Phage predation and antibiotic exposure are inversely associated with disease severity and shape pathogen genetic diversity in cholera patients

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ABSTRACT

Nearly a century has passed since d'Hérelle and colleagues discovered phages that infect *Vibrio cholerae* and influence the clinical and epidemiologic trajectories of cholera outbreaks. Despite an increasingly detailed picture of the molecular mechanisms of resistance and counter-resistance that shape phage and bacterial interactions, we lack an understanding of how these interactions play out during natural infection, how they are affected by antibiotic exposure, and how they relate to clinical outcomes. To fill these gaps, we conducted a nation-wide study of diarrheal disease patients in the cholera-endemic setting of Bangladesh. A total of 2574 stool samples were collected from enrolled patients at hospital admission and screened for *V. cholerae* and virulent phages (ICP1, 2, or 3). All 282 culture positive samples, and an additional 107 culture negative but PCR positive samples, were analyzed by shotgun metagenomic sequencing. From these metagenomes, we estimated the relative abundances of *V. cholerae,* phages, and members of the gut microbiome, while accounting for antibiotic exposure determined by quantitative mass spectrometry. Consistent with d'Hérelle's thesis, we observed higher phage to *V. cholerae* ratios in patients with mild dehydration, demonstrating in the modern era that phages are indicative of disease severity. Antibiotics were associated with less *V. cholerae* and with mild disease; ciprofloxacin in particular was associated with several known antibiotic resistance genes. Phage resistance genes in the *V. cholerae* integrative conjugative element (ICE) were associated with lower phage to *V. cholerae* ratios. In the absence of detectable ICEs, phages shaped genetic diversity, selecting for nonsynonymous point mutations in the *V. cholerae* genome. Together, our results point to both antibiotics and phages as inverse correlates of disease severity, while also selecting for resistance genes or mutations within cholera patients.

INTRODUCTION

The secretory diarrheal disease cholera is caused by the Gram negative bacterium *Vibrio cholerae* (*Vc*) and can progress to life-threatening hypovolemic shock in less than 12 hours (Andrews et al. 2017). Cholera remains a major public health problem due to inadequate sanitation and safe drinking water. Global estimates of cholera burden are 1.3-4.0 million cases and 21,000 to 143,000 deaths annually (Ali et al. 2015). As of January 2023, there were over 30 countries with active outbreaks necessitating the WHO to escalate the response to its highest level (grade three) ("Cholera – Global Situation" report, 2023). Rehydration is the primary life-saving maneuver for cholera patients. With effective rehydration, mortality rates fall from 20% and above to less than 1%; antibiotics reduce stool volume and duration of diarrhea but are considered supportive and reserved for patients with more severe disease to reduce selection for antibiotic resistance (Islam et al. 1995; Dromigny et al. 2002; Weill et al. 2017; Nelson et al. 2011, 2009). Nevertheless, antibiotic-resistant *V. cholerae* have emerged and spread globally (Das et al. 2020; Weill et al. 2017; Lassalle et al. 2022). Mechanisms of resistance are diverse and reside in the core genome and on mobile genetic elements, including the SXT integrative conjugative element (ICE) (Rivard, Colwell, and Burrus 2020). SXT is a ~100 kb ICE and can harbor resistance to sulfamethoxazole and trimethoprim, ciprofloxacin (qnr_{vc}), trimethoprim (*dfra31*) and streptomycin (*aph(6)*) (Waldor, Tschäpe, and Mekalanos 1996; Dalsgaard et al. 1999; Beaber, Hochhut, and Waldor 2002; Creasy-Marrazzo et al. 2022). Recent work has also shown that the ICE can encode diverse phage resistance mechanisms (LeGault et al. 2021).

V. cholerae is targeted by numerous lytic bacteriophages (phages) (Nelson et al. 2008; Boyd et al. 2021; K. D. Seed et al. 2011). With rising levels of antibiotic resistance, phages are a promising alternative or complementary therapy. The first clinical trials of phage therapy cocktails occurred during the Cholera Phage Inquiry from 1927 to 1936 (Summers 1993; D'Herelle and Malone 1927). In a *proto* randomized controlled trial, the Inquiry found the odds of mortality were reduced by 58% among those with phage therapy, with an absolute reduction in

76 mortality of 10% (95% CI 0.27-0.64) (Pasricha, de Monte, and O'Flynn 1936) (reanalyses by E.
77 J. Nelson). Despite these early findings, there is a lack of evidence in the modern era that links
78 phage predation with disease severity during *natural* infection in humans. A collection of
79 tangentially related studies support the hypothesis that phage mitigate severity:
80 Environmentally, phages in the aquatic environment are negatively correlated with cholera
81 cases in Dhaka, Bangladesh over time, suggesting a role for phages in influencing epidemic
82 dynamics (Faruque, Naser, et al. 2005). Clinically, a higher percentage of cholera patients shed
83 phage towards the end of an outbreak (Faruque, Islam, et al. 2005). Computationally, models
84 predict that phage can dampen outbreaks (Jensen et al. 2006). Experimentally, animal studies
85 found phage exposure was negatively associated with burden of colonization and disease
86 severity (Zahid et al. 2008; Jaiswal et al. 2013; Yen, Cairns, and Camilli 2017; Nelson et al.
87 2008). In this setting, a key unanswered mechanistic question is how phage, antibiotics, and
88 associated resistance factors interact to impact disease severity.
89 To address this question, we conducted a national prospective longitudinal study in the
90 cholera endemic setting of Bangladesh. Stool samples were collected at hospital admission
91 from diarrheal disease patients and screened for *V. cholerae* or *V. cholerae* phage. From these
92 positive samples, we sequenced shotgun metagenomes to quantify the relative abundances of
93 *V. cholerae,* phages, and gut microbiome taxa, while accounting for antibiotic exposure
94 determined by mass spectrometry. We demonstrate that severe dehydration is inversely
95 associated with phage ICP1. We also find evidence for non-linear predator-prey interactions
96 within infected hosts, but these interactions are suppressed at high levels of azithromycin.
97 Known phage-resistance elements (ICEs) are associated with lower phage:*V. cholerae* ratios. In
98 the absence of detectable ICEs, phages select for nonsynonymous point mutations in the *V.*
99 *cholerae* genome, with potential evolutionary consequences. Together, our results support a
100 hierarchy of selective pressures and resistance mechanisms evolving within individual cholera
101 patients.

RESULTS & DISCUSSION

Study overview. A total of 2574 stool samples were collected from enrolled participants from March to December 2018; collection continued to April 2019 at one site (icddr,b). A total of 282 samples (10.9%) were culture-positive for *V. cholerae*. Among culture-negative samples, 10% of samples were randomly selected and screened by PCR for *V. cholerae* or phage (ICP1,2,3) which generated an additional 107 positive samples (**Table S1**). Stool metagenomes were successfully sequenced from 88.4% (344/389) of these samples; 35%(122) were from the icddr,b. Detection rates for *V. cholerae,* ICP1, ICP2, and ICP3 were 55% (190), 18% (61), 1% (4) and 8% (28), respectively. Select antibiotics (**Table S2**) were quantified in stool using liquid chromatography-mass spectrometry.

Metagenomic correlates of disease severity and succession. In the context of enrollment at hospital admission, we expected to sample patients at different stages in their disease progression, beginning with high levels of *Vc* followed by *E. coli* and, in most cases, a return to a normal microbiome in which anaerobic bacteria dominate (David et al. 2015). We hypothesized that this ecological succession would be accompanied by a progression from severe to mild dehydration. Consistent with this hypothesis, we identified *Vc* as an indicator species of severe dehydration (Methods). *Vc was* more likely to be present and higher in relative abundance in patients with severe dehydration, while two *Bifidobacterium* species and *E. coli* were indicators of mild dehydration (**Figure 1A, Table S3**). In a time series of cholera patients, the phage ICP1 peaked on the first day of infection (David et al. 2015) yet intriguingly ICP1 was an indicator of mild dehydration in our analysis (**Figure 1A**). This contrasts with ICP3, which despite being less frequently detected in our study (28 samples with >0.1% ICP3 reads, compared to 61 samples with >0.1% ICP1), was an indicator of severe dehydration. This suggests that different phages can have contrasting disease associations. For subsequent analyses, we focus on the more prevalent ICP1 phage.

127 Although the distribution of ICP1 relative abundance is variable and less clearly
128 associated with dehydration status than *Vc* (**Figure S1**), higher ratios of ICP1 to *Vc* were
129 associated with mild dehydration (**Figure 1B**). This ratio is particularly informative, as it provides
130 insight into how strongly ICP1 might suppress its bacterial host. These analyses suggest that
131 ICP1 plays a role in modulating disease severity, or that it could be associated with later stages
132 of diseases than previously thought. Our study design prevents us from confidently
133 disentangling these possibilities; however, we recorded self-reported duration of diarrhea,
134 providing an approximate control for disease progression. We found higher relative abundances
135 of ICP1 associated with mild dehydration at early stages of disease (duration of diarrhea <72h)
136 but not at late stages (**Figure S1B and D**). We therefore cannot exclude a model in which ICP1
137 suppresses *Vc* at early disease stages to reduce disease severity; nor can we exclude ICP1 as
138 a non-causal biomarker of dehydration severity. In either case, ICP1 could provide a clinically
139 useful indicator of disease severity.
140

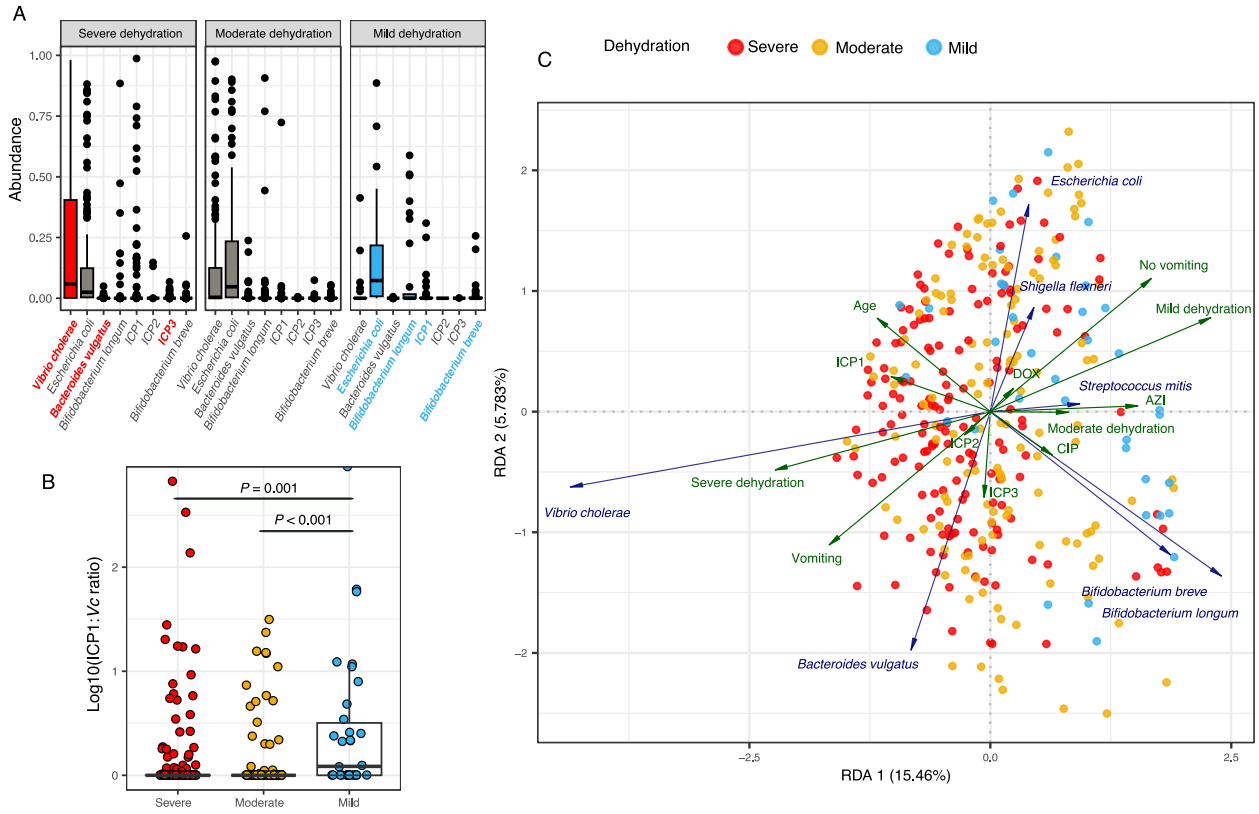

Figure 1. Dehydration severity is inversely associated with higher ICP1:*Vc* ratios in stool metagenomes. (A) Relative abundances of the most prevalent species in patients with severe, moderate, or mild dehydration; these conventions equate to the World Health Organization (WHO) conventions of severe, some and no dehydration, respectively. Significant indicator species for severe or mild dehydration are shown in red or blue bold text, respectively. See Table S3 for full indicator species results. (B) The ICP1:*V. cholerae* ratio is higher in patients with mild dehydration. *P*-values are from a Kruskal-Wallis test with Dunn's post-hoc test, adjusted for multiple tests using the Benjamini-Hochberg (BH) method. Only significant *P*-values (<0.05) are shown. Only 323 samples with *V. cholerae*>0% of metagenomic reads were included, with 165 from severe, 128 from moderate, and 30 from mild cases. A pseudocount of one was added to the ratio before log transformation. In (A) and (B) the solid horizontal line is the median and the boxed area is the interquartile range. (C) Redundancy analysis (RDA) showing relationships among the seven most dominant bacterial species and explanatory variables: phages, patient metadata, and antibiotic concentration. Angles between variables (arrows) reflect their correlations; arrow length is proportional to effect size. Samples (points) are colored by dehydration severity. All displayed variables are significant (*P*<0.05, permutation test) except ICP2, ICP3, and doxycycline (Table S4). For the RDA: R^2=0.25 and adjusted R^2=0.184, permutation test *P* = 0.001). To improve readability, collection date and location are not shown (see Figure S3 for these details).

159 ***Antibiotic exposure is associated with mild disease and resistance genes.*** To visualize the
160 complex relationships between disease severity, bacteria, and phage in the context of antibiotic
161 exposure, we used redundancy analysis (RDA; **Figure 1C; Table S4**). For simplicity, the seven
162 most dominant bacterial species identified by principal components analysis were included
163 (**Figure S2**). As explanatory variables, we used the most important clinical data, chosen by
164 forward selection beginning with phages and antibiotic concentrations (**Figure S3**). In
165 accordance with the indicator species analysis (**Figure 1A, Table S3**), *Vc* was positively
166 correlated with severe dehydration (**Figure 1C**). ICP1 was moderately associated with *Vc*,
167 consistent with a phage's reliance on its host for replication, but less associated with severe
168 dehydration. Both azithromycin (AZI) and ciprofloxacin (CIP) were negatively correlated with *V.*
169 *cholerae* and severe dehydration, suggesting their role in suppressing cholera infection and
170 disease.

171 To test the hypothesis that antibiotic exposure selects for antibiotic resistance genes
172 (ARGs), we assessed the relative abundances of 634 known ARGs conferring resistance to 34
173 antibiotic classes using deepARG (Arango-Argoty et al. 2018) applied to the metagenomic data.
174 To identify associations between these ARGs and gut microbes, we ran a multiple factor
175 analysis (MFA) with the 37 most dominant species from a PCA (20 highest coordinates on both
176 axes, Methods) and all the ARGs. ARGs were primarily associated with three different
177 taxonomic clusters dominated by (i) *V. cholerae*, (ii) *Escherichia* spp. and *Shigella* spp., and (iii)
178 *Prevotella* spp. and *Bifidobacterium* spp. (**Figure S4**). The *V. cholerae* cluster was associated
179 with 12 ARGs: *ompT, ompU*, *varG, dfrA1, dfrA16, aph3, aph6, mexI, floR, tet34, tet35*, and a
180 chloramphenicol exporter. Several of these targets were previously linked to antibiotic
181 resistance in *V. cholerae* (Creasy-Marrazzo et al. 2022; Monir et al. 2023). To determine if these
182 ARGs correlated with antibiotic exposure, we compared the relative abundance of ARGs
183 (normalized to *V. cholerae* reads) at different levels of antibiotic exposure: above or below the
184 minimum inhibitory concentration (MIC) set by experiments under aerobic vs anaerobic

185 conditions (**Table S5**) (Methods). CIP exposure was associated with higher relative abundance
186 of *dfrA16* (Wilcoxon test, BH corrected *P*=0.0178 and 0.0026 for anaerobic and aerobic MIC,
187 respectively), *aph6* (BH corrected *P*=0.084 for anaerobic and 0.0552 for aerobic MIC) (**Figure**
188 **S5)**. No significant correlations were observed with azithromycin or doxycycline. Together, these
189 results provide evidence that exposure to certain antibiotics selects for resistance genes in the
190 human gut.
191
192 ***Predator-prey dynamics between phage and pathogen.*** Offset oscillations between *Vc* and
193 its phages in the aquatic environment have been observed at the broader population level in
194 Bangladesh, supporting the hypothesis that these predator-prey dynamics influence the
195 progression of epidemics (Faruque, Islam, et al. 2005; Jensen et al. 2006). This hypothesis has
196 not been revisited in nearly two decades, and it remains unclear if the dynamics of aquatic
197 phages are mirrored by phages within infected patients. To address these questions, we
198 focused on a one-year time series from our most sampled site, icddr,b, in Dhaka, and tracked
199 the mean relative abundances of *Vc* and ICP1 across patients over time. Within the context of a
200 limited sample size, the abundance of *V. cholerae* was high from March through July 2018;
201 ICP1 abundance was low in March 2018 at the beginning of the epidemic, increased in July,
202 and spiked in August as *V. cholerae* declined (**Figure 2A, B**). ICP1 and *V. cholerae* were
203 inversely correlated from September 2018 onward, with a spike in *V. cholerae* corresponding to
204 a decline of ICP1 in September, and a peak in ICP1 associated with a decline of *V. cholerae* in
205 November. *V. cholerae* and ICP1 were inversely correlated within patients over time (Spearman
206 r=-0.25, P=0.017; **Figure 2C**), mirroring the trend previously observed between recorded
207 cholera cases and environmental phage concentrations (Faruque, Naser, et al. 2005). Sampling
208 sites outside Dhaka had sparser time series, but also generally supported alternating peaks of
209 *Vc* and ICP1 (**Figure S6**). Our results suggest that phages may contribute to suppressing *Vc*
210 not just in the environment, but also within patients. The degree of coupling between phage

concentrations in the environment and within patients could therefore be relevant to epidemic dynamics and deserves further study.

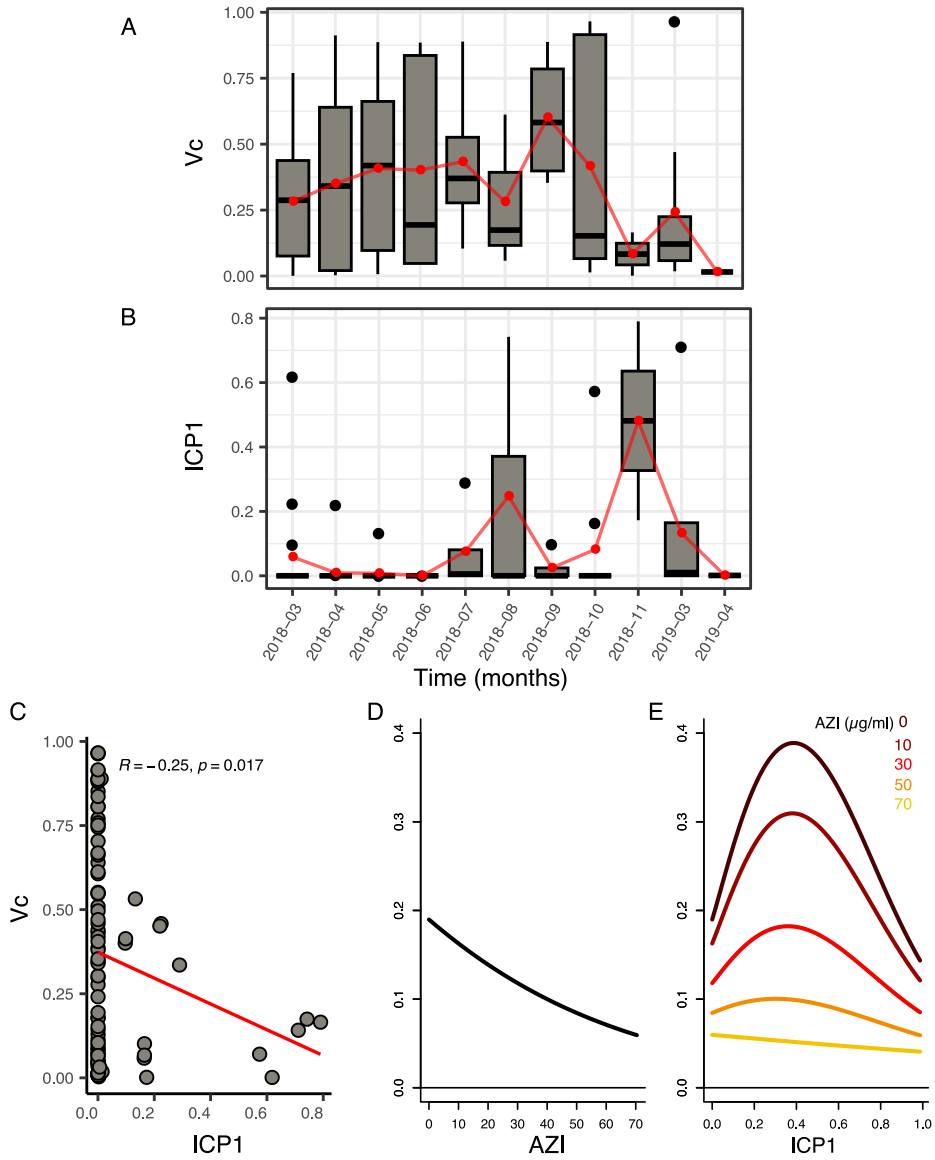

Figure 2. Offset temporal dynamics of *V. cholerae* and phage ICP1. Relative abundances of (A) *V. cholerae* and (B) ICP1 over time (binned by month) in metagenomes sampled from the icddr,b (94 samples with *Vc*>0.5% or ICP1>0.1% of metagenomic reads). Red line shows the mean. The boxplots contain 16 samples from 2018-03, 25 samples from 2018-04,17 samples from 2018-05, 5 samples from 2018-06, 4 samples from 2018-07, 3 samples from 2018-08, 4 samples from 2018-09, 9 samples from 2018-10, 2 samples from 2018-11, 8 samples from 2019-03, and 1 sample from 2019-04. (C) Spearman correlation between *V. cholerae* and ICP1 in the same samples as in A and B. The solid horizontal line is the median and the boxed area is the interquartile range. Panels (D) and (E) show GAM results, fit to data from all 344 samples. (D) *V. cholerae* declines in relative abundance with higher relative abundance of ICP1. (E) The relationship between ICP1 and *V. cholerae* is affected by azithromycin (AZI) concentration. The illustrated AZI concentrations show regular intervals between the minimum (0 ug/ml) and maximum (78 ug/ml) observed values.

227 ***Antibiotic exposure suppresses predator-prey dynamics.*** Our data provide a unique
228 opportunity to determine if and how antibiotics interact with phage to suppress *V. cholerae*
229 within patients. To do so, we modeled the relationships between ICP1, *V. cholerae* and
230 antibiotic exposure within each patient. We fit a generalized additive model (GAM) for *V.*
231 *cholerae* relative abundance as a function of ICP1, antibiotic exposure, and their interaction,
232 including dehydration status as a random effect. We fit GAMs with all antibiotics and their
233 interaction with ICP1, as well as separate models with each antibiotic, alone or in combination,
234 and compared them based on their AIC (**Table S6**). The most parsimonious model (with the
235 lowest AIC) included the interaction between AZI and ICP1 as a predictor of *V. cholerae*
236 abundance. *V. cholerae* was affected by AZI both directly (GAM, P=0.00236, Chi-square test)
237 and in interaction with ICP1 (GAM, P=0.026, Chi-square test); the effect of ICP1 alone was not
238 significant. Consistent with previous reports that AZI suppresses *V. cholerae (Alexandrova et al.*
239 *2019)* and with our RDA results (**Figure 1C**), we found a negative relationship between *V.*
240 *cholerae* and AZI (**Figure 2D**). This is also consistent with our observation that no annotated
241 ARGs were associated with AZI (**Figure S7**), suggesting that *V. cholerae* in our samples is
242 generally AZI-sensitive. The relationship between *V. cholerae* and ICP1 was quadratic: at low
243 ICP1 abundance, the relationship was positive but became negative at higher ICP1 abundance
244 (**Figure 2E**). This alternation between positive and negative correlations is consistent with
245 predator-prey dynamics within infected patients (Carr et al. 2019). However, at higher
246 concentrations of AZI, the quadratic relationship was flattened, effectively suppressing the
247 phage-bacteria interaction.

248 ***Integrative and conjugative elements (ICEs) are associated with phage suppression.***
249 SXTs are ~100kb integrative and conjugative elements (ICEs) that have been associated with
250 resistance to both antibiotics and phages and were first discovered in *V. cholerae* (Waldor,
251 Tschäpe, and Mekalanos 1996). SXTs have conserved 'core' genes along with variable

252 ‘hotspots’ encoding different genes; for example, hotspot 5 is a ~17kb region associated with
253 phage resistance. At the time of our sampling, ICEVchInd5 and ICEVchind6 were the two most
254 prevalent ICEs in Bangladesh (LeGault et al. 2021). These ICEs differ in their anti-phage
255 systems: ICEVchInd5 (henceforth “ind5”) encodes a type 1 bacteriophage exclusion (BREX)
256 system while ICEVchInd6 (“ind6”) encodes several other restriction-modification systems
257 (LeGault et al. 2021).

258 We enumerated ICEs in metagenomes by mapping reads against reference sequences
259 for ind5 (NCBI accession GQ463142.1) and ind6 (accession MK165649.1). An ICE was defined
260 as present when 90% of its length was covered by at least one metagenomic read (**Figure**
261 **S8A**). We found that 59% (144/244) of the samples contained ICEVchind5, 10.6% (26/244)
262 contained ICEVchind6, and 22.1% (54/244) had no detectable ICE. The lack of ICE detections
263 was not due to the lack of *V. cholerae* in a metagenome because ICE-negative samples did not
264 contain fewer *V. cholerae* reads (**Figure S8B**).

265 Resistance mechanisms on ICEs have been shown to suppress phage *in vitro*, but their
266 relevance within human infection remains unclear. We found that metagenomes without a
267 detectable ICE (denoted as ICE-) were associated with higher phage to *V. cholerae* ratios
268 (**Figure 3**). The effect was strongest for ICP1, which had the largest sample size (**Figure 3A**).
269 This observation is consistent with ICE-encoded mechanisms suppressing phage within
270 patients. Higher ICP1:*Vc* ratios, which tend to occur in ICE- patients, were also associated with
271 mild dehydration (**Figure 3B**). The phage:*Vc* ratios varied according to the precise phage-ICE
272 combination, suggesting that different ICE hotspots might confer resistance to different phages
273 (**Figure 3, Figure S9**). Together, these results demonstrate a role for ICEs in suppressing
274 phages during human infections, complementing and generally confirming the predictions of
275 laboratory experiments (LeGault et al. 2021). That said, the suppression is not complete, and
276 further experiments are needed to dissect the underlying causal relationships.

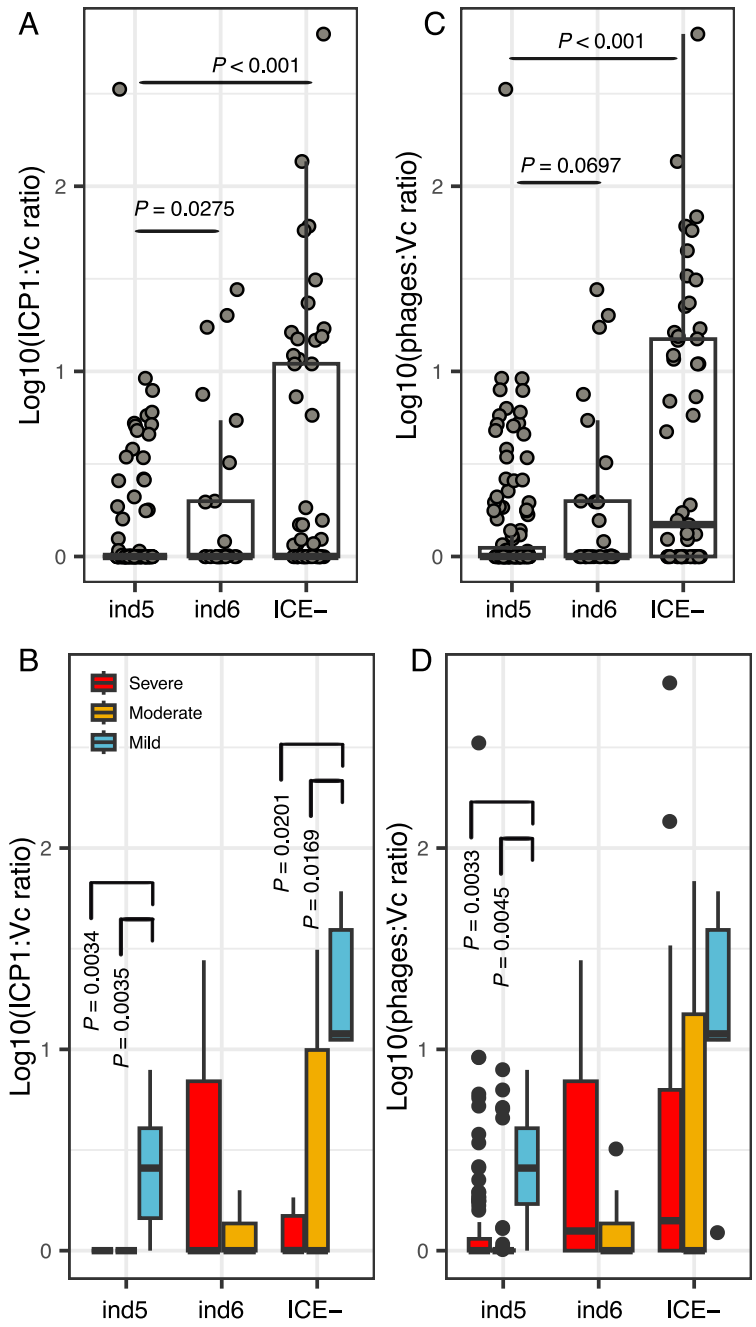

Figure 3. Integrative conjugative elements (ICEs) are associated with lower ICP1:*V. cholerae* ratios in patient metagenomes. (A) Distribution of ICP1:*Vc* ratios across patients with different ICE profiles. (B) The same data as (A) binned into boxplots according to dehydration status. (C) Distribution of phage:*Vc* ratios, including the sum of all phages (ICP1, ICP2, ICP3). (D) The same data as (C) binned into boxplots according to dehydration status. *P*-values are from a Kruskal-Wallis test with Dunn's post-hoc test adjusted with the Benjamini-Hochberg (BH) method. Only *P*-values < 0.1 are shown. Only samples with sufficient *Vc* or ICP1 were included (224 samples with *Vc*>0.5% or phages >0.1% of metagenomic reads), of which 54 samples were ICE-, 26 were ind6+ and 144 were ind5+. For clarity, the Y-axes were log10 transformed after adding one to the ratios. The solid horizontal line is the median and the boxed area is the interquartile range.

289 ***Hypermutation generates V. cholerae genetic diversity.*** In addition to variation in gene
290 content in ICEs and other mobile elements, resistance to phages and antibiotics may be
291 conferred by point mutations (single nucleotide variants; SNVs) that existed before or emerged
292 *de novo* during infection. Although we cannot exclude mixed infections by different *Vc* strains as
293 a source of diversity, there was no evidence for more than one strain co-infecting a patient in
294 our study population (**Figure S10**). We previously found a generally low level of *V. cholerae*
295 genetic diversity within individual cholera patients (Levade et al. 2017), with the exception of
296 hypermutation events characterized by DNA repair defects and hundreds of SNVs in the *Vc*
297 genome, primarily transversion mutations (Levade et al. 2021). Here, with a much larger sample
298 size, we can better quantify the frequency of hypermutator phenotypes, and test if within-host *V.*
299 *cholerae* diversity is associated with phages or antibiotics – both of which could potentially
300 select for resistance mutations. After quality filtering the metagenomic reads, we used InStrain
301 (Olm et al. 2021) to quantify within-host *V. cholerae* diversity in 133 samples that passed the
302 post-instrain filter (Methods). To identify likely hypermutators, we independently counted
303 samples with defects (nonsynonymous mutations) in any of 65 previously defined DNA repair
304 genes (Jolivet-Gougeon et al. 2011) or a high rate of SNVs (25 or more) (Levade et al. 2021).
305 We found that 35% of samples (47/133) had both a high SNV count and nonsynonymous
306 mutations in DNA repair genes – making them likely hypermutator samples. Higher SNV counts
307 were significantly associated with DNA repair defects (Fisher's exact test, P=2.2e-16),
308 consistent with these defects resulting in higher mutation rates within patients. The number of
309 SNVs was not significantly confounded by *V. cholerae* genome coverage (**Figure S11A**).
310 Consistent with our previous study (Levade et al. 2021), putative hypermutators had a distinct
311 mutational profile, enriched in transversions (**Figure S11B**). This suggests that hypermutation is
312 common and detectable in over one third of cholera patient stool metagenomes. We also
313 observed that samples with high numbers of SNVs but no DNA repair defects had somewhat
314 higher levels of phage ICP1 (**Figure S11D**). This suggested that phage could select for

315 resistance mutations even in non-mutators. Nevertheless, for subsequent analysis, we
316 considered all SNVs together, regardless of whether they were generated by hypermutation or
317 not.
318
319 ***Phages, not antibiotics, are associated with V. cholerae within-host diversity.*** We asked if
320 *V. cholerae* within-host diversity was associated with phage or antibiotics – both likely strong
321 selective pressures on bacterial populations. To do so, we fit GLMMs with phages and
322 antibiotics as predictors of the number of high-frequency nonsynonymous (NS) SNVs in the *V.*
323 *cholerae* genome. We focused on higher-frequency SNVs (>10% within a sample) as more
324 likely to be beneficial, and NS SNVs as those more likely to have fitness effects. We fit several
325 models with different combinations of predictors: from a model with all antibiotics and their
326 interaction with ICP1 to separate models with each antibiotic and its interaction with ICP1, we
327 added *V. cholerae* abundance as a fixed effect to the model to control for any coverage effect
328 (**Table S7**). The most parsimonious model included *V. cholerae* abundance and the interaction
329 between *V. cholerae* and ICP1 as predictors of the number of high-frequency NS SNVs. Adding
330 antibiotics or their interaction with ICP1 did not improve the model (**Table S7**), suggesting a
331 limited role for antibiotics in selecting for point mutations within patients.
332 In the model, *V. cholerae* relative abundance and the interaction between *V. cholerae*
333 and ICP1 both had significant effects (GLMM, Wald test, P=0.00246 and P=0.00494
334 respectively). The negative relationship between *V. cholerae* and the number of high-frequency
335 NS SNVs (**Figure 4A**) is not easily explained by coverage artifacts, since the total number of
336 SNVs is not associated with *V. cholerae* relative abundance (**Figure S11A**). The number of
337 high-frequency NS SNVs rises with increasing ICP1 – but only when *V. cholerae* abundance is
338 high (**Figure 4A**). As a control, we ran the same GLMM on NS SNVs without a minimum
339 frequency cutoff and found no significant effects, suggesting that the interaction between ICP1
340 and *V. cholerae* on SNV count is specific to high-frequency mutations, rather than low-

341 frequency mutations that are more likely neutral or deleterious. These data support a scenario in
342 which ICP1 selects for NS SNVs when the *V. cholerae* population is large enough to respond
343 efficiently to selection – for example, at the beginning of an infection or in the absence of
344 antibiotics.

345 If phage select for beneficial mutations, we would expect these mutations to increase in
346 frequency at higher phage abundance. We lack time-series data from individual patients, but the
347 relative abundance of phage provides a proxy for the combined effect of time and strength of
348 phage selection. To test this expectation, we fit a GAM with the average within-sample
349 frequency of SNVs as a function of ICP1, antibiotics, and their interactions. We included the
350 fixed effect of ICE presence/absence as another factor that could provide phage or antibiotic
351 resistance, as well as mutation type to differentiate among non-synonymous (NS), synonymous
352 (S), and intergenic (I) mutations. We fit GAMs with all antibiotics and their interaction with ICP1,
353 as well as simpler models with each antibiotic separately, and compared them based on AIC
354 (**Supplementary Table S8**). The most parsimonious model included the interaction between
355 ICP1, ICE and mutation type, but not antibiotics. ICP1 was a strong predictor of higher
356 frequency NS SNVs in the absence of a detectable ICE (**Figure 4B**). Samples in this analysis
357 were unambiguous in terms of their ICE presence/absence patterns (**Figure S12A, B**). To
358 confirm this model prediction, we compared the distribution of the average frequency of NS
359 SNVs between ICP1-positive and ICP1-negative samples. We found that NS SNV frequency
360 was significantly higher in ICP1-positive samples when the ICE was not detected (Wilcoxon test,
361 P=0.0094) (**Figure 4C**). This effect was strongest for NS mutations, making them the likely
362 targets of ICP1 selection. The slight (not significant, P>0.05) trends for synonymous and
363 intergenic SNVs could be due to genetic linkage with nonsynonymous mutations. Together,
364 these results suggest that, in the absence of detectable ICE-encoded phage resistance, ICP1
365 selects for non-synonymous point mutations that may confer alternative modes of resistance.
366

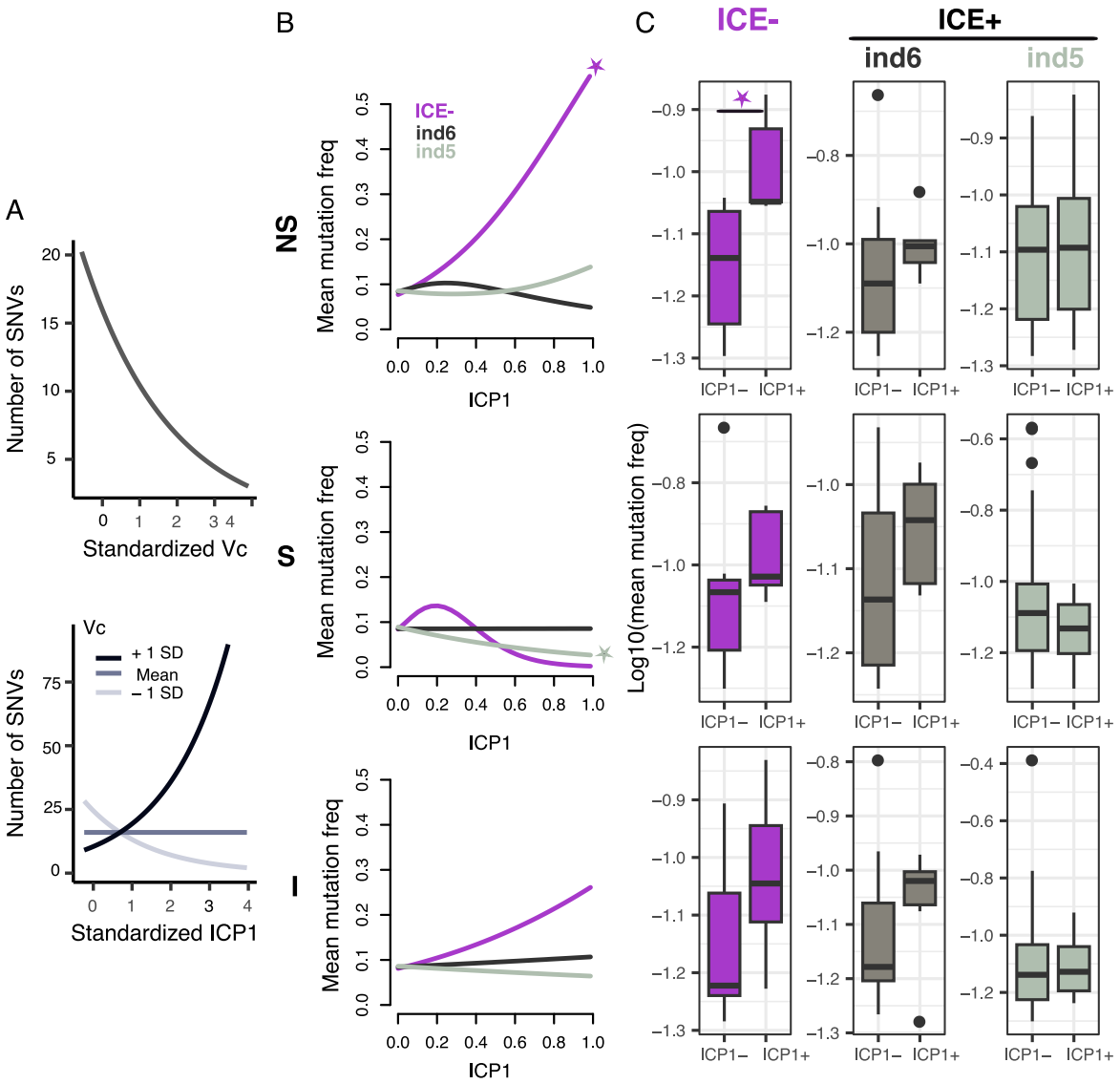

Figure 4. ICP1 selects for non-synonymous point mutations in the *V. cholerae* genome in the absence of ICE. (A) Results of a GLMM modeling high frequency nonsynonymous SNV counts as a function of *V. cholerae* (*Vc*) and ICP1 standardized relative abundances. In the bottom panel, shades of gray indicate *Vc* relative abundance at the mean or +/- 1 standard deviation (SD) across samples. (B) GAM output with the mean mutation frequency as a function of the interaction between ICP1, ICE and mutation type (non-synonymous; NS, synonymous; S, or intergenic; I). Significant effects are shown with a star (P<0.05). The model was fit using 130 samples that passed the post-Instrain filter (Methods). (C) Boxplots of mutation frequency in the presence or absence of ICP1 and/or ICEs. The single significant comparison is indicated with a star (Wilcoxon test, P=0.0094). Boxplots include 130 samples, of which 32 are ICP1+ (ICP1>=0.1% of reads) and 98 are ICP- (ICP1<0.1% of reads). The solid horizontal line is the median and the boxed area is the interquartile range.

380 To explore which point mutations might be specifically associated with phage resistance,
381 we focused on the *V. cholerae* genes that accumulated repeated mutations under ICP1
382 pressure. We previously observed that the secreted hemolysin toxin gene *hlyA* was mutated
383 more often than expected by chance in cholera patients, but we were unable to identify an
384 underlying selective pressure (Levade et al. 2021). In the larger sample reported here, *hlyA* was
385 among the genes most frequently mutated in patients with relatively high *Vc* to ICP1 ratios
386 (**Table S9**). This gene, along with others mutated at either high or low levels of phage (**Table**
387 **S9; Table S10**) – including several with membrane or virulence-related functions – provide
388 candidate phage resistance mechanisms that can be explored in future experiments.

389

390 **CONCLUSION**

391 The tripartite interactions between pathogens, phage, and antibiotics have been studied in the
392 laboratory, *in silico* with mathematical models, and to a lesser extent in the field, but how these
393 factors interact during human infection remains an open question. Our objective was to
394 characterize these interactions in the context of cholera. We analyzed more than 300 stool
395 metagenomes from symptomatic patients enrolled at hospital admission across Bangladesh
396 during an entire seasonal outbreak period. We discovered that higher levels of phage (ICP1)
397 relative to *V. cholerae* were associated with mild dehydration, providing a biomarker of disease
398 severity. The presence of phage alone did not strongly correlate with severity. However, the
399 ratio of phage to *V. cholerae* did strongly correlate with severity, suggesting that "effective
400 predation" is a key metric to be used when considering phage as a biomarker.

401 As predicted by laboratory experiments showing that phage resistance elements on
402 ICEs can protect against ICP1 (LeGault et al. 2021), we found that patient samples without
403 detectable ICEs were associated with higher phage to *V. cholerae* ratios. While we have
404 previously reported loss of ICE genes within cholera patients (Levade et al. 2017), lack of ICE

405 detection in metagenomes could also be explained by colonization of a strain lacking the ICE or
406 encoding an ICE variant with low similarity to those previously sequenced. In the absence of
407 detectable ICE, phage (ICP1) were associated with increased rates of nonsynonymous point
408 mutations in the *V. cholerae* genome. Many of these mutations likely arose by hypermutation,
409 which generates deleterious mutations – but may also rapidly confer phage resistance, as
410 observed in experiments with *Pseudomonas fluorescens (Pal et al. 2007)*. *V. cholerae* can
411 evolve resistance to phage ICP2 within patients, yet the resistance mutations in a surface
412 protein reduce its potential for onward transmission (K. S. Seed et al. 2014). Whether the
413 mutations associated with ICP1 in our study mediate similar fitness tradeoffs for *V. cholerae*
414 remains unknown. Antibiotic exposure was not associated with increased rates of point
415 mutations, but was associated with less *V. cholerae* and less severe disease. Azithromycin
416 appeared to be particularly effective at suppressing *V. cholerae* and was not associated with
417 any known resistance genes in metagenomes. By contrast, ciprofloxacin exposure was
418 associated with several known resistance genes and was less effective at suppressing *V.*
419 *cholerae*. These predictions can be tested in genome-wide association studies, among other
420 approaches.

421 Our study has several limitations which provide opportunities for future research. First,
422 samples were collected at a single time point (hospital admission) which allows us to establish
423 statistical correlations, but in the absence of time-series or interventional experiments, we
424 cannot infer causality. Notably, our results are consistent with ICP1 suppressing *V. cholerae*
425 and reducing disease severity, but time-series studies of individual patients, or randomized
426 controlled trials, will be needed to show causality. Second, shotgun metagenomic sequencing
427 provides rich data on bacteria and phages in the gut microbiome, but is limited to the most
428 abundant taxa, such as ICP1 and *V. cholerae*. Phages ICP2 and ICP3 were less prevalent in
429 our study population, and less abundant within patients, making it challenging to infer their
430 interactions with *V. cholerae* and associations with disease severity. Short read sequencing also

431 made it challenging to link ICE variants with *V. cholerae* genomes, so we relied on read
432 mapping approaches. From a clinical perspective, quality of assessments of dehydration may
433 have varied by provider and location which could represent potential confounders and reduce
434 effect sizes. Finally, our study lacked information about host genetics or immunity, which also
435 contribute to disease outcomes (Harris et al. 2008; Nelson et al. 2009). Future studies
436 combining rich patient metadata, time series, clinical interventions, long-read metagenomics,
437 and isolate genome sequencing will complement and expand upon our study.

438 Despite these limitations, our study implicates both phages and antibiotics as
439 determinants of cholera disease severity, and paves the way for future enquiries into their
440 interacting roles in disease progression and recovery. We propose a hierarchy of selective
441 pressures acting on *V. cholerae* in the gut. In the absence of resistance genes, antibiotics are
442 effective at suppressing cholera and reducing disease severity. In the absence of effective
443 antibiotics, lytic phages suppress *V. cholerae* when the bacteria do not encode phage
444 resistance in the ICE. Finally, in the absence of ICE-encoded resistance, phages may select for
445 point mutations conferring phage resistance and potentially longer-term fitness consequences.
446

447 **MATERIALS AND METHODS**

448 **Ethics Statement**. The samples analyzed were collected within the confines of two previously
449 published IRB approved clinical studies conducted in Bangladesh: (i) The mHealth Diarrhea
450 Management (mHDM) cluster randomized controlled trial (IEDCR IRB/2017/10; icddr,b
451 ERC/RRC PR-17036; University of Florida IRB 201601762) (Khan et al. 2020a). (ii) The
452 National Cholera Surveillance (NCS) study (icddr,b ERC/RRC PR-15127) (Khan et al. 2020b);
453 See supplementary materials for further details.

454 **Study Design.** The study design was a prospective longitudinal study of patients presenting
455 with diarrhoeal disease at five Bangladesh Ministry of Health and Family Welfare district

456 hospitals (both mHDM and NCS sites) and two centralized NCS hospitals (BITID; icddr,b) from
457 March 2018 to December 2018. See supplementary materials.
458 **Sample collection.** Stool samples were collected at hospital admission. Aliquots for transport
459 and subsequent culture were stabbed into Cary-Blair transport media; aliquots for molecular
460 analysis were preserved in RNAlater. See supplementary materials.
461 **Microbiological and molecular analysis.** Culture was performed via standard methods
462 (Balows 2003); total nucleic acid (tNA) was extracted from the RNAlater preserved samples
463 using standard methods. Cholera samples for subsequent metagenomic analysis were identified
464 by screening all samples by culture for *V. cholerae*. Among culture negative samples, a random
465 10% of the remaining samples were screened for *V. cholerae* specific phage (ICP1, 2, 3) by
466 PCR using total nucleic acid (tNA) extracts. From samples positive by culture or PCR,
467 sequencing libraries were prepared using the NEB Ultra II shotgun kit and sequenced on
468 illumina NovaSeq 6000 S4, pooling 96 samples per lane, yielding a mean of >30 million paired-
469 end 150bp reads per sample.
470 **Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS).** Those
471 cholera samples identified for metagenomic analysis were also analyzed by qualitative and
472 quantitative LC-MS/MS for antibiotics (Alexandrova et al. 2019; Creasy-Marrazzo et al. 2022).
473 While the target list for the qualitative analyses was broad, the list for the quantitative analyses
474 was narrow: Ciprofloxacin, Doxycycline/Tetracycline, and Azithromycin. Standard curves were
475 made for each quantitative target by preparing a dilution series of the three native and isotopic
476 forms of the quantitative targets (Ciprofloxacin, Doxycycline, Azithromycin); for quantitative LC-
477 MS/MS, clinical samples were spiked with the isotopes as internal references. See
478 supplementary materials.
479 **Metagenomic data analysis.** We taxonomically classified short reads using Kraken2 (Wood,
480 Lu, and Langmead 2019) and Bracken v.2.5 (Lu et al. 2017). Reads were assembled using
481 MEGAHIT v.1.2.9 (Li et al. 2015) and binned with DAS tool (Sieber et al. 2018). To characterize

482 diversity within *V. cholerae*, we used StrainGE (van Dijk et al. 2022) and InStrain v.1.5.7 (Olm et
483 al. 2021). To identify antibiotic resistance genes in metagenomes, we used deepARG v 1.0.2
484 (Arango-Argoty et al. 2018). See supplementary materials for details.

485 **Statistical analyses.** Statistics and visualizations were done in R studio version 1.2.5042. See
486 supplementary materials for details.

487 **Data availability.** All sequencing data are deposited in the NCBI SRA under BioProject
488 PRJNA976726. See supplementary materials for further information.

489 **Code availability.** Computer code for this paper will be available at
490 https://github.com/Naima16/Cholera-phage-antibiotics.

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