Title: Phage predation, disease severity and pathogen genetic diversity in cholera patients

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Abstract

Despite an increasingly detailed picture of the molecular mechanisms of phage-bacterial interactions, we lack an understanding of how these interactions evolve and impact disease within patients. Here we report a year-long, nation-wide study of diarrheal disease patients in Bangladesh. Among cholera patients, we quantified *Vibrio cholerae* (prey) and its virulent phages (predators) using metagenomics and quantitative PCR, while accounting for antibiotic exposure using quantitative mass spectrometry. Virulent phage (ICP1) and antibiotics suppressed *V. cholerae* to varying degrees and were inversely associated with severe dehydration depending on resistance mechanisms. In the absence of anti-phage defenses, predation was 'effective,' with a high predator:prey ratio that correlated with increased genetic diversity among the prey. In the presence of anti-phage defenses, predation was 'ineffective,' with a lower predator:prey ratio that correlated with increased genetic diversity among the predators. Phage-bacteria coevolution within patients should therefore be considered in the deployment of phage-based therapies and diagnostics.

One Sentence Summary

A survey of cholera patients in Bangladesh identifies phage predation as a biomarker of disease severity and driver of coevolution within patients.

MAIN TEXT

Introduction

Cholera is caused by the Gram negative bacterium *V. cholerae* (*Vc*) and can progress to life-threatening hypovolemic shock in less than 12 hours (*1*). Cholera remains a major public health problem because of inadequate sanitation and restricted access to safe drinking water. Global estimates of the cholera burden are 1.3-4.0 million cases and 21,000-143,000 deaths annually (*2*). In 2023, there were over 30 countries with active outbreaks necessitating the WHO to escalate the response to its highest level (*3*). Rehydration is the primary life-saving intervention for cholera patients. With adequate rehydration, mortality rates fall from over 20% to less than 1%. Antibiotics reduce stool volume and duration of diarrhea but are generally reserved for patients with more severe disease to reduce selection for antibiotic resistance (*4-8*). Nevertheless, antibiotic-resistant *Vc* have emerged globally (*6, 9, 10*). Mechanisms of resistance are diverse and reside in the core genome, plasmids of the incompatibility type C (*11, 12*) and on mobile genetic elements, including a ~100kb integrative conjugative element (ICE), which can harbor resistance to sulfamethoxazole and trimethoprim, ciprofloxacin (qnr_{vc}), trimethoprim (*dfra31*) and streptomycin (*aph(6)*) (*13-16*). Recent work has also shown that the ICE can encode diverse phage resistance mechanisms, with distinct hotspots of gene content variation encoding different resistance genes (*17*).

With rising levels of antibiotic resistance, virulent bacteriophages (phages) are a promising alternative or complementary therapy to antibiotics. Phages and bacteria coevolve, with each partner selecting for adaptations in the other that generates genetic diversity for both predator and prey (*18, 19*). Coevolution likely explains the extensive arsenal of resistance and counter-resistance mechanisms among *Vc* and its phages (*17, 19-24*). Yet it remains unclear how these interactions impact disease severity during natural infection, with and without antibiotic exposure. Virulent phages specifically targeting *Vc* include ICP1 (*Myoviridae*), ICP2 (*Podoviridae*), and ICP3 (*Podoviridae*) (*21, 25, 26*). These common phages are found in

73 symptomatic and asymptomatic cholera patients during acute infection or the convalescent
74 period.

75 The first clinical trials of phage therapy occurred during the Cholera Phage Inquiry from
76 1927 to 1936 (*27, 28*). In a *proto* randomized controlled trial, the Inquiry found the odds of
77 mortality were reduced by 58% among those given phage therapy, with an absolute reduction in
78 mortality of approximately 10% (*29*). Despite these early findings, there is a lack of evidence in
79 the modern era linking phage predation with disease severity during natural infection in humans.
80 However, indirect studies support a link. Environmentally, virulent phages in aquatic
81 environments have been negatively correlated with cholera cases in Bangladesh over time,
82 suggesting a role for phages in influencing epidemic dynamics (*30*). Clinically, a higher
83 percentage of cholera patients shed virulent phages towards the end of an outbreak period (*31*),
84 suggesting that outbreaks may collapse because of phage predation (*8)*. Theoretically, models
85 predict that phage predation can dampen cholera outbreaks (*32*). Experimentally, animal
86 studies found phage predation was inversely associated with colonization and severity (*25, 33-*
87 *35*). The key unanswered question is if and how virulent phages, antibiotics, and bacterial
88 evolution interact to impact infection in a meaningful manner for clinicians, public health officials,
89 and most importantly patients.

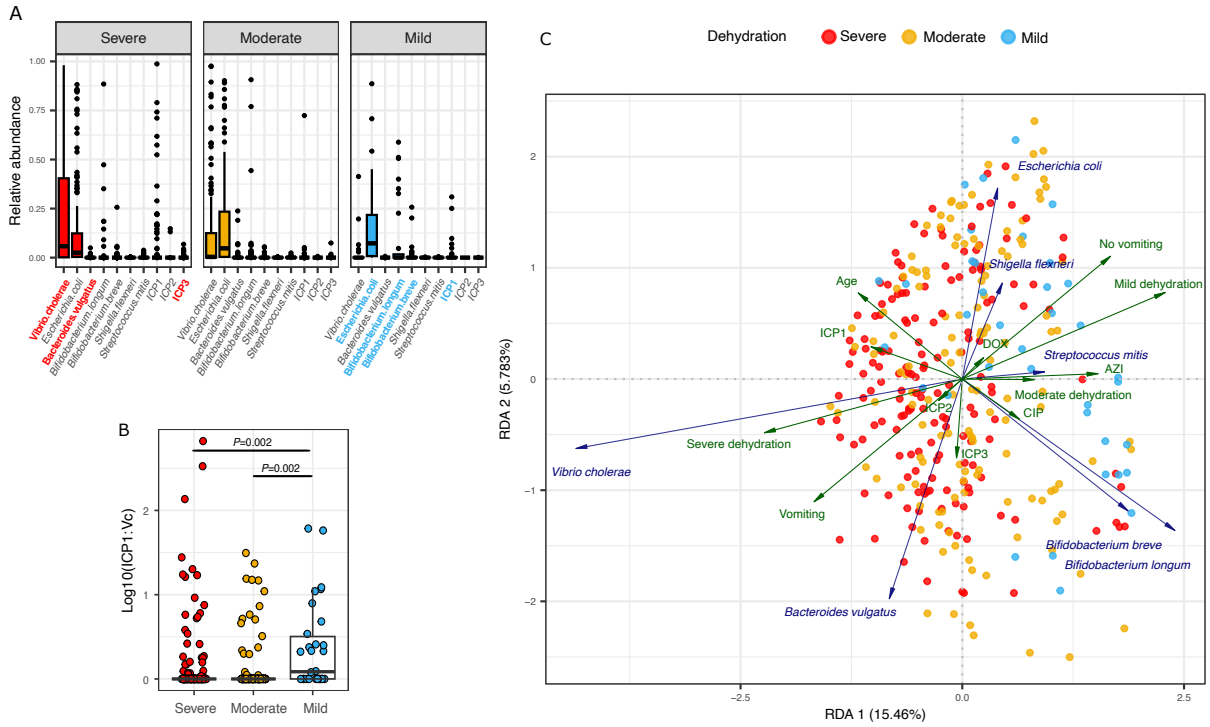
90 To address this question, we conducted a national prospective longitudinal study in the
91 cholera endemic setting of Bangladesh. Stool samples were collected at hospital admission
92 from diarrheal disease patients and screened for *Vc*, antibiotics, and cholera phages, focusing
93 on the obligately lytic and commonly prevalent phage ICP1. Patients in this setting routinely self-
94 medicate with antibiotics before arriving at the hospital, hence the need to measure antibiotics in
95 stool. We hypothesized that: (i) virulent phages and antibiotics would suppress *Vc* and be
96 inversely associated with disease severity, (ii) suppression by phage would be lifted for *Vc*
97 encoding anti-phage genes on ICEs, (iii) phages would select for other resistance mutations in
98 the absence of ICE-encoded resistance, and (iv) phages would be under selection to escape

99 suppression by ICEs. We provide broad support for these hypotheses, paving the way for
100 mechanistic experimental studies, the development of the phage:pathogen ratio as a biomarker
101 of disease severity, and further dissection of the longer-term consequences of phage predation
102 on pathogen evolution.
103

104 **Results**

105 ***Study overview.*** A total of 2574 stool samples were collected from enrolled participants
106 admitted for diarrheal disease at seven hospitals across Bangladesh from March to December
107 2018; collection continued until April 2019 at one site (icddr,b). Three groups of cholera samples
108 were analyzed: (i) *Vc* culture-positive (282/2574; 10.9%), (ii) *Vc* culture-negative but phage
109 (ICP1,2,3) PCR-positive (127/2292; 5.5%; 80 included; 47 excluded for DNA < 1ng/µl), and (iii) a
110 random 10% of *Vc* culture-negative and phage PCR-negative samples that were *Vc* PCR-
111 positive (14.8%; 37/250; 27 included; 10 excluded for DNA < 1ng/µl; see **Table S1** for PCR
112 primers). Stool metagenomes were sequenced from 88.4% of samples (344/389, with the
113 remainder failing library preparation) from these three groups, 35% of which were from the
114 icddr,b site. Based on metagenomic read mapping to a taxonomic database, detection rates for
115 *Vc,* ICP1, ICP2, and ICP3 were 55%, 18%, 1% and 8%, respectively. These detection rates
116 were supported by an analysis of *Vc* phages identified in metagenomic assemblies. As
117 expected, the prophage encoding the cholera toxin (CTXphi) was identified in most assemblies,
118 with ICP1 being the most prevalent obligately virulent phage. While some additional putative
119 phages were detected, none were prevalent enough to merit further analysis (**Fig. S1**). For both
120 *Vc* and ICP1, relative abundances in metagenomes correlated with absolute quantification by
121 qPCR (**Fig. S2**). Five antibiotics (**Table S2**) were prioritized for detection in stool using liquid
122 chromatography-mass spectrometry (LCMS); of these, azithromycin, ciprofloxacin, and
123 doxycycline were quantified.

124 ***Metagenomic correlates of disease severity and succession.*** At hospital admission, we
125 expected patients to present at different stages of disease, with an ecological succession of *Vc*
126 followed by the facultative anaerobe *Escherichia coli,* then by a flora of mostly anaerobic
127 bacteria (*36*). We hypothesized that these stages of succession would be associated with
128 changes in disease severity. As expected, we identified *Vc* as an indicator species of severe
129 dehydration. *Vc* was relatively more abundant in patients with severe dehydration, while two
130 *Bifidobacterium* species, *E. coli*, and ICP1 were indicators of mild dehydration (**Fig. 1A, Table**
131 **S3**). ICP3 was an indicator of severe dehydration despite being less frequently detected in our
132 study (28 samples with >0.1% ICP3 reads, compared to 61 samples with >0.1% ICP1). This
133 shows that different phages can have contrasting disease associations.



134
135 **Fig. 1. Dehydration severity is inversely associated with higher ICP1:*V. cholerae* ratios in stool**
136 **metagenomes**. (A) Relative abundances of phages and the seven most dominant bacterial species
137 identified with PCA (Fig. S5) in patients with severe, moderate, or mild dehydration; these conventions
138 equate to the World Health Organization (WHO) conventions of 'Severe', 'Some' and 'No' dehydration,
139 respectively. Significant indicator species for severe or mild dehydration are shown in red or blue bold
140 text, respectively (P<0.05 in a permutation test with 9999 iterations as implemented in the indicator
141 species function in R). See Table S3 for indicator species results applied to all 37 species selected in the
142 PCA dimensionality reduction (Fig. S5; Methods). (B) The ICP1:*Vc* ratio from metagenomics 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 out of 344 samples were included (*Vc*>0% of metagenomic reads), with 165 from severe, 128 from moderate, and 30 from mild cases. A pseudocount of one was added to the ratio before log transformation. For supporting analyses using qPCR data, see Fig. S4. 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 identified with PCA (Fig. S5) and explanatory variables: phages (ICP1, ICP2, ICP3), patient metadata: age in years, vomiting state (yes or no), dehydration status (severe, moderate or mild), the location where the sample was collected, and date of sampling; and antibiotic concentration (μg/ml) from quantitative mass spectrometry for azithromycin (AZI), ciprofloxacin (CIP) and doxycycline (DOX). Angles between variables (arrows) reflect their correlations; arrow length is proportional to effect size. Samples (points) are colored by dehydration severity. All displayed variables have a significant effect (*P*<0.05, permutation test) except for 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 Fig. S6 for these details). Color code in all panels: blue: mild dehydration, orange: moderate, and red: severe.

We focused on ICP1 for subsequent analyses given its prevalence. The distribution of ICP1 relative abundance was variable and less clearly associated with dehydration status than *Vc* (**Fig. S3**). Deeper investigation revealed that it was not simply the presence of phage that mattered, but the ratio of ICP1 to *Vc*. Higher ratios were inversely associated with dehydration severity (**Fig. 1B**); the same results were obtained using qPCR rather than metagenomics to quantify the ratio (**Fig. S4**). This simple ratio is therefore a potential biomarker of 'effective' phage predation that could be used in clinical, diagnostic, and epidemiological applications.

As a preliminary proof of concept, we tested the hypothesis that the ICP1:*Vc* ratio could be used to delineate the dehydration status. We used a bootstrapping method to identify an optimal ratio to differentiate between patients with dehydration (moderate or severe) versus patients without dehydration by WHO clinical measures (Methods). This step is clinically important because patients with moderate and severe dehydration ('positives' in this analysis) require rehydration treatment. The analyses yielded a threshold ratio of 0.18 (approximately 1 ICP1 to 5 *Vc*). At this threshold, the approach had a sensitivity of 85% (95%CI 80% to 89%) and positive predictive value (PPV) of 95% (95%CI 92-96%) to identify dehydrated patients; the specificity was 53% (95%CI 24% to 72%) and negative predictive value was 26% (95%CI 19% to 35%). The samples were distributed as 248 true positives, 16 true negatives, 14 false

177 positives, and 45 false negatives. Clinically, a high sensitivity is preferred over high specificity in
178 order to not 'miss' dehydrated patients; the high PPV gives justification to expend resources for
179 a fluid resuscitation. The results demonstrate the potential utility of the phage:bacteria ratio as a
180 biomarker to differentiate severity status and requires an independent study for validation and
181 further evaluation of the low NPV.

182 We next sought to identify potential interactions between ICP1 and temporal stages of
183 disease. Previously, ICP1 was found to be associated with early, rather than late stages of
184 disease, peaking on the first day of infection in cholera patients sampled over time (*36*). Given
185 that we collected one sample per patient at hospital admission, we were unable to determine
186 with certainty whether ICP1 suppresses *Vc* or whether it is a non-causal marker of late-stage
187 disease when patients are recovering. Despite this limitation, we recorded self-reported duration
188 of diarrhea, providing a proxy for disease progression. We found that higher relative
189 abundances of ICP1 were associated with mild dehydration at early stages of disease (duration
190 of diarrhea <72h) but not at later stages (**Fig. S3B and D**). We therefore favor a scenario in
191 which ICP1 suppresses *Vc* at early disease stages in a way that reduces disease severity.
192 However, further time series studies will be required to establish causality.

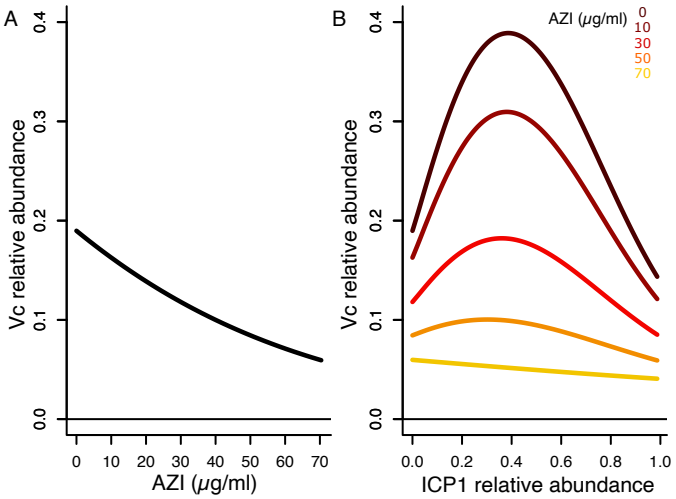
193 ***Antibiotics in stool are inversely associated with disease severity.*** To visualize the
194 complex relationships between disease severity, bacteria, and phages in the context of
195 antibiotic exposure, we used redundancy analysis (RDA; **Fig. 1C; Table S4**). For simplicity, the
196 seven most dominant bacterial species identified by principal component analysis were included
197 (**Fig. S5**). As explanatory variables, we visualized clinical data with strong effects, chosen by
198 forward selection and starting with phages and antibiotic concentrations (**Fig. S6**). In
199 accordance with the indicator species analysis (**Fig. 1A, Table S3**), higher *Vc* relative
200 abundance was positively correlated with severe dehydration (**Fig. 1C**). ICP1 was moderately
201 associated with *Vc*, consistent with a phage's reliance on its host for replication, but less
202 associated with severe dehydration. The antibiotics azithromycin (AZI) and to a lesser extent

203 ciprofloxacin (CIP) were negatively correlated with *Vc* and severe dehydration, suggesting their
204 role in suppressing cholera infection and disease. Supporting previous reports that AZI
205 suppresses *Vc* (*37*), AZI was most strongly anticorrelated with *Vc* in our cohort (**Fig. 1C**). We
206 did not identify annotated antibiotic resistance genes associated with AZI exposure (**Fig. S7**) at
207 established thresholds (**Table S5**. In contrast, CIP exposure was significantly associated with
208 the resistance genes *dfrA* and *aph6* (**Fig. S8),** which are both associated with *Vc* in our
209 metagenomes (**Fig. S9**) and have previously been linked with CIP resistance in *Vc (16, 38)*.
210 Taken together, these results suggest CIP exposure selects for resistance genes within
211 patients, potentially explaining why CIP may be less effective at suppressing *Vc* than AZI.
212 ***Azithromycin suppresses predator-prey dynamics.*** We next asked if and how antibiotics
213 interact with phages to suppress *Vc* within patients. To do so, we modeled the relationships
214 between ICP1, *Vc* and antibiotic exposure within each patient. We fit generalized additive
215 models (GAMs) of *Vc* (relative abundance from metagenomics or absolute abundance from
216 qPCR) as a function of ICP1, antibiotic concentrations, and their interaction, including
217 dehydration status as a random effect. We fit GAMs with all quantified antibiotics and their
218 interaction with ICP1, as well as separate models for each antibiotic, alone or in combination,
219 and compared them based on their Akaike Information Criterion (AIC; **Tables S6, S7**). The most
220 parsimonious model (with the lowest AIC), using either metagenomics or qPCR data, showed a
221 significant negative relationship between *Vc* and AZI (**Fig. 2A, S10**). This result is consistent
222 with the redundancy analysis (RDA) results (**Fig. 1C**) and with known patterns of *Vc*
223 suppression by AZI during infection (*37*). The relationship between *Vc* and ICP1 was quadratic
224 in both metagenomics- and qPCR-based models: at low ICP1 abundance, the relationship was
225 positive but became negative at higher ICP1 abundance (**Fig. 2B, S10**). This alternation
226 between positive and negative correlations is consistent with predator-prey dynamics within
227 infected patients (*39*). However, at higher concentrations of AZI, the quadratic relationship
228 flattened, effectively suppressing the phage-bacteria interaction, likely because AZI kept *Vc* at

229 low density. In the future, these interactions could be interrogated using patient time-series and

230 laboratory experiments challenging *Vc* with antibiotics and phages.

231



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233 **Fig. 2. Interactions between *V. cholerae*, phage ICP1, and azithromycin**. Generalized additive models
234 (GAM) results, fit with relative abundance of *Vc* as a function of antibiotic concentrations (μg/ml) and ICP1
235 relative abundance in 344 metagenomes. (A) *Vc* declines in relative abundance with higher abundance of
236 azithromycin (AZI) in μg/ml. (B) The relationship between ICP1 and *Vc* is affected by AZI concentration
237 (μg/ml); the illustrated AZI concentrations show regular intervals between the minimum (0 μg/ml) and
238 maximum (70 μg/ml) observed values. Both effects of AZI (A) and the ICP1-AZI interaction (B) are
239 significant (Chi-square test, $P<0.05$). For details on GAM outputs see Table S6. Relative abundances are
240 from metagenomics; see Fig. S10 for equivalent analyses using qPCR data.

241

242 ***Integrative and conjugative elements (ICEs) are associated with phage suppression.*** The

243 ICE is a region of the *Vc* genome that encodes resistance to both antibiotics and phages (*13*).

244 ICEs have conserved 'core' genes along with variable 'hotspots' encoding different genes; for

245 example, hotspot 5 is a ~17kb region associated with phage resistance. At the time of our

246 sampling, ICEVchInd5 (abbreviated here as *ind5*) and ICEVchInd6 (*ind6*) were the two most

247 prevalent ICE types in Bangladesh (*17*). These ICEs differ in their anti-phage systems: *ind5*

248 encodes a type 1 bacteriophage exclusion (BREX) system while *ind6* encodes several other

249 different restriction-modification systems (*17*).

250 We screened for ICEs in metagenomes by mapping reads against reference sequences

251 for *ind5* (NCBI accession GQ463142.1) and *ind6* (accession MK165649.1). An ICE was defined
252 as present when 90% of its length was covered by at least one metagenomic read (**Fig. S11A**).
253 We found that 64% (144/224) of samples with *Vc*>0.5% or ICP phages >0.1% of metagenomic
254 reads contained *ind5*, 12% (26/224) contained *ind6*, and 24% (54/224) had no detectable ICE.
255 The lack of ICE detections was not due to the lack of *Vc* in a metagenome because ICE-
256 negative samples did not contain fewer *Vc* reads (**Fig. S11B**).

257 Resistance mechanisms on ICEs have been shown to suppress phage *in vitro (17)*, but
258 their relevance within human infection remains unclear. We found that metagenomes without a
259 detectable ICE (denoted as ICE-) were associated with higher phage:*Vc* ratios (**Fig. 3**). The
260 effect was strongest for ICP1, which had the largest sample size (**Fig. 3A**). This observation is
261 consistent with ICE-encoded mechanisms suppressing phage within patients. Higher ICP1:*Vc*
262 ratios, which occurred more often in ICE- patients, were also associated with mild dehydration
263 (**Fig. 3B**). ICP1 is more strongly suppressed by *ind5* than by *ind6* (**Fig. 3**), while ICP3 appears
264 to be better suppressed by *ind6* than *ind5*, albeit with borderline statistical significance **(Fig.**
265 **S12**). We next compared ratios by phage resistance genotype (*ind5, ind6* vs ICE-) and
266 dehydration status. For patients with mild dehydration, we observed lower ICP1:*Vc* ratios in *ind5*
267 compared to ICE- samples (Kruskall-Wallis test with Dunn's post-hoc correction, $p = 0.022$).
268 Despite this difference, some patients with mild disease and *ind5* still had non-zero ICP1:*Vc*
269 ratios (**Fig. 3B**), indicating the ICP1 is imperfectly suppressed by *ind5*. In the severe group, *ind5*
270 patients also had lower ICP1:*Vc* ratios than ICE- patients (Dunn's post-hoc test with BH
271 correction, P=0.0035). In the moderate group, patients carrying *ind5* had lower ICP1:*Vc* ratios
272 compared to patients with *ind6* (Dunn's post-hoc test with BH correction, P=0.048), consistent
273 with *ind5* more effectively suppressing ICP1. The same associations were evident using qPCR-
274 based quantification of phage:*Vc* ratios (**Fig. S13**). Together, these results implicate ICEs in
275 phage resistance during human infections, complementing and generally confirming the

predictions of earlier laboratory experiments (*17*). That said, the suppression is not complete, and further experiments are needed to dissect the underlying causal relationships.

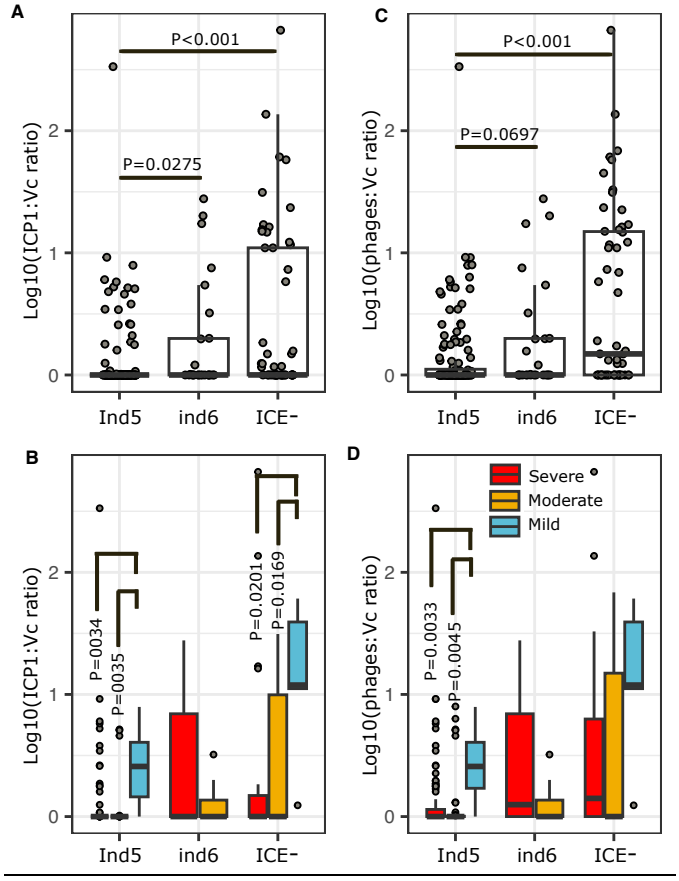

Fig. 3. Integrative conjugative elements (ICEs) are associated with lower ICP1:*V. cholerae* ratios in patient metagenomes. (A) Distribution of ICP1:*Vc* ratios across patients with different ICE profiles. (B) The same data as (A) binned into boxplots according to dehydration status: mild (blue), moderate (orange) and severe (red). (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 appreciable *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*+. 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. Relative abundances are from metagenomics; see Fig. S13 for supporting analyses using qPCR data.

Hypermutation generates V. cholerae genetic diversity. In addition to variation in gene content in ICEs and other mobile elements, we hypothesized that resistance to phages and antibiotics could be conferred by point mutations (single nucleotide variants; SNVs) that existed before or emerged *de novo* during infection. Although we cannot fully exclude mixed infections

295 by different *Vc* strains as a source of within-patient diversity, we found no evidence for more
296 than one strain co-infecting a patient in our study population (**Fig. S14**). We previously found a
297 low level of *Vc* genetic diversity within individual cholera patients (*40*) – on the order of 0-3
298 detectable SNVs – with the exception of hypermutation events characterized by DNA repair
299 defects and dozens of SNVs in the *Vc* genome, primarily transversion mutations (*41*).
300 Hypermutation generates deleterious mutations, but may also rapidly confer phage resistance,
301 as shown experimentally with *Pseudomonas fluorescens (18)*. Here, we were able to better
302 quantify the frequency of hypermutators in a larger sample size, and test if within-host *Vc*
303 diversity is associated with phage or antibiotic exposure – both of which could potentially select
304 for resistance mutations to each factor.

305 To identify hypermutators in metagenomes, we tallied *Vc* populations with defects
306 (nonsynonymous mutations) in any of 65 previously defined DNA repair genes (*42*) or with a
307 relatively high number of SNVs (25 or more) (*41*). We used InStrain (*43*) to quantify *Vc* within-
308 host diversity in 133 samples passing stringent sequencing quality filters (Methods) and found
309 that 35% of samples (47/133) had both a high SNV count and nonsynonymous mutations in
310 DNA repair genes – making them likely to contain hypermutators. Higher SNV counts were
311 significantly associated with DNA repair defects (Fisher's exact test, P<2.2e-16), consistent with
312 these defects yielding higher mutation rates within patients. The number of SNVs was not
313 confounded by *Vc* genome coverage (**Fig. S15A**). Consistent with our previous study (*41*),
314 putative hypermutators had a distinct mutational profile enriched in transversions (**Fig. S15B,C**).
315 For subsequent analysis, we considered all SNVs together, regardless of whether they were
316 generated by hypermutation.

317 ***Phages, not antibiotics, are associated with* Vc *within-host diversity.*** We hypothesized that
318 *Vc* within-host diversity would be shaped by potential selective pressures, namely phages or
319 antibiotics within patients. To test this hypothesis, we fit generalized linear mixed models

320 (GLMMs) with phages and antibiotics as predictors of the number of high-frequency
321 nonsynonymous (NS) SNVs in the *Vc* population within a patient. We focused on higher-
322 frequency SNVs (>10% within a sample) as likely beneficial mutations (unlikely to rise to such
323 high frequency by chance if neutral or deleterious) and on NS SNVs as more likely to have
324 fitness effects. We fit several models with different combinations of predictors: a model with all
325 antibiotics and their interaction with ICP1 and separate models with each antibiotic and its
326 interaction with ICP1. We added *Vc* abundance as a fixed effect to the model to control for any
327 coverage bias in SNV calling (**Table S8**). The most parsimonious model included *Vc* abundance
328 and the interaction between *Vc* and ICP1 as predictors of the number of high-frequency NS
329 SNVs. Adding antibiotics, or their interaction with ICP1, did not improve the model (**Table S8**),
330 suggesting a limited role for antibiotics in selecting for *Vc* point mutations within patients.

331 In the model, *Vc* relative abundance and the interaction between *Vc* and ICP1 both had
332 significant effects (GLMM, Wald test, P=0.00246 and P=0.00494 respectively). The negative
333 relationship between *Vc* and the number of high-frequency NS SNVs (**Fig. 4A**) was not easily
334 explained by sequencing coverage, since the total number of SNVs is not associated with *Vc*
335 relative abundance (**Fig. S15A**). The number of high-frequency NS SNVs rose with increasing
336 ICP1 – but only when *Vc* abundance was relatively high (**Fig. 4A**). As a control, we ran the
337 same GLMM on NS SNVs without a minimum frequency cutoff and found no significant effects,
338 suggesting that the interaction between ICP1 and *Vc* on SNV count is specific to high-frequency
339 mutations, rather than low-frequency mutations that are more likely selectively neutral or
340 deleterious. These data support a scenario in which ICP1 selects for NS SNVs when the *Vc*
341 population is large enough to respond efficiently to selection – for example, at the beginning of
342 an infection.

343 If phages select for beneficial mutations, we expect these mutations to increase in
344 frequency at higher intensity of phage predation. We lack time-series data from individual
345 patients, but the relative abundance of phage provides a proxy for the combined effects of the

strength and duration of phage selection. To test this hypothesis, we fit a GAM with the average within-sample frequency of SNVs as a function of ICP1, antibiotics, and their interactions. We included the fixed effect of ICE (*ind5*, *ind6,* or ICE-) as another factor that could provide phage or antibiotic resistance, as well as mutation type to differentiate among non-synonymous (NS), synonymous (S), and intergenic (I) mutations. We fit GAMs with all antibiotics and their interaction with ICP1, as well as models with or without each antibiotic separately (**Table S9**). The most parsimonious model included the interaction between ICP1, ICE and mutation type, but not antibiotics. ICP1 was a strong predictor of higher frequency NS SNVs in the absence of a detectable ICE (**Fig. 4B**). Samples in this analysis were unambiguous in terms of their ICE presence/absence patterns (**Fig. S16**).

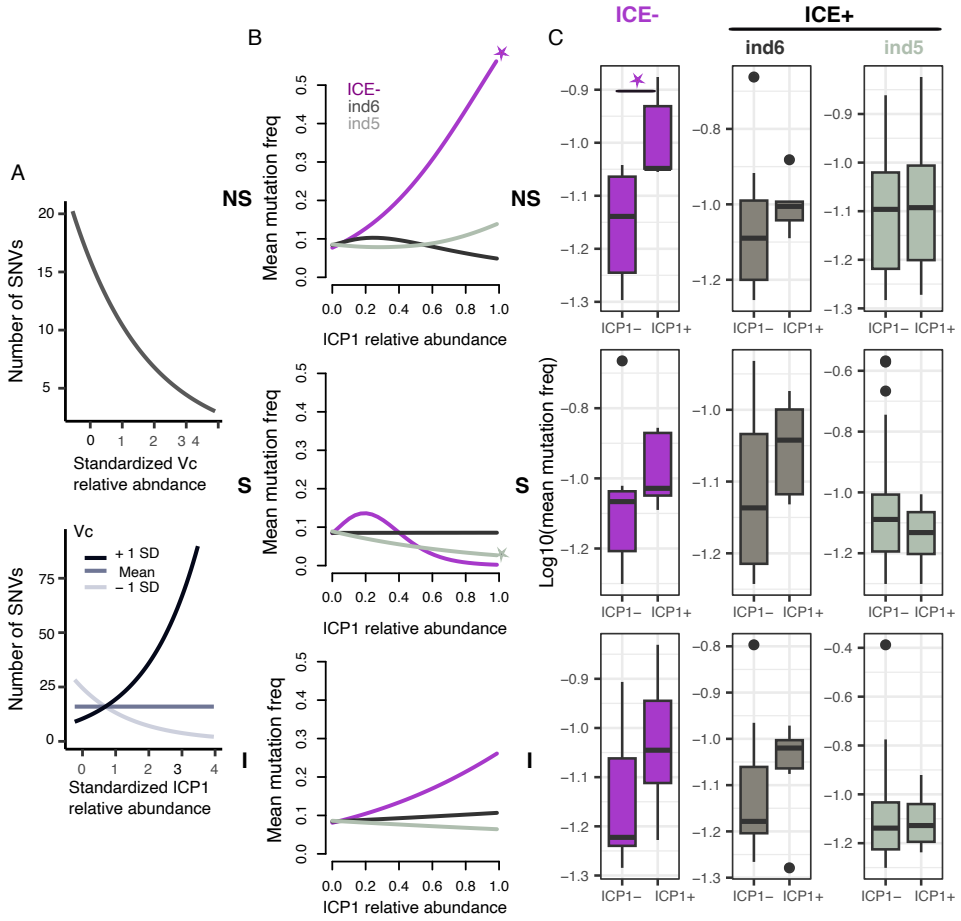

Fig. 4. ICP1 selects for non-synonymous point mutations in the *V. cholerae* genome in the absence of ICE. (A) Results of a GLMM modeling high frequency nonsynonymous SNV counts as a

359 function of *Vc* and ICP1 standardized relative abundances. In the bottom panel, shades of gray indicate
360 *Vc* relative abundance at the mean or +/- 1 standard deviation (SD) across samples. Both *Vc* and the
361 interaction between *Vc* and ICP1 have significant effects (Wald test, $P<0.05$), the model was fit using 68
362 samples in which InStrain identified NS mutations at frequency > 10%. (B) GAM results with the mean
363 mutation frequency as a function of the interaction between ICP1, ICE and mutation type (non-
364 synonymous; NS, synonymous; S, or intergenic; I). Significant effects are shown with a star (Chi-square
365 test, $P<0.05$). The model was fit using 130 samples that passed the post-InStrain filter for SNV quality
366 (Methods). (C) Boxplots of mutation frequency in the presence or absence of ICP1 and/or ICEs. The only
367 significant comparison is indicated with a star (Wilcoxon test, $P=0.0094$). Boxplots include 130 samples,
368 of which 32 are ICP1+ (ICP1>=0.1% of reads) and 98 are ICP- (ICP1<0.1% of reads). The solid
369 horizontal line is the median and the boxed area is the interquartile range. For supporting analysis using
370 qPCR data, see Fig. S17.
371

372 To confirm and visualize this model prediction, we compared the distribution of the
373 average frequency of NS SNVs between ICP1-positive and ICP1-negative samples. Consistent
374 with the model, NS SNV frequency was significantly higher in ICP1-positive samples when the
375 ICE was not detected (Wilcoxon test, $P=0.0094$) making this SNV category likely to contain
376 targets of selection by ICP1 predation (**Fig. 4C**). Qualitatively similar results were found when
377 ICP1 was quantified by qPCR instead of metagenomics (**Fig. S17**). Together, the results
378 suggest that, in the absence of detectable ICE-encoded phage resistance, ICP1 selects for
379 nonsynonymous point mutations instead. We identified several *Vc* genes, including some with
380 membrane or virulence-related functions, that were mutated at higher ICP1:*Vc* ratios (**Table**
381 **S10**); these provide candidate phage resistance mechanisms that can be explored in future
382 experiments. In contrast, the secreted hemolysin gene, *hlyA,* that we previously observed to be
383 mutated more often than expected within cholera patients (*41*) was among the genes most
384 frequently mutated in patients with relatively low ICP1:*Vc* ratios (**Table S11**). This suggests that
385 *hlyA* sequence evolution may be affected directly or indirectly by phage predation, through
386 mechanisms that remain unclear.

387 As *Vc* evolves as a function of ICP1, we expect ICP1 evolution to be impacted by *Vc.*
388 Specifically, we hypothesized that ICP1 may evolve to infect *Vc* in the presence of *ind5*,
389 explaining some of the variation in both ICP1:*Vc* ratios and disease severity (**Fig. 3**). Despite
390 the generally low genetic diversity of ICP1 (*21)*, we were able to quantify SNVs in the ICP1

genome in 45 samples. This sample size was too low to fit sophisticated models, but simple correlations allowed us to draw tentative conclusions. First, we ruled out sequencing coverage as source of bias in SNV calling (Spearman correlation between ICP1 relative abundance and number of SNVs, $p > 0.1$ for all SNV categories). Next, we observed a negative correlation between the ICP1:*Vc* ratio and the number of NS SNVs in the ICP1 genome – a correlation that is only significant when *Vc* encodes an *ind5* ICE (**Fig. 5A, S18**). This is consistent with our observation that *ind5* is associated with lower ICP1:*Vc* ratios in our cohort (**Fig. 3**), potentially suppressing ICP1 and applying selection to escape suppression via NS mutations. Some of these NS mutations may be beneficial to the phage, rising to high frequency along with ICP1 – which is indeed what we observe: the mean frequency of NS SNVs in ICP1 increases with the ICP1:*Vc* ratio, but only in the presence of *ind5* (**Fig. 5B, S19**). Several ICP1 genes, mostly hypothetical proteins, repeatedly acquired NS mutations in the presence of *ind5*, providing candidate escape mutations to test in future work (**Table S12**).

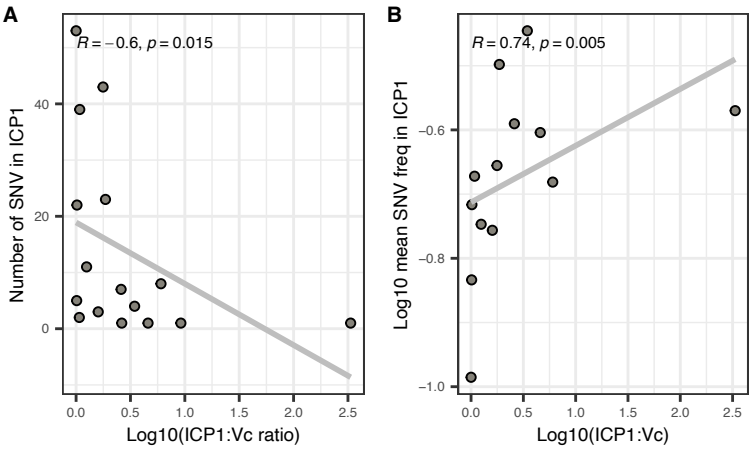

Fig. 5. ICP1 evolution in samples containing ICE *ind5*. (A) The number of nonsynonymous (NS) SNVs detected in the ICP1 genome is negatively correlated with the ICP1:*Vc* ratio in the presence of *ind5*. (B) The mean frequency of NS SNVs in the ICP1 genome is positively correlated with the ICP1:*Vc* ratio in the presence of *ind5.* The X-axes were log10 transformed after adding one to the ratios. The Spearman correlation coefficients and *p*-values are shown. See Figures S18 and S19 for equivalent plots in ICE- and *ind6* samples, and for synonymous and intergenic SNVs.

412 **Discussion**

413 The tripartite interactions between pathogens, phages, and antibiotics have been studied in the
414 laboratory, *in silico* with mathematical models, and to a lesser extent in the field, but how these
415 factors interact during human infection remains an open question. Our objective was to
416 characterize these interactions in the context of cholera. We analyzed more than 300 stool
417 metagenomes from cholera patients enrolled at hospital admission across Bangladesh during
418 an entire outbreak season. We found that high predator (ICP1) to prey (*Vc*) ratios were inversely
419 associated with disease severity and provide a proof of concept for translational applications.
420 We demonstrated how *Vc* and ICP1 interact within patients, with ICP1 selecting for potential
421 phage resistance point mutations in the absence of ICE-encoded anti-phage defenses, and *Vc*
422 selecting for point mutations in the phage genome in the presence of *ind5*. This apparent
423 coevolution between predator and prey likely has longer-term consequences for cholera
424 infection and transmission. Antibiotics, particularly azithromycin, also played a role in
425 suppressing *Vc* and could mask phage-bacteria interactions. Ciprofloxacin was associated with
426 known antibiotic resistance genes, but we found no evidence that antibiotics select, as ICP1
427 does, for high-frequency nonsynonymous point mutations. Thus, although resistance
428 mechanisms to certain phages and antibiotics colocalize to the ICE (*17),* they impose distinct
429 selective pressures that could be exploited to improve the efficacy of antibiotics by combining
430 them with phage therapy.

431 Our study has several limitations. First, samples were collected at a single time point at
432 hospital admission which allowed us to establish statistical correlations, but we cannot infer
433 causality in the absence of time-series or interventional clinical studies. Second, our cohort
434 allowed us to study the interaction between *Vc* and ICP1, but the sample size for ICP2 and
435 ICP3 was insufficient for most statistical analyses. Third, we prioritized common antibiotics for
436 mass spectrometry, but we cannot exclude a role for other unmeasured antibiotics. Fourth, due
437 to logistical limitations, we extracted DNA from a bacterial pellet plus a small amount of

supernatant from each sample. This allowed us to capture both intracellular and cell-bound phages, along with free phage particles, but sequencing each fraction separately could yield further insights into distinct phage populations. From a clinical perspective, the measurement of dehydration status was categorical and could be improved in future studies by incorporating digital tools to quantify the degree of dehydration (*44-46*). Finally, our study lacked information about host genetics or immunity, which also influence disease severity (*8, 47*). Future studies combining rich patient metadata, time-series design, long-read metagenomics, and isolate genome sequencing will complement and build upon these findings.

CONCLUSION

We propose that an index of effective phage predation, quantified as the phage:bacteria ratio, might be used as a tool for physicians to assess disease severity, and potentially prognosticate a disease course. We show here that this ratio is associated with cholera disease severity, but its predictive value should be studied in larger cohorts sampled over the course of infection. Whether this biomarker can be generalized to phages other than ICP1 and diseases other than cholera, and whether the association with disease severity changes as predator and prey coevolve over time, remains unclear. The potential of phage therapy and prophylaxis has long been recognized, and a combination of ICP1, 2, and 3 prevents cholera in animal models (*35*). However, just as hypermutators can drive the evolution of resistance to combinations of antibiotics (*48*), they may also help pathogen populations to survive combinations of phages, while increasing their potential to evolve resistance to future antimicrobial treatments. Phage therapy cocktails will therefore need to be updated regularly to remain effective against currently circulating coevolved bacteria, and creative new strategies are needed to minimize the unwanted evolution of phage, and possibly antibiotic, resistance.

463 **Materials and Methods Summary**

464 **Ethics Statement**. The samples analyzed were collected within two previously published IRB
465 approved clinical studies in Bangladesh: (i) The mHealth Diarrhea Management (mHDM) cluster
466 randomized controlled trial (IEDCR IRB/2017/10; icddr,b ERC/RRC PR-17036; University of
467 Florida IRB 201601762) (*46*). (ii) The National Cholera Surveillance (NCS) study (icddr,b
468 ERC/RRC PR-15127) (*49*); See supplementary materials for further details.

469 **Study Design.** The study design was a prospective longitudinal study of patients presenting
470 with diarrheal disease at five Bangladesh Ministry of Health and Family Welfare district hospitals
471 (both mHDM and NCS sites) and two centralized NCS hospitals (BITID; icddr,b) from March
472 2018 to December 2018. Sites were distributed geographically nation-wide (*50*). See
473 supplementary materials.

474 **Sample collection.** Stool samples were collected at hospital admission. Aliquots for transport
475 and subsequent culture were stabbed into Cary-Blair transport media; aliquots for molecular
476 analysis were preserved in RNAlater (Invitrogen). See supplementary materials.

477 **Microbiological and molecular analysis.** Culture was performed via standard methods (*51*);
478 total nucleic acid (tNA) was extracted from the RNAlater preserved samples using standard
479 methods. Criteria for subset subsequent shotgun metagenomic sequencing were: (i) culture
480 positivity, (ii) phage (ICP1,2,3) detection by PCR among culture-negative samples, or (iii) *Vc*
481 detection by PCR among a random 10% of culture-negative and phage (ICP1,2,3) negative
482 samples. Sequencing libraries were prepared using the NEB Ultra II shotgun kit and sequenced
483 on Illumina NovaSeq 6000 S4, pooling 96 samples per lane, yielding a mean of >30 million
484 paired-end 150bp reads per sample. Among samples identified for metagenomic analysis,
485 qPCR was performed to determine absolute abundances of *Vc*, ICP1, ICP2, and ICP3.

486 **Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS).** Those
487 cholera samples identified for metagenomic analysis were analyzed by qualitative and

488 quantitative LC-MS/MS for antibiotics. Based on prior research (*16, 37*), the target list prioritized
489 5 common antibiotics: ciprofloxacin, doxycycline/tetracycline, and azithromycin were analyzed
490 quantitatively, and metronidazole and nalidixic acid were analyzed qualitatively. Standard
491 curves were made for each quantitative target by preparing a dilution series of the three native
492 and isotopic forms of the quantitative targets; clinical samples were spiked with the isotopes of
493 the quantitative targets as internal references. See supplementary materials.
494 **Metagenomic data analysis.** We taxonomically classified short reads using Kraken2 (*52*) and
495 Bracken v.2.5 (*53*). Reads were assembled using MEGAHIT v.1.2.9 (*50*) and binned with DAS
496 tool (*54*). We inferred probable phage contigs using geNomad v1.7.0 (*55*) and predicted their
497 likely bacterial hosts with iPhoP v1.3.1 (*56*). To characterize intra-patient *Vc* diversity, we used
498 StrainGE (*57*) and InStrain v.1.5.7 (*43*). To identify antibiotic resistance genes in metagenomes,
499 we used deepARG v 1.0.2 (*58*). See supplementary materials for details.
500 **Statistical analyses.** Statistics and visualizations were done in R version 3.6.3 and R studio
501 version 1.2.5042. See supplementary materials for details.

502 **Competing interests:** Authors declare that they have no competing interests.

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

505 **Code availability.** Computer code needed to reproduce figures and results in this study is
506 available on Github at https://github.com/Naima16/Cholera-phage-antibiotics. DOI:
507 10.5281/zenodo.10573867 (77).

508

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703 **Acknowledgements**: We thank the patients for participating in this study as well as the clinical
704 and laboratory teams who collected the samples. We are grateful to S. Flora and colleagues at
705 the Institute of Epidemiology, Disease Control and Research (IEDCR), Ministry of Health and
706 Family Welfare, Government of Bangladesh who collaborated on the original clinical studies in
707 which the samples analyzed herein were collected. We are also grateful to R. Autrey, B.
708 Johnson and K. Berquist for their administrative expertise at the University of Florida. AIK and
709 FQ were the principal investigators (PI) in Bangladesh and PIs of the ERC/RRC approvals at
710 the icddr,b. EJN was the PI and obtained IRB approval at the University of Florida. Associated
711 protocol numbers/registrations are: IEDCR IRB/2017/10; icddr,b ERC/RRC PR-17036;
712 University of Florida IRB 201601762; clinicaltrials.gov NCT03154229. This collective research

713 infrastructure and support was invaluable to the success of this study. We thank members of the
714 Nelson, Khan, Qadri and Shapiro labs, and Frédérique Le Roux, for discussions that improved
715 the manuscript.

716 **Funding:** This work was supported by the National Institutes of Health grants to EJN
717 [R21TW010182] and KBB [S10 OD021758-01A1] and internal support from the Emerging
718 Pathogens Institute at the University of Florida and the Departments of Pediatrics/ Children's
719 Miracle Network (Florida). BJS and NM were supported by a Canadian Institutes for Health
720 Research Project Grant. AC was supported by a Postdoctoral Mobility Fellowship of the Swiss
721 National Science Foundation [P500PB_214356]. The funders had no role in study design, data
722 collection and analysis, decision to publish, or preparation of the manuscript.

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737

Supplementary materials:

Materials and methods

Figures: S1 to S19

Tables: S1 to S12

Data Files S1 to S5

References 59-77