 5×10^3 CFU in Panel B were excluded from
270 Panel C. P-values were determined by the Mann-Whitney U test.

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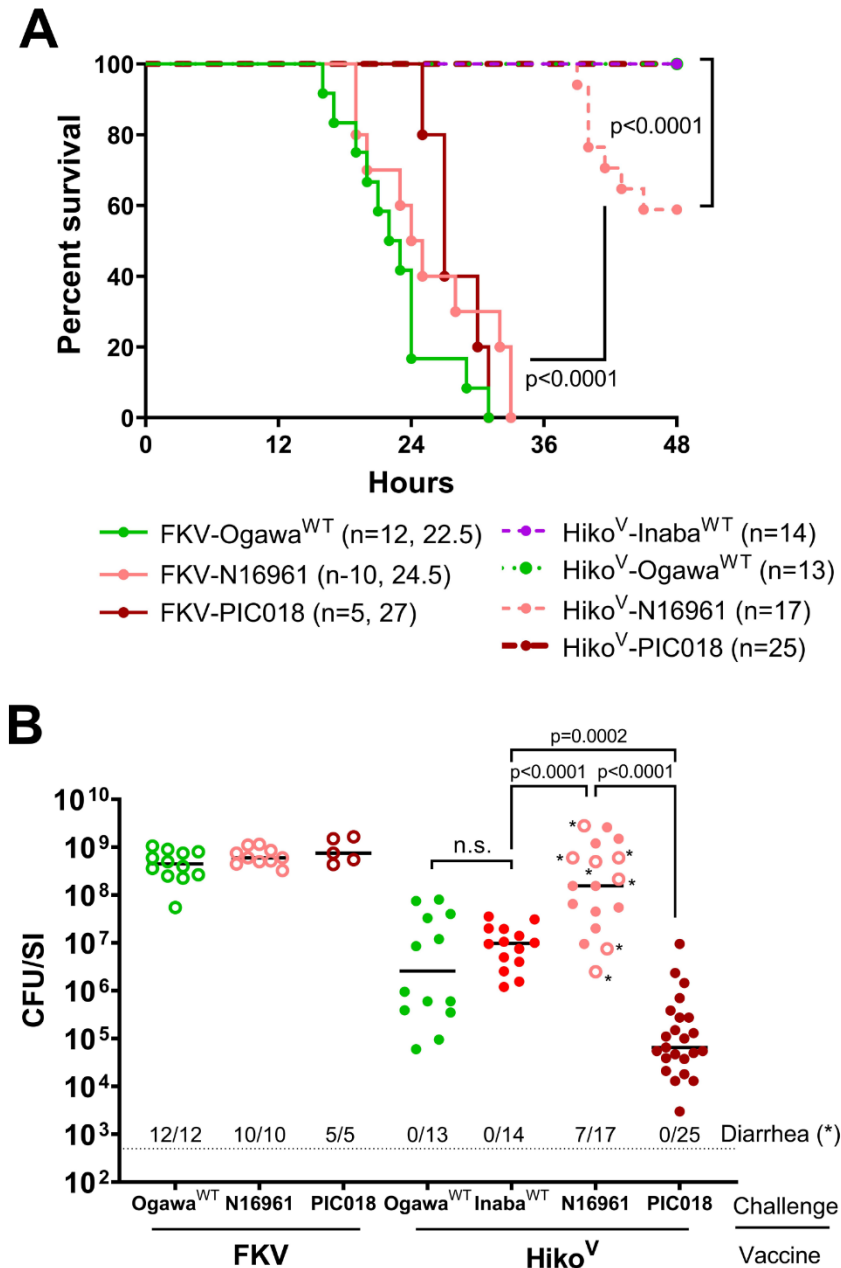
276 **Serotype-independent challenge strain properties impact the protective efficacy of Hiko^V**

277 To further explore the protective scope of the bivalent Hiko^V strain against diverse *V. cholerae*
278 challenges, we immunized an additional cohort of GF mice with formalin-inactivated or live Hiko^V.
279 Hiko^V stably colonized the mice and the vaccinated animals gained weight and developed similar
280 serum VATs as described above (Supplementary Figure 4). We challenged litters from these
281 vaccinated dams with 7PET clinical strains from the last several decades to capture the
282 evolutionary spectrum of the 7th cholera pandemic. These included N16961, an Inaba
283 Bangladeshi isolate from 1971⁴¹, PIC018, an Inaba Bangladeshi isolate from 2007⁴² along with
284 the Haiti^{WT} (isolated in 2010) derived strains Ogawa^{WT} and Inaba^{WT}, used above (Supplementary
285 Figure 2). Consistent with data from the previous cohort (Figure 3), pups from FKV-vaccinated
286 dams were not protected against disease or colonization (Figure 6). In contrast, pups from live
287 Hiko^V-immunized dams were completely protected against the three contemporary isolates
288 (PIC018, Ogawa^{WT} and Inaba^{WT}) (Figure 6A). None of these animals developed diarrhea or died.
289 Despite the equivalent clinical protection from these three challenge strains, Hiko^V vaccination
290 suppressed colonization by PIC018, an Inaba strain, more potently than either Haitian serotype
291 (Figure 6B). Conversely, pups from Hiko^V-immunized dams displayed significantly lower levels of
292 clinical protection (7/17 with diarrhea/died, $p=0.0087$ vs. Inaba^{WT}) and colonization suppression
293 when challenged with the early 7PET strain N16961 (Figure 6). These findings strongly suggest
294 that strain-specific factors in addition to serotype-specific immune responses determine the
295 protective efficacy of OCVs.

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299 **Figure 6. Diverse single strain challenges in pups from dams immunized with Hiko^V.** (A)
 300 Survival kinetics in P3-4 pups from dams immunized with formalin-killed (FKV) or live Hiko^V
 301 challenged with the indicated WT *V. cholerae* strain. The sample size and median survival times,
 302 where calculable, are indicated. (B) Intestinal *V. cholerae* burden in the pups from Panel A at the
 303 time of death (open circles) or at assay endpoint (48 hpi, closed circles). Burden is plotted as
 304 CFU/SI and pups with visible signs of diarrhea at the time of sacrifice are marked with an asterisk.
 305 P-values were determined by the Mann-Whitney U test.

306

307 **Discussion**

308 The Inaba and Ogawa serotypes of O1 serogroup *V. cholerae*, which were initially described over
309 a century ago, continue to cause virtually all pandemic cholera⁴³. Based on knowledge that the
310 O1 O-antigen is a critical target of protective immunity against cholera and that the methylation
311 that distinguishes Ogawa from Inaba strains can impact anti-*V. cholerae* immune responses, we
312 engineered genetically matched serotype variants of a live OCV candidate, HaitiV, as well as
313 isogenic Ogawa and Inaba WT challenge strains, to determine which, if any, O1 serotype would
314 be the most immunogenic and protective. We hypothesized that the bivalent Hikojima serotype
315 would be the most effective OCV formulation, as it presents both Inaba and Ogawa antigens. We
316 found that all three HaitiV variants - Inaba^V, Ogawa^V, and Hiko^V - were both immunogenic and
317 protective in the GF mouse model. Given that we observed relatively minor differences between
318 the vaccines, we suggest that the impact of O1 serotype variation in OCV design may be minimal,
319 and our data did not consistently identify a superior serotype across the assays we performed.
320 However, a striking result from this study was that all three live vaccines were far more
321 immunogenic than a formalin killed version of Hiko^V, strongly supporting the idea that a live OCV
322 has great potential for control of cholera.

323 In the challenge assays, all three live vaccines protected nearly all pups from Inaba^{WT} challenge,
324 but Ogawa^V was superior to either Inaba^V or Hiko^V in the animals challenged with Ogawa^{WT}, even
325 though the latter two vaccines elicited nearly equal anti-Ogawa VATs as Ogawa^V. Despite
326 protecting as well as Inaba^V and Hiko^V against Inaba^{WT} challenge, Ogawa^V induced lower anti-
327 Inaba VATs than the other two vaccines, underscoring the complexities of experimental markers
328 of protective immunity and suggesting that finer-scale metrics to evaluate vaccine efficacy would
329 be valuable. This is especially important since immune and protection metrics both report on
330 vaccine potency, but the translational insight imparted by these assays in relation to each other
331 is not entirely clear. Given the tight range of protective efficacy of all three vaccines (85-100%), it

332 was not possible to correlate VAT level and protection in the animals in our studies, as had been
333 done in humans, beyond the observation that seroconversion was tightly associated with
334 protection^{44,45}. The split-litter challenges showed that a surrogate measure of efficacy, the FROI
335 bacterial replication value, correlated with the level of circulating vibriocidal antibodies in the dam.
336 This is consistent with knowledge that immunity to cholera is primarily driven by anti-bacterial
337 effects⁴⁶. Similarly, our use of mixed isogenic challenge strains allowed us to directly measure the
338 relative replication (CI) of Inaba and Ogawa *V. cholerae* strains against each other in the same
339 intestinal environment. This experimental format revealed that immunization with Hiko^V elicited
340 immune responses that skewed the relative expansion of the Ogawa and Inaba challenge strains.
341 In conjunction with the observation of serotype-bias in FROI, these data support the idea that anti-
342 O-antigen antibodies are the direct effectors responsible for vaccine-mediated suppression of
343 colonization^{5,47} and suggest that determination of the molecular bases of serotype-biased *V.*
344 *cholerae* intestinal replication is warranted.

345 An important caveat underlying our findings is that GF mice lack commensal gut microbes and
346 could have altered immune responses to immunization, especially considering recent studies that
347 have highlighted how *V. cholerae*-microbiota interactions can influence colonization, disease, and
348 development of anti-*V. cholerae* immunity⁴⁸⁻⁵². However, the strong association of VAT induction
349 with protection and superior efficacy of live over inactivated vaccine strains in this model, and our
350 similar findings of OCV function in mice with transiently disrupted microbiomes reinforces the idea
351 that the GF mouse live OCV model holds substantial translational promise^{36,37}.

352 Investigations of the influence of the O1 serotypes on natural *V. cholerae* infection or OCV
353 function in the literature are sparse, and sometimes conflicting. Surveillance-based natural
354 infection studies conducted in Bangladesh, where cholera is endemic, have suggested that Inaba
355 cholera infections confer stronger protection against future exposure to Ogawa *V. cholerae* than
356 the reverse heterologous re-infection^{25,26}. Conversely, an early volunteer challenge study with WT

357 *V. cholerae* suggested that Ogawa-stimulated immunity against both homologous and
358 heterologous serotype re-challenge was non-inferior to that conferred by Inaba⁴¹. In addition,
359 recent analyses of immune responses during cholera suggest that Ogawa infections may elicit
360 stronger cross-serotype reactive immune responses than Inaba infections⁵³. There are several
361 reasons that may explain why these studies have yielded disparate conclusions. First, the
362 relatively small numbers of re-infected subjects in both challenge and surveillance studies limit
363 their robustness. Additionally, serotype dynamics during cholera epidemics can range from clonal
364 domination to co-circulation of Inaba and Ogawa strains, with local outbreaks often “switching”
365 from Ogawa to Inaba and back again, complicating the interpretation of serotype-specific disease
366 incidence in surveillance studies by impacting serotype-specific re-infection frequencies^{24,25,54–57}.
367 This is further confounded by the knowledge that serotype switching can be driven by selective
368 pressure from serotype-specific antibodies, sometimes within the same host, making it likely that
369 changes in population-level serotype-specific immunity drive serotype switching during
370 outbreaks^{29,58,59}. Lastly, no study has directly compared the immunogenicity or protective efficacy
371 of Inaba and Ogawa OCVs with otherwise identical genetic backgrounds, a crucial control in the
372 context of a continuously evolving pathogen such as *V. cholerae*. Our findings thus augment
373 epidemiological observations and provide a needed framework to benchmark the performance of
374 all three known O1 *V. cholerae* serotypes as OCVs.

375 Since we cannot predict *a priori* which serotype will cause an outbreak, and given that the reports
376 described above conflict on whether Ogawa or Inaba vaccines are preferable, our data suggest
377 that the development of a live Hikojima OCV would be the most risk-averse approach to next-
378 generation OCV design. Hiko^V was largely non-inferior to Inaba^V and Ogawa^V in our study,
379 presents both Ogawa and Inaba antigens, and, as a single strain formulation, could simplify the
380 OCV manufacturing process. It will be of interest to investigate how different Hikojima-generating

381 alleles of *wbeT* that give rise to skewed (not 1:1) Inaba:Ogawa O-antigen ratios influence serotype
382 switching, immunogenicity and OCV efficacy.

383 Finally, the controls and experimental schemes we used should be valuable for the design of
384 future investigations of OCVs, not only in GF mice, but potentially also in humans, as cholera is
385 one of the few infectious diseases for which there is a human challenge model⁶⁰. For example,
386 using mixed-challenge inocula (e.g. Inaba and Ogawa *V. cholerae*) could enable assessment of
387 the within-host relative fitness of the two major *V. cholerae* serotypes in immunized human
388 volunteers. Our observation that the apparent potency of OCVs is challenge strain-dependent
389 (Figure 6) also emphasizes the need for careful selection of relevant strains in animal and human
390 investigations of OCVs. Similar considerations were taken when the early 7PET isolate N16961
391 was introduced as an updated challenge in 1980⁴¹. This isolate has since been used as the
392 predominant human challenge strain, including the most recently reported volunteer challenge
393 OCV trial⁴⁵. Recent 7PET evolution over the last three decades has led to strains with altered
394 virulence traits, and potentially immunogenicity^{61–64}. Our data suggest that currently circulating
395 late 7PET *V. cholerae*, which have never been used as challenge strains, may interact differently
396 with OCV-induced immunity than early 7PET and 6th pandemic isolates, and thus should be
397 considered for use not only as next-generation live OCVs, but as challenge strains in future human
398 volunteer studies. This change may advance the development of efficacious OCV candidates as
399 well as yield mechanistic insights into immune responses to contemporary pandemic *V. cholerae*.

400

401 **Methods**

402 **Bacterial strains and growth conditions**

403 Bacteria were grown in lysogeny broth (LB) supplemented with the indicated
404 antibiotics/compounds at the following concentrations: streptomycin (Sm, 200µg/mL), kanamycin

405 (200µg/mL), carbenicillin (Cb, 50µg/mL), chloramphenicol (Cm, 0.75µg/mL)
406 sulfamethoxazole/trimethoprim (SXT, 80 and 16µg/mL) and 5-bromo-4-chloro-3-indolyl-β-d-
407 galactopyranoside (X-gal, 60µg/mL). For growth on plates, LB + 1.5% agar was used. Unless
408 otherwise noted, strains were grown in liquid media at 37°C shaking at 200rpm. All *V. cholerae*
409 strains in this study were spontaneous SmR derivatives of the wild-type. Bacterial stocks were
410 stored at -80°C in LB with 35% glycerol. Strains and plasmids used in this study are listed in
411 Supplementary Table 1 and 2, respectively.

412 **Serotype agglutination assay**

413 To serotype vaccine and WT strains, triplicate 10µL drops of saturated overnight single colony
414 cultures of each strain were spotted onto a glass slide and mixed with 5µL of anti-Inaba or anti-
415 Ogawa sera (BD Difco) or a vehicle control (0.85% NaCl). Drops were then mixed with a sterile
416 pipette tip and gently rocked for 30 seconds. Anti-Inaba serum strongly agglutinates Inaba and
417 Hikojima *V. cholerae*. Anti-Ogawa serum strongly agglutinates Ogawa and Hikojima *V. cholerae*.
418 Bacteria were imaged with a Nikon Eclipse TS100 inverted microscope at 100x magnification.

419 **Engineering HaitiV and HaitiWT derivatives**

420 HaitiV and HaitiWT variants were created by conventional allelic exchange techniques as
421 previously described³⁵. Briefly, the HaitiV or HaitiWT *V. cholerae* derivative of interest was
422 conjugated with SM10λpir *E. coli* bearing the suicide plasmid pCVD442 or pDS132 carrying the
423 allele to be exchanged as well as 700bp of up- and downstream homology to the targeted genomic
424 region. Conjugations were performed at a 1:1 donor:recipient ratio for 4 hours at 37°C and single
425 crossovers were isolated by plating reactions on LB + Sm/Cb (pCVD442) or LB + Cm (pDS132)
426 agar plates. To select for double crossovers, single crossover colonies were re-streaked on LB +
427 10% overnight at 30°C or were grown in LB + Sm/Cb for 4 hours at 37°C and then sub-cultured
428 1:100 statically in LB + 10% sucrose overnight at room temperature, followed by plating on LB +

429 Sm. Colonies were then checked for Cb resistance by duplicate patching. Sm^R/Cb^S colonies were
430 screened by colony PCR (for *hlyA*, *lacZ* and *recA*) or Sanger sequencing (for *wbeT*) to identify
431 colonies with the correct double crossover.

432 **GF mouse oral immunization scheme and sample collection**

433 3-6-week old GF female C57BL/6 mice were obtained from the Massachusetts Host-Microbiome
434 Center and housed in autoclaved cages with food and water given *ad libitum* in a non-gnotobiotic
435 BL-2 facility on a 12-hour light/dark cycle for the duration of the study. On Day 0, mice were
436 anesthetized with isoflurane and orally gavaged with 10⁹ CFU of overnight culture of one of the
437 three serotype variants of live or formalin-killed (inactivated, FKV) HaitiV in 100uL 2.5% NaHCO₃.
438 Formalin inactivation was performed as previously described³⁵. Immunized mice were monitored
439 daily and weighed weekly. At the indicated timepoints in Figure 1A, a blood sample was collected
440 by submandibular puncture for immunological assays and a fresh fecal pellet was collected from
441 each mouse for plating on LB + Sm to enumerate HaitiV shedding. Blood samples were clotted
442 at room temperature for 1 hour, centrifuged at 20000 x g for 5 minutes and supernatant (serum)
443 stored at -20°C for subsequent analyses. Mice were co-housed according to vaccine group until
444 Day 42, when mice designated for mating were re-housed with age-matched GF C57BL/6 males
445 to initiate the mating and infant challenge study phase.

446 **Quantification of vibriocidal antibody titers**

447 A complement-mediated cell lysis assay was performed to quantify vibriocidal responses in serum
448 samples as previously described⁴⁰. The clinical isolates PIC018 and PIC158 were used as the
449 Inaba or Ogawa *V. cholerae* target, respectively. Seroconversion was defined as ≥4x increase in
450 titer relative to the first measurement. A characterized mouse monoclonal antibody targeting *V.*
451 *cholerae* O1 O-specific polysaccharide was used as a positive control for the vibriocidal assay³⁶.

452 Titers are reported as the dilution of serum causing a 50% reduction in target optical density
453 compared to control wells with no serum added.

454 **Infant mouse single strain lethal challenge assay**

455 Lethal dose single strain challenge assays were performed as previously described³⁶. Pregnant
456 dams were singly housed at E17-20 for delivery. At P3 (third day of life), pups were orally
457 inoculated with 10^7 CFU of the indicated WT *V. cholerae* strain in 50 μ L LB and returned to their
458 dam. In split litters receiving Inaba^{WT} or Ogawa^{WT}, pups were randomly assigned to each
459 inoculum. Infected pups were monitored every 4-6 hours for onset of diarrhea and reduced body
460 temperature. Once signs of disease were observed, monitoring was increased to 30-minute
461 intervals until moribundity was reached, at which point pups were removed from the nest and
462 euthanized for dissection, homogenization and plating of the small intestine (SI) on LB + Sm/X-
463 gal for CFU enumeration. Pups that were alive at 48 hours post inoculation (hpi) were deemed
464 protected from the challenge. Upon removal of the final pup in each litter, a submandibular blood
465 sample was collected from the dam for vibriocidal antibody titer quantification. Fold replication
466 over inoculum (FROI) values were calculated by dividing the total *V. cholerae* SI CFU burden at
467 time of sacrifice by the inoculum. Mean FROI values were calculated by averaging FROIs from
468 both surviving and succumbed pups in a given subgroup (i.e. Inaba or Ogawa-inoculated pups in
469 a given litter or all pups in the litter). We excluded pups rejected by their dams from analyses due
470 to our inability to attribute mortality to infection alone.

471 **Infant mouse mixed strain non-lethal competitive index assay**

472 For non-lethal, competitive index (CI) infections, P5 (fifth day of life) pups were separated from
473 their dams and orally inoculated with a 1:1 mix (total 10^5 CFU) of HaitiWT Ogawa *lacZ*- and
474 HaitiWT Inaba *lacZ*+ *V. cholerae*, a dose insufficient to cause disease or mortality but sufficient
475 for robust intestinal colonization⁶⁵. At 20 hpi, pups were euthanized for dissection and CFU plating

476 of the SI on LB+Sm/X-gal for blue/white colony counting. CIs were obtained by dividing the ratio
477 of white:blue (Ogawa:Inaba) colonies in the SI to the ratio of white:blue colonies in the inoculum.

478 **Statistical analysis**

479 Statistical analyses were performed with Prism 8 (Graphpad). Survival curves were analyzed with
480 the log-rank (Mantel-Cox) test and CFU burdens and CIs were compared with the Mann Whitney
481 U test. Correlations between fold replication and VATs were generated by linear regression and
482 statistically tested with the F-test. Bacterial replication within the same litter (FROI) was analyzed
483 with the Wilcoxon signed-rank matched pair test. Differential incidence of diarrhea in challenged
484 pups was analyzed with two-tailed Fisher's exact tests. A p-value <0.05 was considered
485 statistically significant.

486 **Animal use statement**

487 This study was performed in accordance with the NIH Guide for Use and Care of Laboratory
488 animals and was approved by the Brigham and Women's Hospital IACUC (Protocol
489 2016N000416). Infant (P14 or younger) mice were euthanized by isoflurane inhalation followed
490 by decapitation. Adult mice were euthanized at the end of the study by isoflurane inhalation
491 followed by cervical dislocation.

492

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501

502 **Author Contributions**

503 Conceptualization: BS, BF, MKW. Methodology: BS, BF, MKW. Investigation: BS, BF, TZ, GB.
504 Supervision: MKW. Visualization: BS. Writing (Original Draft Preparation): BS, BF, MKW. Writing
505 (Review and Editing): BS, BF, TZ, GB, MKW.

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