Anaerobic conditions unmask antimicrobial resistance

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Abstract

Antimicrobial resistance (AMR) in Gram negative enteropathogens is an urgent threat to the antibiotic formulary. These taxa undergo anaerobic respiration within the host, yet little is known about how anaerobic conditions influence antimicrobial resistance (AMR). The facultative enteropathogen *Vibrio cholerae* was chosen as a model to determine the impact of anaerobic growth on AMR because cholera is one of the few non-invasive diarrhoeal diseases for which antibiotics are indicated, albeit conditionally. *V. cholerae* isolates from a single outbreak were tested for resistance by minimal inhibitory concentrations (MIC) under aerobic and anaerobic conditions against clinically relevant antibiotics. Here we show that the odds of classifying isolates as resistant under anaerobic compared to aerobic conditions increased over 20 times for ciprofloxacin and 50 times for azithromycin, yet for doxycycline, all isolates remained below the breakpoint for resistance. Genome-wide association studies (GWAS) found significant associations between known and unknown genetic elements and AMR phenotypes that varied by oxygen exposure and antibiotic concentrations. In most cases, AMR phenotypes were more heritable – and more genes significantly associated with AMR were discovered – under anaerobic conditions compared to aerobic conditions. These findings challenge the paradigm of testing facultative enteropathogens for AMR under aerobic conditions alone. This experimental approach establishes a new, more sensitive framework to track and investigate mechanisms of AMR.

INTRODUCTION

Antimicrobial resistance (AMR) in Gram negative pathogens arises by point mutation(s) and AMR gene acquisition and is selected by high rates of inappropriate antibiotic use. Fast and accurate approaches to identify AMR are needed to gauge the extent to which the antibiotic formulary is threatened. However, knowledge gaps remain on the factors and mechanisms that result in AMR. There are also debates surrounding how well conventional *in vitro* assays represent *in vivo* physiology. For example, facultative anaerobic pathogens experience hypoxia and anoxia within the gastrointestinal tract[1,2], yet standard *in vitro* assays test for AMR under aerobic conditions alone[3]. How a factor as fundamental as oxygen exposure effects AMR is poorly understood. To investigate this question, we chose the facultative anaerobe *Vibrio cholerae* as a model system. In *V. cholerae* mechanisms of AMR and anaerobic growth are well characterized *in vivo* and *in vitro*[4-11], and cholera is one of the few non-invasive diarrhoeal diseases for which antibiotics are indicated, albeit conditionally.

Rehydration is the definitive intervention for acute diarrhoeal disease[12]. Antibiotics are supportive and indicated for only a few diarrhoeal diseases, including cholera[13,14]. The World Health Organization (WHO) recommends ciprofloxacin, azithromycin or doxycycline for cholera patients with severe dehydration[12,14-16]. Antibiotics shorten the frequency and duration of diarrhoea[16]. In practice, guideline adherence in cholera endemic regions may be low out of clinical concern that a patient ‘might have cholera’ and that the patient may develop severe dehydration, contributing to rates of inappropriate antibiotic usage that can rise above 90%[17,18]. Strong regional associations between antibiotic use and rise of AMR have been observed across enteric taxa,

44 including *V. cholerae*[19-21]. Given that AMR genetic elements frequently co-localize on
45 mobile elements[22], inappropriate single-agent therapy poses a risk of multidrug-
46 resistance (MDR) selection.

47 Associations between AMR phenotypes and genotypes are well known for the three
48 antibiotics recommended to treat cholera; the cognate AMR mechanisms share
49 commonality across Gram negative taxa. Ciprofloxacin (a fluoroquinolone) resistance
50 mechanisms include mutations in genes encoding type II topoisomerases (e.g. *gyrA,*
51 *gyrB, parC, parE*)[23,24]. Synergy occurs such that mutations in *gyrA* and *parC* increase
52 ciprofloxacin resistance 60-fold, but the individual effects are 10- to 16-fold and 0-fold,
53 respectively[23,25-28]. Efflux pump upregulation, porin loss and Qnr expression also convey
54 fluoroquinolone resistance[23,24]. Resistance can increase over 30-fold when strains
55 harbor *qnr* family genes[23,29,30]. In *V. cholerae*, $qnr_{V.cholerae}$ resides on an integrative and
56 conjugative element (ICE; 'SXT' in *V. cholerae*)[22,31-34]. Azithromycin (a macrolide)
57 resistance mechanisms are similarly diverse and include mutations in the 23S ribosomal
58 RNA (rRNA) target genes and ribosomal protein genes. Macrolide resistance is also
59 conveyed by carriage of rRNA methyltransferase genes (*erm*) and associated induction
60 mechanisms, *cis*-acting peptides, efflux systems (e.g. *mef, msr)*, macrolide esterases
61 (e.g. *ere*), and macrolide phosphotransferases (e.g. *mphA,* which often resides in the *V.*
62 *cholerae* SXT element)[35]. Doxycycline (a tetracycline) resistance is conferred by
63 mutations in the 16S rRNA component of the 30S ribosomal subunit[36]. Additional
64 mechanisms include tetracycline-specific ribosomal protection proteins (RPPs),
65 tetracycline specific efflux pumps (e.g. *tet(59)*) that can reside on SXT element in *V.*

66 *cholerae*, intrinsic efflux pumps, AraC-transcriptional activators, and cytoplasmic ATP-
67 dependent serine proteases[36].

68 Complex associations between AMR phenotypes and genotypes have been studied by
69 random mutagenesis, phenotypic screening, and network analyses[37-42], and applied in
70 *V. cholerae*[43]. These studies uncovered how the effect of an antibiotic is shaped by a
71 large number of often more subtle physiologic perturbations, including altered DNA
72 synthesis/repair, central metabolism/growth, and SOS response[44-46]. A limitation of AMR
73 assays conducted under aerobic conditions alone is that they may not reflect these
74 physiologic perturbations experienced in the host. Aerobic oxidative phosphorylation
75 generates reactive oxygen species (ROS) that are lethal unless a sufficient defense is
76 mounted by factors like superoxide dismutase, catalase, and glutathione systems[47,48].
77 Under anaerobic conditions, growth rate typically slows and proton motive force is
78 reduced[49,50], which can have both synergistic and antagonistic effects on antibiotics
79 [46,51]. In *Escherichia coli,* ROS are generated after fluroquinolone (bactericidal) treatment
80 under aerobic conditions[52] and fluoroquinolone tolerance increases under anaerobic
81 conditions[53-55]. The extent to which tetracyclines and macrolides (both bacteriostatic)
82 induce ROS and how anaerobiosis influences tolerance is less known[44,56,57].

83 The objective of this study was to compare AMR phenotypes with underlying genotypes
84 under aerobic and anaerobic conditions. We hypothesized that rates of AMR would
85 differ between aerobic and anaerobic conditions, and that distinct genetic elements
86 would be associated with phenotypes under the different conditions. To test these two
87 hypotheses, *V. cholerae* isolates were collected from cholera patients[58]. We found that
88 phenotypic resistance to ciprofloxacin and azithromycin, but not to doxycycline,

89 increased significantly under anaerobic conditions. Testing under anaerobic conditions
90 also allowed us to discover novel genes associated with AMR. Taken together, our
91 results show how the genetic basis of AMR differs between aerobic and anaerobic
92 conditions, with implications for AMR surveillance in public health, research and clinical
93 settings.

94 **METHODS**

95 **Clinical sample collection.** The clinical samples in this study were collected as part of
96 a previously published IRB approved study [58]. Recruitment, consent, enrollment and
97 procedures have been described previously[58]. In brief, stool samples were obtained
98 during the spring cholera outbreak period of 2006 at the International Centre for
99 Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh. Samples
100 were collected prior to hospital administration of antibiotics; patient histories were
101 negative for known antibiotic exposure. The library consists of 67 *V. cholerae* isolates
102 (Supplementary Table 1). Paired stool supernatant was available for 50 isolates.

103 **Antimicrobial resistance testing.** Growth kinetics and MIC determinations for
104 ciprofloxacin, azithromycin, and doxycycline were performed in LB broth with twelve
105 two-fold serial dilutions with concentrations approximated for CLSI MIC breakpoints[3] for
106 *V. cholerae* (ciprofloxacin = 2 μg/ml; azithromycin = 4 μg/ml; doxycycline = 16 μg/ml)[3].
107 Isolates were prepared and grown aerobically at 37˚C in 15-ml tubes containing 5-ml LB
108 broth at 220 r.p.m. Bacteria were back-diluted to a final OD_{600} of 0.01 (200 μl/well) in LB
109 with or without the respective antibiotic dilution-series in black Corning CoStar clear-
110 bottom 96-well plates. Plates were placed in a BioTek Synergy H1 reader pre-warmed

111 to 37˚C with the lid on. Anaerobic conditions were generated using a continuous
112 chamber flow (5% CO_2, 95% N_2) and a BioTek CO_2/O_2 gas controller; anaerobic growth
113 plates were given a 10-minute equilibration period. OD_{600} was measured every 2
114 minutes for 8 hours at 37˚C with orbital shaking at 220 r.p.m. A standard logistic
115 equation was fit to growth curve data using the R package *growthcurver* version 0.3.0[59].
116 Outcome measures were generation time, carrying capacity (k), and area under the
117 curve (AUC). The MIC was determined using a logistic fit for growth over the twelve,
118 two-fold serial dilutions of the test antibiotic. Binary phenotypic resistance categories
119 were set in concordance with CLSI standards. Intermediate and resistant isolates were
120 scored as resistant.

121 **Use of catalase to determine if ROSs contribute to antibiotic sensitivity.** To test if
122 the reduction of reactive oxygen species (ROSs) was associated with increased
123 tolerance to antibiotics under aerobic conditions, MICs were determined for two select
124 strains (E7946, EN160) with/without catalase added to the media (10 U/ml). Catalase
125 quenches H_2O_2 that freely diffuses across the cell envelope. Controls consisted of
126 adding 10 mM H_2O_2 to media with catalase, bacteria, or catalase and bacteria. The
127 catalase and H_2O_2 concentrations were chosen based on optimization experiments in
128 which *V. cholerae* were cultured with a gradient of catalase (0 ,0.001, 0.01, 0.1, 1, 10
129 U/ml) and a gradient H_2O_2 (0, 0.1, 1, 10 mM). Growth curves were performed with
130 viable counts as endpoints to determine the minimum dose for lethality by H_2O_2 or
131 protection by catalase (data not shown).

132 **Whole-genome sequencing.** Genomic DNA was extracted from *V. cholerae* isolates
133 using the Qiagen DNeasy Blood and Tissue Kit. Library construction was completed

134 using the Illumina Nextera XT v.2 DNA Library Preparation Kit. Twenty-four genomes
135 were pooled and sequenced on an Illumina MiSeq for 500 cycles per run. Using CLC
136 Genomics Workbench v20, raw reads were filtered by length, trimmed, and mapped to
137 the reference genome (*V. cholerae* O1 El Tor E7946) to identify single-nucleotide
138 variants. To identify genes not present in the reference genome, contigs were
139 assembled *de novo* using CLC Genomics Workbench v20. Known resistance genes
140 were identified using the comprehensive antibiotic resistance database (CARD)[60,61] and
141 ResFinder[62,63].

142 **Genome-wide association studies.** A series of genome-wide association studies
143 (GWAS) were conducted to detect genomic variants associated with antibiotic
144 resistance phenotypes under both aerobic and anaerobic conditions; a total of 30
145 GWAS were generated by 3 antibiotics x 5 antibiotic concentrations x 2 oxygen
146 conditions. The sample size for analysis was 67 genomes. To extract genomic variants,
147 which can potentially capture all sources of variation in the genome (i.e single
148 nucleotide variants, indels and gene presence/absence) without *a priori* assumption
149 about the underlying gene content of each sample (e.g. accessory genes or plasmids),
150 unitigs were generated from genomes assembled using GATB[64]. Unitigs are sequences
151 of variable length (unlike k-mers of fixed length *k*) which represent the variations in the
152 population of genomes under study in high-resolution. GWAS were performed using
153 linear mixed models implemented in pyseer v.1.3.6 and adjusted for population
154 stratification using the kinship matrix estimated from the phylogenetic tree[65].

155 To generate the phylogenetic tree, genome alignments consisting entirely of variable
156 nucleotides were produced from whole genome SNP data generated by CLC Genomics

157 Workbench v20 using VCF-kit 0.1.6[66]. The tree was then inferred by RaxML under the
158 general time reversible (GTR) model with rate variation across sites following a GAMMA
159 distribution[67]. We used the linear-mixed model approach to adjust for population
160 stratification and linkage disequilibrium in microbial GWAS[68]. Heritability (h^2), an
161 estimate of the proportion of the phenotype variance that can be explained by total
162 genomic variation represented in the unitigs, was also calculated using pyseer v.1.3.6.
163 Likelihood-ratio test *p*-values for the association tests were adjusted for multiple-testing
164 by Bonferroni correction (at a genome-wide false discovery rate of 0.05) for the number
165 of unique unitig patterns (i.e. only giving one count to a unitig with an identical
166 presence/absence profile across genomes). We also removed unitigs tagged with the
167 errors 'bad-chisq', 'pre-filtering-failed', 'lrt-filtering-failed', 'firth-fail' and 'matrix-
168 inversionerror' after the analysis. To further remove false positive GWAS hits, we
169 removed any considerable clusters of unitigs (> 20) with identical *p*-values, as these are
170 likely to be lineage-specific markers or markers with strong linkage disequilibrium
171 comprised of mostly non-causal variants linked on the same clonal frame. GWAS hits
172 were annotated by mapping the unitigs to two reference genomes of *V. cholerae*,
173 namely, E7946 (NCBI assembly accession number: GCA_002749635.1) and O1
174 biotype El Tor strain N16961 (NCBI assembly accession number: GCA_003063785.1)
175 using BWA. Statistically significant GWAS hits were further annotated with the CARD
176 resistance gene identifier (RGI) after filtering the 'loose' hits and hits with identity <0.90.

177 **Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS).**
178 This approach was based on former studies[69]. Stool supernatants were centrifuged and
179 filtered (0.2 μM surfactant-free cellulose acetate; Thermo Scientific Nalgene). Proteins

180 were precipitated (1:7 ratio (v/v) of water::methanol). Supernatants were diluted with
181 methanol and water (1:1 v/v) in 0.1% formic acid for liquid chromatography, and 5 μl of
182 supernatant was injected for analysis. LC/MSMS was performed on a 2.1 x 150-mm
183 Hypersil Gold aQ column (particle size, 3 μm) using a high-performance liquid
184 chromatography system (Thermo UltiMate 3000 series) with an LTQ XL ion trap mass
185 spectrometer (Thermo Fisher Scientific). Mobile phases were 1% formic acid in water
186 (A) and 1% formic acid in methanol (B) and held at a constant 5%B for 2min before
187 ramping to 95%B at 15 min where it was held for an additional minute before returning
188 to starting conditions for a total run time of 25 min.

189 Eluent was ionized using electrospray ionization (ESI) in positive mode at a spray
190 voltage of 5 kV, a nitrogen sheath gas flow rate of 8 L min^{-1}, and capillary temperature
191 of 300°C. Two scan events were programmed to perform an initial scan from *m/z* 100 to
192 1000, which was followed by targeted collision induced dissociation based on a
193 retention time and mass list. Retention time windows ranged from 0.35 minutes to 6.50
194 min, depending on the elution range of the standards at high and low concentrations.
195 Masses were targeted for the most abundant adduct or ion associated with each
196 antibiotic (typically the $[M+H]^+$ ion) with a *m/z* 1 window. Data analysis for the
197 amoxicillin, sulfamethoxazole/trimethoprim, azithromycin, tetracycline, doxycycline,
198 metronidazole, nalidixic acid, and ciprofloxacin was performed manually using extracted
199 ion chromatograms and MSMS matching with an in-house antibiotic MSMS library using
200 Xcalibur 2.2 SP 1.48 (Thermo Fisher Scientific).

201 **Statistical analysis.** Bivariate analyses of categorical data were analyzed using
202 Fisher's Exact Test, and continuous data were analyzed using the Mann-Whitney U
203 Test (alpha = 0.05). McNemar's test was used to analyze paired data (alpha = 0.05).

204 **RESULTS**

205 **Comparison of antimicrobial resistance under aerobic and anaerobic conditions.**
206 Pairwise analysis for baseline growth parameters found that carrying capacity, AUC,
207 and growth velocity were significantly lower under anaerobic compared to aerobic
208 conditions (Supplementary Table 2). In this physiologic context, the distribution of
209 resistance differed depending on aerobic versus anaerobic conditions (Fig. 1). The MIC
210 modes for ciprofloxacin were 7 µg/ml (min= 0.0016 µg/ml; max= 32 µg/ml) and 2 µg/ml
211 (min= 0.004 µg/ml; max= 8 µg/ml) under anaerobic and aerobic conditions, respectively
212 (Supplementary Table 3). The rates of resistance under anaerobic (93%; N= 62/67) and
213 aerobic (54%; N=36/67) conditions were significantly different (Fig. 1a; Supplementary
214 Table 4). For azithromycin, the MIC modes were 32 µg/ml (min= 8 µg/ml; max= 128
215 µg/ml) and 4 µg/ml (min= 1 µg/ml; max= 32 µg/ml) under anaerobic and aerobic
216 conditions respectively (Supplementary Table 3). The rates of resistance under
217 anaerobic (n=67/67; 100%) and aerobic (n=15/67; 22%) conditions were significantly
218 different (Fig. 1b; Supplementary Table 4). For doxycycline, the MIC modes were 1
219 µg/ml under both aerobic and anaerobic conditions, respectively (Supplementary Table
220 3); only two isolates were found to be resistant under anaerobic conditions and one
221 under aerobic conditions. Taken together, the odds of classifying isolates as resistant
222 under anaerobic compared to aerobic conditions increased over 20 times for

223 ciprofloxacin (OR= 27; 95% CI= 4.45-1105) and over 50 times for azithromycin (OR =
224 53; 95% CI= 9.10-2134; Supplementary Table 4).

225 **Addition of catalase to test if reactive oxygen species effect antibiotic**
226 **tolerance/sensitivity under aerobic conditions.** For ciprofloxacin, the MICs for the
227 sensitive reference strain E7946 (Cip^S, Azi^S, Dox^S) and the resistant clinical isolate
228 EN160 (Cip^R, Azi^R, Dox^S) remained unchanged when catalase was added to the media
229 under aerobic conditions. The addition of catalase was not associated with differences
230 in AUCs for both E7946 and EN160 in media containing ciprofloxacin, azithromycin, or
231 doxycycline at 2-fold below the MIC. The AUCs in LB media with and without catalase
232 alone for E7946 and EN160 were not statistically different (Supplementary Table 5).

233 **Characterization of known AMR genetic elements under aerobic and anaerobic**
234 **conditions**. The distribution of known AMR genetic elements (Fig. 2) was grouped by
235 point mutations (likely transmitted vertically, not on an established mobilizable element)
236 and horizontal transmission (on an established mobilizable element).

237 *Point mutations in known AMR genes.* For ciprofloxacin, the most identified known
238 resistance mutations were those in the topoisomerase encoding genes *gyrA*
239 (VC1258/RS06370) (Ser83Ile) and *parC* (VC2430/RS12340) (Ser85Leu). These were
240 present in 60% (40/67) and 70% (47/67) of all isolates, while 54% (36/57) contained
241 both. For azithromycin, we found no mutations in known resistance genes encoding
242 ribosomal proteins L4 (*rplD;*VC2595/RS13175) and L22 (*rplV;*VC2591/RS13155) [70,71].
243 For tetracycline (proxy for doxycycline), one of the 9 total 16S rRNA genes (VCr001)
244 was found to have a single nucleotide insertion of a G nucleotide at position 327 within

245 the sequences of 15% (10/67) of isolates; the significance is unknown. No mutations
246 were detected in the 30S ribosomal protein encoded by genes *rpsJ* (VC2597/RS13185)
247 or *rpsC* (VC2590/RS13150), whose mutations have been associated with tetracycline
248 class resistance in other Gram negative organisms [72-74].

249 *AMR genes on mobile genetic elements.* The integrative conjugative element (ICE)
250 SXT/R391 was found in 90% (60/67) of isolates. The ICE contained a pentapeptide
251 repeat protein conferring fluoroquinolone resistance (qnr_{Vc}), a macrolide-inactivating
252 phosphotransferase (*mph*(A)), and a major facilitator superfamily (MFS) efflux pump
253 conferring tetracycline resistance (*tet*(59)) [75-78]. The genes qnr_{Vc}, *mph*(A), and *tet*(59)
254 were also found in 78% (52/67), 33% (22/67), and 78% (52/67) of isolates, respectively.

255 We examined to what extent these point mutations and genes were associated with
256 resistance. Ciprofloxacin resistance under both anaerobic and aerobic conditions was
257 significantly associated with qnr_{VC}, *gyrA* and *parC* (Supplementary Table 6).
258 Identification of the known azithromycin AMR gene *mph(A)* was significantly associated
259 with resistance under aerobic conditions alone (P<0.001). The gene *tet(59)* was not
260 associated with doxycycline resistance under aerobic or aerobic conditions (both
261 p=0.566).

262 **Discovery of AMR associated genetic elements under aerobic and anaerobic**
263 **conditions**. To more broadly explore the genetic basis of AMR, we conducted genome-
264 wide association studies (GWAS). This approach used the phenotype of area under the
265 curve (AUC) to represent 'growth' with or without exposure to the three antibiotics at five
266 concentrations under either aerobic or anaerobic conditions. Phenotypes (AUCs) at

267 similar antibiotic concentrations were positively correlated within aerobic and anaerobic
268 conditions for all three antibiotics (Fig. 3). Phenotypes were also positively correlated
269 between aerobic and anaerobic conditions for ciprofloxacin (Fig. 3a). However, AUCs
270 were weakly, or even negatively, correlated between aerobic and anaerobic conditions
271 for azithromycin and doxycycline (Fig. 3b,c). These results reinforce the idea that
272 anaerobic and aerobic growth are distinct under antibiotic pressure.

273 The heritability of the AMR phenotypes (AUCs) was estimated prior to the GWAS.
274 Heritability (h^2) was defined as the proportion of phenotypic variation explained by
275 genetic variation, measured as unique contiguous tracts of the genome (unitigs) that tag
276 both single nucleotide variants, indels, and gene content changes (Methods). We found
277 relatively high heritability (h^2 in the range 0.60-0.99) of growth across concentrations of
278 ciprofloxacin under both aerobic and anaerobic conditions, yielding statistically
279 significant GWAS hits (Table 1; Supplementary Data Files). In contrast, heritability
280 tended to be much lower under aerobic compared to anaerobic conditions for both
281 azithromycin and doxycycline, yielding significant GWAS hits only under anaerobic
282 conditions (Table 1; Supplementary Data Files).

283 AMR genes identified by GWAS were diverse (Fig. 4; Supplementary Data Files). These
284 candidates included known AMR genes, such as *qnrVC* and *dfrA,* which were
285 associated with ciprofloxacin resistance under both aerobic and anaerobic conditions.
286 We identified seven genes associated with ciprofloxacin resistance under anaerobic
287 conditions alone (including the stress response gene *barA* and a *radC* homolog involved
288 in DNA repair), and ten genes under aerobic conditions alone (including *rtxB*). Under
289 anaerobic conditions, most genes were identified at ciprofloxacin concentrations at or

290 above 0.25 µg/ml; however, four genes, including *barA*, were identified under one of the
291 lowest tested ciprofloxacin concentrations (0.13 µg/ml). GWAS hits for doxycycline and
292 azithromycin resistance were found only under anaerobic conditions. For azithromycin,
293 two genetic elements were identified: *mphA* and a region between *ompT* and *dinG*
294 (*ompT-dinG)*. For doxycycline, 23 genes were shared across concentrations; however,
295 the gene discovery rate was highest at the lower concentrations (n=53 at 0.13 µg/ml; n
296 =26 µg/ml). GWAS hits included the major facilitator superfamily antibiotic efflux pump
297 *tet(59)* (Fig. 4, Supplementary Data Files). The majority of genetic elements identified
298 by GWAS for all three antibiotics have unknown function, yet the rate of detection of
299 known AMR genes suggests that the genes of unknown function may indeed play a role
300 in AMR.

301 **Associations between antibiotics detected in stool by LC-MS/MS and rates of**
302 **AMR at the genotypic and phenotypic levels.** A total of 198 antibiotics were detected
303 in the 51 stool supernatants tested (Fig. 5). At least one antibiotic was detected in 98%
304 (n=50/51), at least two antibiotics were detected in 94% (n=48/51), and 3 or more
305 antibiotics were detected in 90% (n=46/51) of stool supernatants (Fig. 5). Antibiotics
306 detected were ciprofloxacin (n=48/51; 94%), nalidixic acid (n=41/51; 80%),
307 metronidazole (n=37/51; 73%), tetracycline (n=24/51; 47%), doxycycline (n=24/51;
308 47%), bactrim (sulfamethoxazole/trimethoprim; n=22/51; 43%), and amoxicillin (n=2/51;
309 43%); azithromycin was not detected. We hypothesized that resistance to ciprofloxacin,
310 azithromycin and/or doxycycline in the isolates would correlate with antibiotic detection
311 in the paired stool sample by LC-MS/MS. For quinolone/fluoroquinolone and
312 tetracycline/doxycycline, detection by LC-MS/MS was not associated with AMR

genotypes or phenotypes (Supplementary Table 7). Associations for azithromycin could not be tested because azithromycin was not detected.

DISCUSSION

In this study, the rate of resistance among clinical isolates differed significantly between aerobic and anaerobic conditions. The extent to which isolates became tolerant to antibiotic exposure under anaerobic conditions differed by antibiotic class. The genetic basis of resistance was uniquely discoverable under anaerobic conditions and differed by antibiotic class. These findings challenge the convention of testing facultative enteropathogens for AMR under aerobic conditions alone. The approach of using AUC growth curve data as a dependent continuous variable for GWAS to identify AMR genetic elements under aerobic and anaerobic conditions provides a new methodologic framework to better track AMR for clinical stewardship and public health surveillance.

Antibiotics from three disparate classes were studied. The experiments revealed commonalities as well as important difference between classes. The odds of classifying isolates as resistant under anaerobic conditions compared to aerobic conditions increased over 20 times for ciprofloxacin and over 50 times for azithromycin. Generalizable physiologic mechanisms for increased tolerance to antibiotics under anaerobic conditions may include reduced doubling-time or growth rate limiting the impact of agents that directly or indirectly disrupt cell envelope integrity. Additional mechanisms include reduction in proton motive force that impacts the capacity to pump agents across the cell envelope, and ROS that induce both intracellular and cell wall stress and can act synergistically to potentiate antibiotic lethality. The assays that

335 utilized catalase to quench hydrogen peroxide were conducted to evaluate this latter
336 possibility. The assays were limited because catalase targets one type of ROS
337 (hydrogen peroxide) in the extracellular space, which freely diffuses from the
338 intracellular space. For the two strains tested, the MIC for all three antibiotics did not
339 increase with the addition of catalase suggesting that the reduction of ROSs alone
340 cannot account for increased MICs observed under anaerobic conditions
341 (Supplementary Table 5).

342 Knowledge gaps on the genetic basis of AMR have decreased dramatically with the
343 advance of whole-genome sequencing. However, this fund of knowledge is susceptible
344 to bias because conventional methods use aerobic conditions to test for AMR among
345 facultative enteropathogens. This study prioritized the factor of oxygen exposure as a
346 determinant of AMR phenotype because facultative enteropathogens experience
347 hypoxia and anoxia in the animal host. The first phase of the analysis focused on known
348 AMR genotypes with known AMR phenotypes. For ciprofloxacin, mutations in *parC* and
349 carriage of qnr_{Vc} significantly associated with phenotypic resistance under aerobic and
350 anaerobic conditions; mutations in *gyrA* significantly associated with resistant
351 phenotypes under anaerobic conditions alone. The lack of a significant association
352 between *gyrA* mutations and phenotype under aerobic conditions may be due to the
353 limited sample size. For azithromycin, *mphA* was identified and significantly associated
354 with AMR phenotypes under aerobic and anaerobic conditions. While *tet*(59) was
355 identified, very few isolates were identified as resistant to doxycycline under aerobic
356 (n=1) or anaerobic conditions (n=2). These data demonstrate that the evaluation of the
357 relationship between of AMR genotype and phenotype may strengthen under anaerobic

358 conditions and may expose an experimental bias in our historical understanding of
359 AMR.

360 The second phase of analysis sought to identify unknown genetic elements associated
361 with AMR. Initial attempts to use categorical data (resistant vs sensitive) in the GWAS
362 resulted in modest gene discovery rates (data not shown). To address this problem, the
363 continuous variable of AUC strengthened the GWAS. Breakpoint and sub-breakpoint
364 concentrations were chosen based on a rationale that different genetic elements might
365 contribute differently to AMR phenotypes at different antibiotic concentrations. GWAS
366 expectedly identified qnr_{Vc} for ciprofloxacin exposure under aerobic and anaerobic
367 conditions, *mphA* for azithromycin exposure under anaerobic conditions alone and
368 *tet*(59) for doxycycline exposure under anaerobic conditions alone. For ciprofloxacin,
369 seven genes were discovered in anaerobic conditions alone; these included a homolog
370 for DNA repair (*radC*), 2-component histidine kinase involved in stress response (*barA*),
371 and an ATP-dependent zinc protease. The genes *dfrA31* and APH(6)-Id were identified
372 for both aerobic and anaerobic conditions and encode a trimethoprim-resistant
373 dihydrofolate reductase and an antibiotic inactivation protein, respectively. These genes
374 are located on SXT with qnr_{Vc} and may therefore be associated due to genetic linkage
375 rather than due to causal roles in ciprofloxacin resistance. For azithromycin, two genetic
376 elements under anaerobic conditions alone were identified: *mphA* (mentioned above)
377 and an intergenic region between *ompT* (porin; known to be associated with AMR)[79,80]
378 and *dinG* (ATP-dependent DNA helicase). For doxycycline, a diverse set of 57 genetic
379 elements under anaerobic conditions alone were identified that included *tet(59)*
380 (mentioned above), *vexK* (efflux RND transporter permease associated with AMR)[81-83],

381 and *zorA* (anti-phage defense system ZorAB subunit A; a putative proton channel that
382 may respond to membrane perturbation by depolarization)[84].

383 Genes associated with an AMR phenotype were found on SXT (e.g. *tet(59), aph(6)-Id,*
384 *radC, dfra1*) and discovered on the Vibrio Pathogenicity Island II (VSPII; N16961
385 VC0506-VC0512 / E7946 loci RS02705-RS02745); these loci are known to be
386 genetically diverse in Bangladesh[85]. The GWAS hits in VSPII encode both biofilm/
387 autoaggregation associated factors as well as an aerotaxis protein (AerB; VC0512)[86];
388 findings consistent with roles in AMR and aerobic/ anaerobic conditions. The GWAS
389 were limited by sample size and were at risk of increased false positive discovery rates
390 at AMR 'hot-spots' like SXT. Despite these limitations, GWAS enabled the discovery of
391 an intriguing list of genetic targets that were associated with AMR and require future
392 molecular analysis to test for causal relationships.

393 LC-MS/MS analysis on paired stool supernatants was conducted to test the hypothesis
394 that the rates of AMR genotypes and phenotypes were higher when the stool sample
395 contained the cognate antibiotic. A quinolone/fluoroquinolone AMR genotype (known) or
396 phenotype was not associated with quinolone or fluoroquinolone detection. A
397 tetracycline/doxycycline AMR genotype (known) or phenotype was not associated with
398 tetracycline/doxycycline detection. These results were limited by a low number of
399 control samples negative for the antibiotic in question. While antibiotics were
400 widespread, the LC-MS/MS data cannot be used to establish prevalence because the
401 original enrollment criteria excluded patients that self-reported antibiotic intake. This
402 would seem like a significant limitation, but a prior study documented that self-reports
403 had no predictive value compared to antibiotics detected in stool by LC-MS/MS[69]. This

404 study found nearly all patients shed at least one antibiotic despite self-reports of no
405 antibiotic intake. The high detection rates of nalidixic acid detection as well as both
406 doxycycline and tetracycline does not match clinical patterns of antibiotic prescription[69].
407 Non-medical sources of antibiotic exposure include known food and water
408 contamination with antibiotics[87]. These data highlight the experimental difficulty of
409 linking exposure to genotype and phenotype when the environment and clinical settings
410 are nearly saturated with antibiotics. These results are a further call to action to increase
411 antibiotic stewardship in healthcare as well as in the food-chain to combat AMR.

412 **Conclusions**

413 Facultative enteropathogens are exposed to antibiotics under aerobic and anaerobic
414 conditions during their life history in both the human gut and in the environment. We
415 used the facultative enteropathogen *V. cholerae* as a model to test for differences in
416 AMR phenotypes under aerobic and anaerobic conditions. The odds of classifying
417 isolates as resistant under anaerobic compared to aerobic conditions increased over 10
418 times for two of the three antibiotics tested. While several known resistance genes were
419 associated with AMR under both conditions, many genes were only associated with
420 AMR under one condition. In general, heritability tended to be higher and more genes
421 associated with resistance under anaerobic conditions, suggesting that key genetic
422 determinants of resistance may be missed when experiments are only performed
423 aerobically. Taken together, these findings challenge the paradigm of testing facultative
424 enteropathogens for AMR under aerobic conditions alone. The experimental approach
425 herein may also establish a new, less biased framework to track and investigate
426 mechanisms of AMR.

427 **Acknowledgements**

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432 Florida. Stephen Calderwood, Jason Harris, and Regina LaRocque were the principal
433 investigators (PI) of the parent study/IRB protocol (Massachusetts General Hospital, Harvard
434 University School of Medicine, USA) under which the samples were collected by EJN when he
435 was a NIH Fogarty Fellow. Andrew Camilli provided additional reagents and insights for this
436 manuscript; FQ was the principal investigator in Bangladesh and PI of the ERC/RRC approvals
437 at the icddr,b. This generous research infrastructure and support was invaluable to the success
438 of this study.

439 **Data availability**

440 Data analyzed in the manuscript have been made available in the online supplementary
441 material.

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448 **Disclaimer**

449 The funders had no role in study design, data collection and analysis, decision to publish, or
450 preparation of the manuscript.

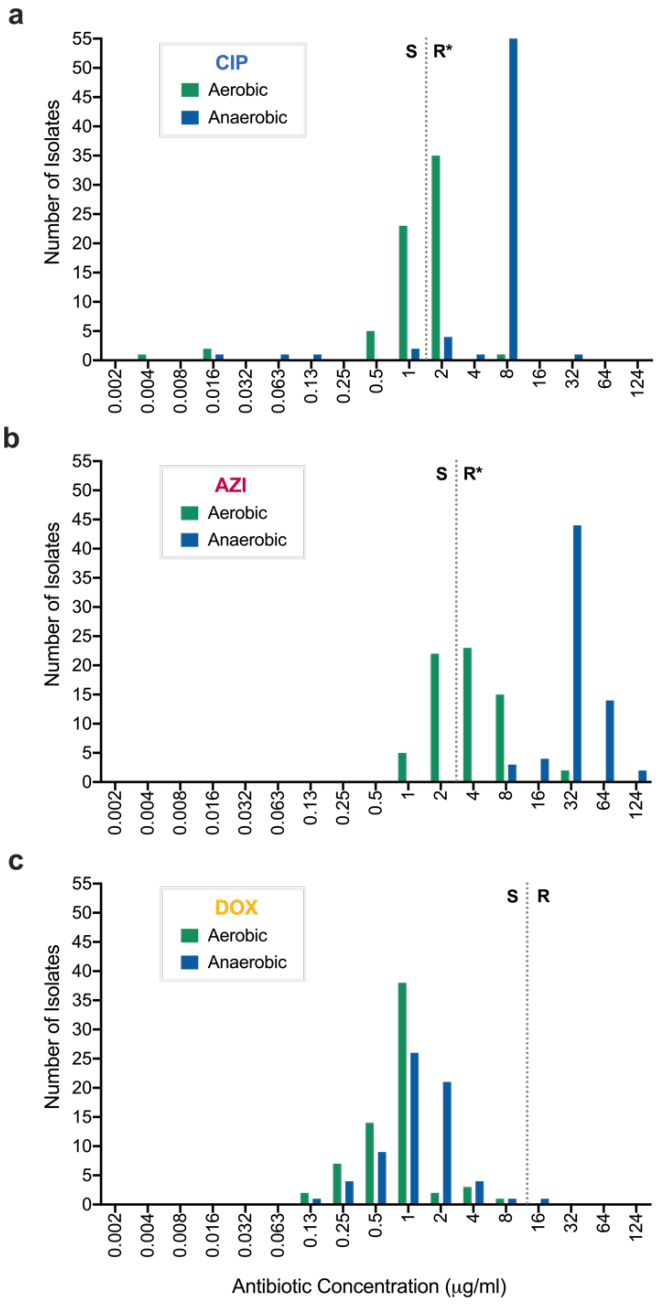
451 **Potential conflicts of interest.**

452 All authors: No reported conflicts.

453 **FIGURES**

454

455 **FIGURE 1**



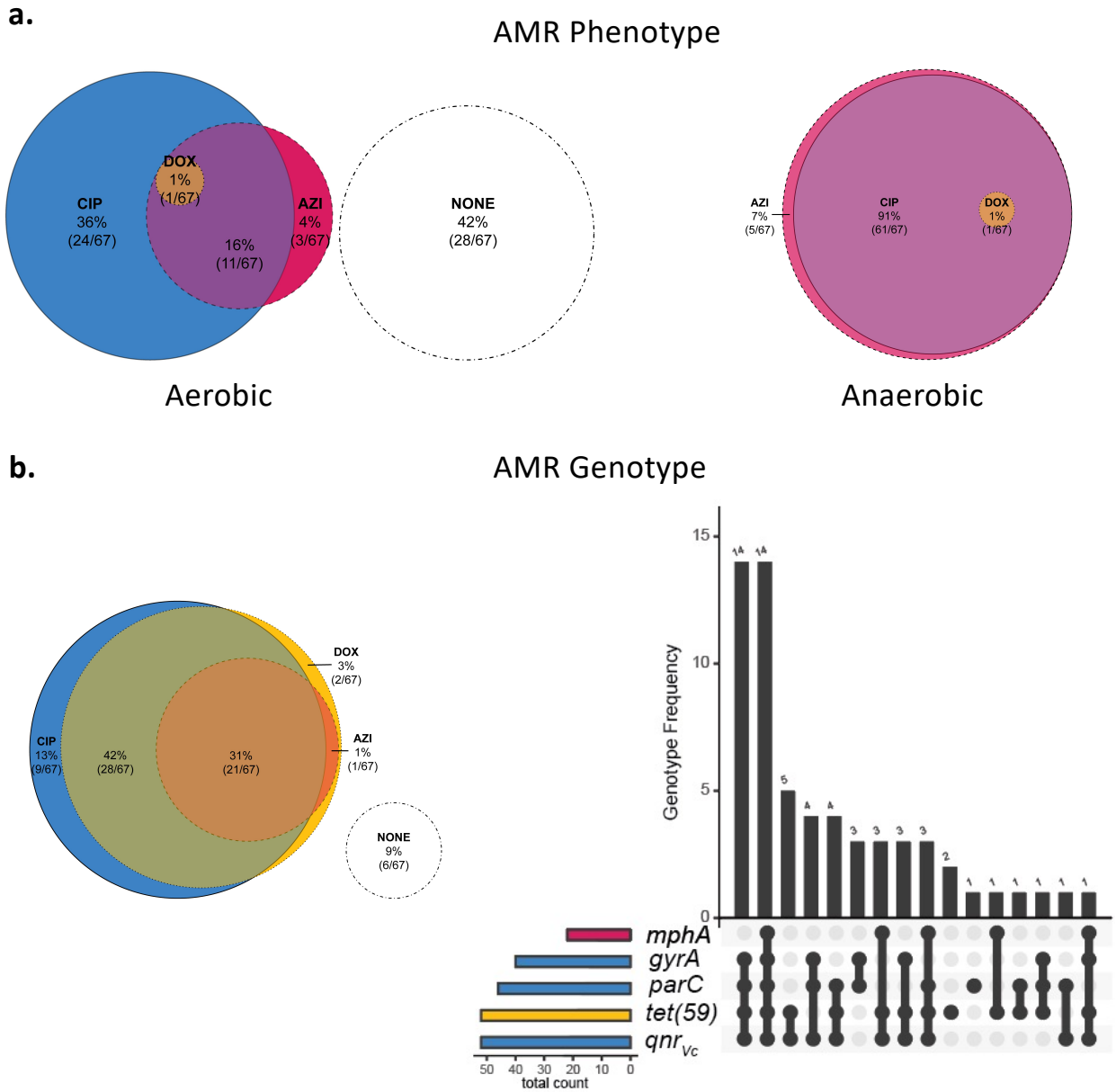
456

457 **Fig. 1. Distribution of antibiotic resistance among clinical isolates under aerobic and anaerobic**
458 **conditions.** Ciprofloxacin (CIP; **a**), Azithromycin (AZI; **b**), and Doxycycline (DOX; **c**). Resistance was
459 determined by MIC under aerobic (green) and anaerobic conditions (blue). Data are from 67 human-shed
460 *V. cholerae* isolates. Dotted line is the breakpoint for resistance per CLSI standards (CIP = 2 µg/ml; AZI =
461 4 µg/ml; DOX = 16 µg/ml). "*" represents a significant difference in the frequency of isolates identified as
462 resistant to ciprofloxacin and azithromycin by McNemar's test (both $p<0.001$). S=sensitive. R = Resistant.

463

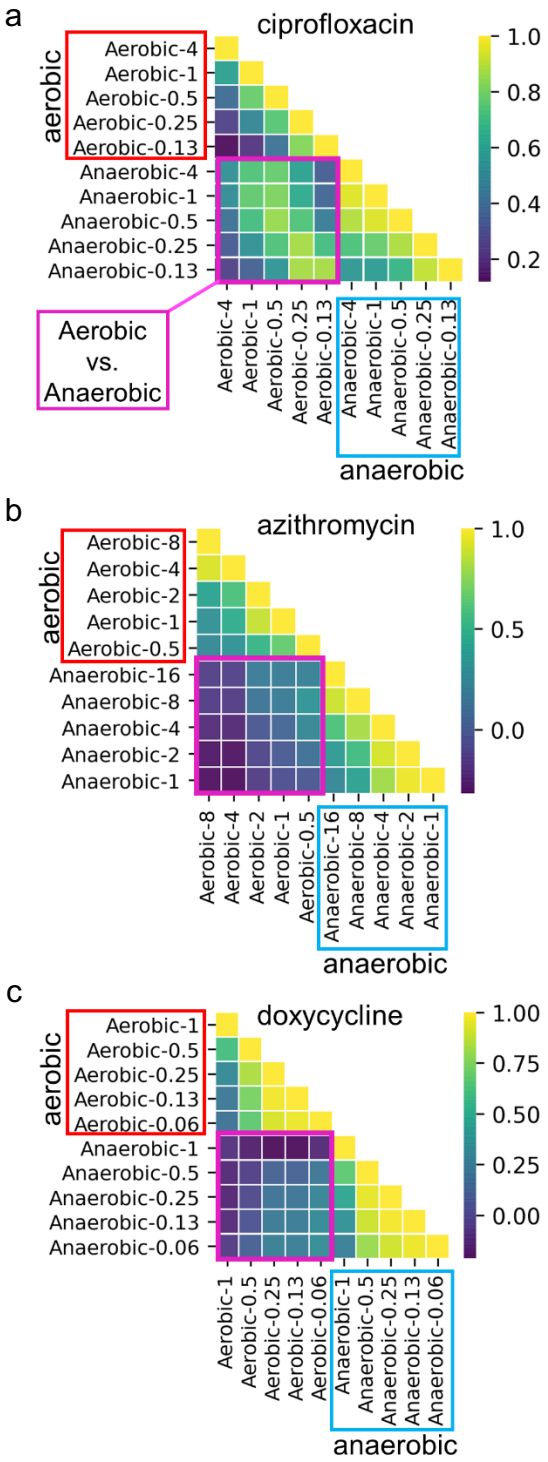
464 **FIGURE 2**

465



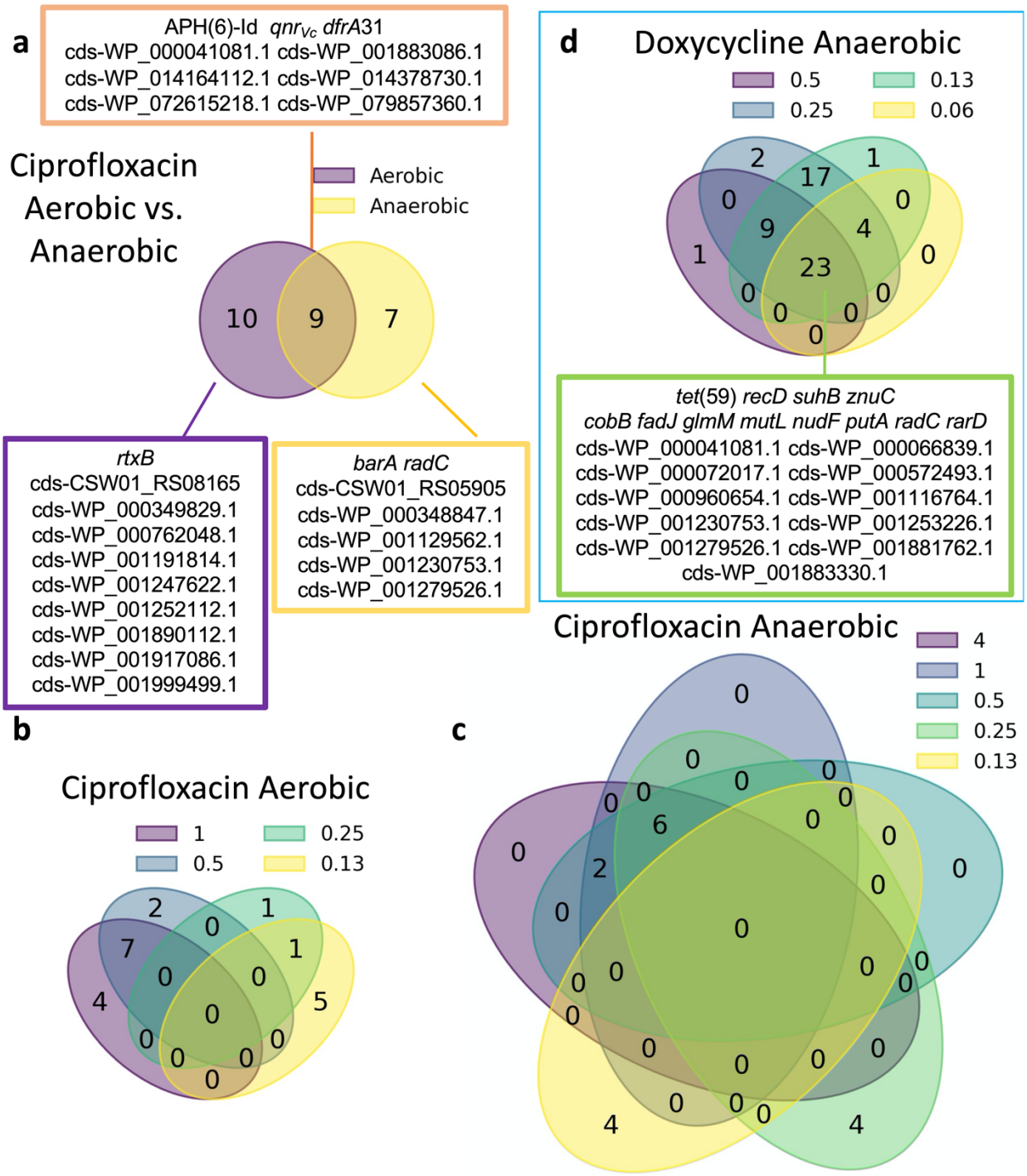
466
467 **Fig. 2. AMR phenotypes and known AMR genetic elements in human-shed *V. cholerae* isolates**. **a,**
468 Proportional Venn diagram (Euler) of AMR phenotypes to azithromycin (AZI), doxycycline (DOX) and/or
469 ciprofloxacin (CIP) under aerobic (left) and anaerobic conditions (right). **b,** Distribution of specific known
470 AMR genetic elements by proportional Venn diagram (Euler; left) and bar chart (right). AMR genetic
471 elements to other antibiotics are not shown.

472 **FIGURE 3**



473
474 **Fig. 3. Correlations among *V. cholerae* growth phenotypes at different concentration of antibiotics**
475 **and respiratory conditions.** Correlations of *V. cholerae* growth phenotypes (AUC) to ciprofloxacin (CIP;
476 **a**), azithromycin (AZI; **b**), and doxycycline (DOX; **c**) at different antibiotic concentrations under aerobic
477 and anaerobic conditions (e.g. “Anaerobic-0.06”). Heatmaps show correlation coefficients (scale bar is to
478 right).
479

480 **FIGURE 4**



481
482 **Fig. 4. Distribution of AMR genes associated with AMR growth phenotypes at different**
483 **concentrations of antibiotics under aerobic and anaerobic conditions.** Venn diagrams show the
484 overlap between genes associated with **(a)** ciprofloxacin (CIP) resistance under aerobic vs. anaerobic
485 conditions, **(b)** ciprofloxacin at different concentrations (µg/ml) under aerobic conditions, **(c)** ciprofloxacin
486 at different concentrations (µg/ml) under anaerobic conditions, and (**d)** doxycycline at different
487 concentrations (µg/ml) under anaerobic conditions.

	CIP	NAL	MET	TET	DOX	BAC	AMO	AZI	ERY	CEF	Detected (N)
EN126	+	+	+	-	+	+	+	-	-	-	6
EN160	+	+	+	-	+	+	+	-	-	-	6
EN127	+	+	+	+	-	+	-	-	-	-	5
EN129	+	+	+	+	-	+	-	-	-	-	5
EN135	+	+	+	+	+	-	-	-	-	-	5
EN149	+	+	+	+	-	+	-	-	-	-	5
EN153	+	+	+	+	-	+	-	-	-	-	5
EN162	+	+	+	+	-	+	-	-	-	-	5
EN164	+	+	+	+	-	+	-	-	-	-	5
EN166	+	+	+	+	-	+	-	-	-	-	5
EN168	+	+	+	+	-	+	-	-	-	-	5
EN178	+	+	+	+	-	+	-	-	-	-	5
EN182	+	+	+	+	-	+	-	-	-	-	5
EN183	+	+	+	+	-	+	-	-	-	-	5
EN185	+	+	+	+	-	+	-	-	-	-	5
EN188	+	+	+	+	-	+	-	-	-	-	5
EN071	+	-	+	-	+	+	-	-	-	-	4
EN078	+	+	-	+	-	+	-	-	-	-	4
EN092	+	+	+	+	-	-	-	-	-	-	4
EN125	+	+	-	+	-	+	-	-	-	-	4
EN143	+	+	-	+	+	-	-	-	-	-	4
EN144	+	+	+	+	-	-	-	-	-	-	4
EN145	+	+	+	-	+	-	-	-	-	-	4
EN148	+	+	+	-	+	-	-	-	-	-	4
EN150	+	+	+	-	+	-	-	-	-	-	4
EN155	+	+	+	-	+	-	-	-	-	-	4
EN156	+	-	+	-	+	+	-	-	-	-	4
EN159	+	+	-	+	-	+	-	-	-	-	4
EN165	+	+	+	-	+	-	-	-	-	-	4
EN167	+	+	+	-	+	-	-	-	-	-	4
EN169	+	+	+	-	+	-	-	-	-	-	4
EN171	+	-	+	-	+	+	-	-	-	-	4
EN174	+	+	-	+	-	+	-	-	-	-	4
EN181	+	+	+	-	+	-	-	-	-	-	4
EN184	+	+	+	+	-	-	-	-	-	-	4
EN189	+	+	+	-	+	-	-	-	-	-	4
EN018	+	-	+	-	+	-	-	-	-	-	3
EN026	+	-	+	-	+	-	-	-	-	-	3
EN027	+	-	+	-	+	-	-	-	-	-	3
EN095	+	+	-	-	+	-	-	-	-	-	3
EN096	+	+	-	-	+	-	-	-	-	-	3
EN133	+	+	-	+	-	-	-	-	-	-	3
EN134	+	+	-	+	-	-	-	-	-	-	3
EN141	+	+	-	-	+	-	-	-	-	-	3
EN147	+	+	-	-	+	-	-	-	-	-	3
EN191	+	-	+	-	+	-	-	-	-	-	3
EN072	+	-	+	-	-	-	-	-	-	-	2
EN173	+	-	+	-	-	-	-	-	-	-	2
EN137	-	+	-	-	-	-	-	-	-	-	1
EN146	-	+	-	-	-	-	-	-	-	-	1
EN132	-	-	-	-	-	-	-	-	-	-	0
Detected (N)	48	41	37	24	24	22	2	0	0	0	198 total detected
Positive (%)	94	80	73	47	47	43	4	0	0	0	98% with antibiotic(s)

Fig. 5. Antibiotic detection in stool supernatants by mass spectrometry (LC-MS/MS). Green with "+" = Detected. White with "-" = not detected. CIP= Ciprofloxacin, NAL = Nalidixic acid, MET = metronidazole, TET = tetracycline, DOX = Doxycycline, BAC = Sulfamethoxazole and/or Trimethoprim, AMO = Amoxicillin, ERY = Erythromycin, CEF = Ceftriaxone. Stool supernatants were not available for EN79,80,86,88,100,103,109,116-120, 123, 124, 130, 131.

494 **TABLE**

495

496

497 **Table 1.** Identification of genetic elements by GWAS that associate with AMR.

Condition	Outcome [b]	Antibiotic Concentration (µg/ml)				
Ciprofloxacin[a]		CIP4	CIP1	CIP0.5	CIP0.25	CIP0.13
Aerobic	Heritability (h^2)	0.99	0.73	0.60	0.74	0.92
	Associated genes	0	11	9	2	6
Anaerobic	Heritability (h^2)	0.81	0.73	0.72	0.72	0.87
	Associated genes	8	8	8	10	4
Azithromycin[a]		AZI16	AZI8	AZI4	AZI2	AZI1
Aerobic	Heritability (h^2)	-	0.00	0.02	0.00	0.11
	Associated genes	-	-	-	-	-
Anaerobic	Heritability (h^2)	0.30	0.77	0.66	0.38	0.00
	Associated genes	2	1	0	0	-
Doxycycline[a]		DOX1	DOX0.5	DOX0.25	DOX0.13	DOX0.06
Aerobic	Heritability (h^2)	0.00	0.27	0.23	0.30	0.50
	Associated genes	-	0	0	0	0
Anaerobic	Heritability (h^2)	0.10	0.61	0.72	0.72	0.77
	Associated genes	0	23	55	54	17

498 [a] Ciprofloxacin = CIP; Azithromycin = AZI; Doxycycline = DOX
499 [b] Heritability is the proportion of phenotypic variation that is explained by genetic variation. Associated
500 genes are all significant GWAS hits after correction for multiple hypothesis testing ($P < 0.05$ after
501 Bonferroni correction).
502

503
504

505 **SUPPLEMENTATRY MATERIAL**

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521
522
523 **Supplementary Data Files (.csv)**
524
525 **Date File S1.** GWAS Hits. Ciprofloxacin resistance phenotype. Aerobic.
526 **Date File S2.** GWAS Hits. Ciprofloxacin resistance phenotype. Anaerobic.
527 **Data File S3.** GWAS Hits. Azithromycin resistance phenotype. Anaerobic.
528 **Data File S4.** GWAS Hits. Doxycycline resistance phenotype. Anaerobic.
529

530 **Supplementary Table 1.** Reference strains and clinical isolates

531

		Aerobic[b]			Anaerobic[b]			Source[c]
	Strain[a]	CIP^R	AZI^R	DOC^R	CIP^R	AZI^R	DOX^R	
Reference	E7946	+	-	-	+	-	-	(1)
Clinical Isolates								
EN018	L_EN1286	-	-	-	-	+	-	(2)
EN026	L_EN1291	-	-	-	-	+	-	(2)
EN027	L_EN1292	-	-	-	-	+	-	(2)
EN071	L_EN1300	+	-	-	+	+	-	(2)
EN072	L_EN1301	-	-	-	+	+	-	(2)
EN078	L_EN1303	+	-	-	+	+	-	(2)
EN079	L_EN1304	-	+	-	+	+	-	(2)
EN080	L_EN1305	+	-	-	+	+	-	(2)
EN086	L_EN1307	+	+	-	+	+	-	(2)
EN088	L_EN1308	+	-	-	+	+	-	(2)
EN092	L_EN1310	+	-	-	+	+	-	(2)
EN095	L_EN1312	+	+	-	+	+	-	(2)
EN096	L_EN1313	+	+	-	+	+	-	(2)
EN100	L_EN1314	+	+	-	+	+	-	(2)
EN103	L_En1315	-	-	-	+	+	-	(2)
EN109	L_EN1319	-	-	-	+	+	-	(2)
EN116	L_EN1325	-	-	-	+	+	-	(2)
EN117	L_EN1326	-	-	-	-	+	-	(2)
EN118	L_EN1327	-	+	-	+	+	-	(2)
EN119	L_EN1328	+	-	-	+	+	-	(2)
EN120	L_EN1329	-	-	-	+	+	-	(2)
EN123	L_EN1330	-	-	-	+	+	-	(2)
EN124	L_EN1331	+	-	-	+	+	-	(2)
EN125	L_EN1332	+	+	-	+	+	-	(2)
EN126	L_EN1333	+	-	-	+	+	-	(2)
EN127	L_EN1334	+	-	-	+	+	-	(2)
EN129	L_EN1335	-	-	-	+	+	-	(2)
EN130	L_EN1336	+	-	-	+	+	-	(2)
EN131	L_EN1337	+	-	-	+	+	-	(2)
EN132	L_EN1338	+	-	-	+	+	-	(2)
EN133	L_EN1339	+	-	-	+	+	-	(2)
EN134	L_EN1340	-	+	-	+	+	-	(2)

EN135	L_EN1341	-	-	-	+	+	-	(2)
EN137	L_EN1343	+	+	-	+	+	-	(2)
EN141	L_EN1344	+	-	-	+	+	-	(2)
EN143	L_EN1346	-	-	-	+	+	-	(2)
EN144	L_EN1347	+	-	-	+	+	-	(2)
EN145	L_EN1348	+	+	-	+	+	+	(2)
EN146	L_EN1349	+	-	-	+	+	-	(2)
EN147	L_EN1350	-	-	-	+	+	-	(2)
EN148	L_EN1351	-	-	-	+	+	-	(2)
EN149	L_EN1352	+	-	-	+	+	-	(2)
EN150	L_EN1353	-	-	-	+	+	-	(2)
EN153	L_EN1355	-	-	-	+	+	-	(2)
EN155	L_EN1357	+	-	-	+	+	-	(2)
EN156	L_EN1358	-	-	-	+	+	-	(2)
EN159	L_EN1360	+	+	-	+	+	-	(2)
EN160	L_EN1361	+	-	-	+	+	-	(2)
EN162	L_EN1363	+	+	-	+	+	-	(2)
EN164	L_EN1365	-	-	-	+	+	-	(2)
EN165	L_EN1366	+	-	-	+	+	-	(2)
EN166	L_EN1367	+	-	-	+	+	-	(2)
EN167	L_EN1368	+	-	-	+	+	-	(2)
EN168	L_EN1369	+	+	-	+	+	-	(2)
EN169	L_EN1370	-	-	-	+	+	-	(2)
EN171	L_EN1371	+	+	-	+	+	-	(2)
EN173	L_EN1372	-	-	-	+	+	-	(2)
EN174	L_EN1374	-	-	-	+	+	-	(2)
EN178	L_EN1377	+	-	-	+	+	-	(2)
EN181	L_EN1379	-	-	-	-	+	-	(2)
EN182	L_En1380	-	-	-	+	+	-	(2)
EN183	L_EN1381	-	-	-	+	+	-	(2)
EN184	L_EN1382	+	-	-	+	+	-	(2)
EN185	L_EN1383	-	-	-	+	+	-	(2)
EN188	L_EN1385	-	-	-	+	+	-	(2)
EN189	L_EN1386	+	+	-	+	+	-	(2)
EN191	L_EN1388	-	-	-	+	+	-	(2)

532 [a] Prefix of "L_" was used to distinguish the strain number in the library from the clinical isolate number, which is
533 maintained to be consistent with prior publications.
534 [b] CIP^R = ciprofloxacin resistance. AZI^R = azithromycin resistance. DOX^R = doxycycline resistance.
535 [c] Sources of strains: (1) Mekalanos, J. J. Duplication and amplification of toxin genes in Vibrio cholerae. Cell 35,
536 253-263, (1983) ; (2) Nelson, E. J. et al. Complexity of rice-water stool from patients with Vibrio cholerae plays a
537 role in the transmission of infectious diarrhea. Proc Natl Acad Sci U S A 104, 19091-19096, (2007).

538 **Supplementary Table 2.** Baseline growth parameters of *V. cholerae* clinical isolates
539 under aerobic and anaerobic conditions
540

Growth parameter [a]	Aerobic median	Anaerobic median	P [b]
K	1.08	0.261	**< 0.001**
AUC	5.15	1.58	**< 0.001**
Velocity	0.011	0.006	**< 0.001**

541 [a] AUC = area under the curve. K = carrying capacity. Media was LB alone without antibiotics. Velocity =
542 growth rate in percent increase per minute at half the carrying capacity.
543 [b] Wilcoxon signed-rank test for growth. Bold = statistically significant (P<0.05).
544

545 **Supplementary Table 3.** Minimal inhibitory concentrations (MICs) for
546 ciprofloxacin, azithromycin, and doxycycline among *V. cholerae* clinical
547 isolates
548

	Ciprofloxacin[b]		Azithromycin[b]		Doxycycline[b]	
µg/ml[a]	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0.002	0	0	0	0	0	0
0.004	1	0	0	0	0	0
0.008	0	0	0	0	0	0
0.016	2	1	0	0	0	0
0.032	0	0	0	0	0	0
0.063	0	1	0	0	0	0
0.13	0	1	0	0	2	1
0.25	0	0	0	0	7	4
0.5	5	0	0	0	14	9
1	23	2	5	0	**38**	**26**
2	**35**	4	22	0	2	21
4	0	1	**23**	0	3	4
8	1	**56**	15	3	1	1
16	0	0	0	4	0	1
32	0	1	2	**44**	0	0
64	0	0	0	14	0	0
124	0	0	0	2	0	0

549 [a] Concentration of antibiotic.
550 [b] Distribution of MICs for clinical isolates grown under aerobic and anaerobic conditions. Bold text
551 signifies the concentration at which the MIC mode was determined among the clinical isolates.
552

553 **Supplementary Table 4.** Comparison of rates of resistance detected under aerobic
554 versus anaerobic conditions among *V. cholerae* clinical isolates
555

	N	R^{Ae}/R^{An}	R^{Ae}/S^{An}	S^{Ae}/R^{An}	S^{Ae}/S^{An}	OR[b]	95% CI[b]	P[b]
Ciprofloxacin[a]	67	36	0	26	5	27	(4.45 - 1105)	**<0.001**
Azithromycin[a]	67	15	0	52	0	53	(9.10 - 2132)	**<0.001**
Doxycycline[a]	67	0	1	1	65	1	(0.01 - 78.5)	1

556 [a] Distribution of paired resistant ('R') and sensitive ('S') phenotypes for isolates under aerobic ('Ae') and
557 anaerobic ('An') conditions.
558 [b] McNemar's Exact Test. Bold = statistically significant (P<0.05)
559

560 **Supplementary Table 5.** Effect of catalase on growth parameters for *V. cholerae*
561 E7946 and EN160 under aerobic conditions
562

Experiment	Strain[a]	Antibiotic[b]	Catalase[c]	AUC mean[d]	AUC IQR[d]	P[e]
1.	E7946	CIP	YES	41.84	1.76	0.136
		CIP	NO	44.2	0.865	
2.	E7946	AZI	YES	196	3.37	0.533
		AZI	NO	197.5	0.294	
3.	E7946	DOX	YES	308.5	6.32	0.959
		DOX	NO	308.9	4.11	
4.	E7946	NO	YES	317.3	4.65	0.818
		NO	NO	318	5.42	
5.	EN160	CIP	YES	138.4	22.1	0.551
		CIP	NO	126.1	25.2	
6.	EN160	AZI	YES	265.8	7.12	0.571
		AZI	NO	269.9	6.75	
7.	EN160	DOX	YES	280.1	19.3	0.895
		DOX	NO	275.8	21.6	
8	EN160	NO	YES	319.5	11.26	0.092
		NO	NO	326.6	8.78	

563 [a] E7946 (Cip^S, Azi^S,Dox^S) is the reference strain and EN160 (Cip^R, Azi^R, Dox^S) is a clinical isolate.
564 Biological replicates in experiments 1-3 and 5-7 were 3, each with 4 technical replicates. Biological
565 replicates for experiments 4 and 8 were 9, each with 4 technical replicates.
566 [b] CIP = ciprofloxacin. AZI = azithromycin. DOX = doxycycline. Assays were run at CIP = 0.5, AZI = 2, and
567 DOX = 0.25 µg/ml for EN160; E7946 was run at CIP = 0.002, AZI = 1, and DOX = 0.013
568 µg/ml.
569 [c] CIP and AZI were tested with 3 biological replicates; DOX with 2 biological replications; LB controls with
570 9 biological replicates.
571 [d] AUC = area under the curve. IQR = interquartile range.
572 [e] Student's t-test. Bold = statistically significant (P<0.05)

573 **Supplementary Table 6.** Comparison of antibiotic resistance phenotypes and known
574 resistance genotypes among *V. cholerae* clinical isolates

Aerobic conditions [a]								
Antibiotic tested	Gene	R/P	R/NP	S/P	S/NP	OR[b]	95% CI[b]	P[b]
Ciprofloxacin	*qnrVc*	36	0	16	15	31	(4.22 - 695)	**<0.001**
	gyrA	28	8	12	19	5.4	(1.86 - 17.9)	**0.002**
	parC	31	5	16	15	5.6	(1.65 - 18.9)	**0.003**
Azithromycin	*mphA*	15	0	7	45	83	(11.2 - 1938)	**<0.001**
Doxycycline	*tet(59)*	1	0	51	15	0.62	(0.046 - 18.9)	0.566
Anaerobic conditions [a]								
Antibiotic tested	Gene	R/P	R/NP	S/P	S/NP	OR[b]	95% Cb[c]	P[b]
Ciprofloxacin	*qnrVc*	52	10	0	5	27	(3.53 - 666)	**<0.001**
	gyrA	40	22	0	5	10	(1.39 - 248)	**0.016**
	parC	47	15	0	5	17	(2.28 - 416)	**0.003**
Azithromycin	*mphA*	22	45	0	0	0.51	(0.0127 - 20.1)	1
Doxycycline	*tet(59)*	1	0	51	15	0.62	(0.046 - 18.9)	0.566

575 [a] R = resistant phenotype, S = sensitive phenotype, P = gene present, NP = gene not present.
576 Enumerations include isolates with at least the specific gene named; isolates may have more than one
577 resistance gene (e.g. *qnrVC, gyrA* and *parC*)
578 [b] Fisher's Exact Test. Bold = statistically significant (P<0.05)
579
580

581 **Supplementary Table 7.** Test of association between antibiotic detection by
582 mass spectrometry and AMR genotypes and phenotypes among *V. cholerae*
583 clinical isolates

Antibiotic detection (D) and AMR genotype present (P) [a]					
	D/P	D/NP	ND/P	ND/NP	*P*[b]
CIP	41	7	3	0	1
CIP + NAL	35	4	9	3	0.334
DOX	16	8	20	7	0.759
DOX + TET	2	0	34	15	1
Antibiotic detection (D) and resistance phenotype (R) under aerobic conditions [a]					
	D/R	D/S	ND/R	ND/S	*P*[b]
CIP	24	24	3	0	0.238
CIP + NAL	22	17	5	7	0.511
DOX	1	23	0	27	0.471
DOX + TET	0	2	1	48	1
Antibiotic detection (D) and resistance phenotype (R) under anaerobic conditions [a]					
	D/R	D/S	ND/R	ND/S	*P*[b]
CIP	44	4	3	0	1
CIP + NAL	38	1	9	3	**0.036**
DOX	1	23	0	27	0.217
DOX + TET	0	2	1	48	1

584 [a] D=detected, ND=not detected, R=resistant by MIC, S=sensitive by MIC, P = Present, NP
585 = Not present.
586 [b] Fisher's Exact Test. Bold = statistically significant (P<0.05)
587

588 **REFERENCES**

589

590 1 Chun, C., Zheng, L. & Colgan, S. P. Tissue metabolism and host-microbial
591 interactions in the intestinal mucosa. *Free Radic Biol Med* **105**, 86-92,
592 doi:10.1016/j.freeradbiomed.2016.09.027 (2017).

593 2 Zheng, L., Kelly, C. J. & Colgan, S. P. Physiologic hypoxia and oxygen
594 homeostasis in the healthy intestine. A Review in the Theme: Cellular Responses
595 to Hypoxia. *Am J Physiol Cell Physiol* **309**, C350-360,
596 doi:10.1152/ajpcell.00191.2015 (2015).

597 3 *Performance Standards for Antimicrobial Susceptibility Testing*. 27th edn,
598 (Clinical and Laboratory Standards Institue (CLSI), 2017).

599 4 Bueno, E., Sit, B., Waldor, M. K. & Cava, F. Genetic Dissection of the
600 Fermentative and Respiratory Contributions Supporting *Vibrio cholerae* Hypoxic
601 Growth. *J Bacteriol* **202**, doi:10.1128/JB.00243-20 (2020).

602 5 Bueno, E., Pinedo, V. & Cava, F. Adaptation of *Vibrio cholerae* to Hypoxic
603 Environments. *Front Microbiol* **11**, 739, doi:10.3389/fmicb.2020.00739 (2020).

604 6 Schild, S. *et al.* Genes induced late in infection increase fitness of *Vibrio cholerae*
605 after release into the environment. *Cell Host Microbe* **2**, 264-277,
606 doi:10.1016/j.chom.2007.09.004 (2007).

607 7 Xu, Q., Dziejman, M. & Mekalanos, J. J. Determination of the transcriptome of
608 *Vibrio cholerae* during intraintestinal growth and midexponential phase in vitro.
609 *Proc Natl Acad Sci U S A* **100**, 1286-1291, doi:10.1073/pnas.0337479100
610 (2003).

611 8 Mandlik, A. *et al.* RNA-Seq-based monitoring of infection-linked changes in *Vibrio*
612 *cholerae* gene expression. *Cell Host Microbe* **10**, 165-174,
613 doi:10.1016/j.chom.2011.07.007 (2011).
614 9 Beaber, J. W., Hochhut, B. & Waldor, M. K. SOS response promotes horizontal
615 dissemination of antibiotic resistance genes. *Nature* **427**, 72-74,
616 doi:10.1038/nature02241 (2004).
617 10 Narendrakumar, L., Gupta, S. S., Johnson, J. B., Ramamurthy, T. & Thomas, S.
618 Molecular Adaptations and Antibiotic Resistance in *Vibrio cholerae*: A Communal
619 Challenge. *Microb Drug Resist* **25**, 1012-1022, doi:10.1089/mdr.2018.0354
620 (2019).
621 11 Das, B., Verma, J., Kumar, P., Ghosh, A. & Ramamurthy, T. Antibiotic resistance
622 in *Vibrio cholerae*: Understanding the ecology of resistance genes and
623 mechanisms. *Vaccine* **38 Suppl 1**, A83-A92, doi:10.1016/j.vaccine.2019.06.031
624 (2020).
625 12 *The treatment of diarrhoea -- A manual for physicians and other senior health*
626 *workers. —4th rev.* 4th Rev. edn, Vol. WHO/CAH/03.7 (World Heath
627 Organization).
628 13 Control, G. T. F. o. C. *Cholera outbreak: assessing the outbreak response and*
629 *improving preparedness*. Vol. WHO/CDS/CPE/ZFK/2004.4 (World Health
630 Organization, 2004).
631 14 Control., G. T. F. o. C. *Use of antibiotics for the treatment and control of cholera*,
632 <https://www.who.int/cholera/task_force/use-of-antibiotics-for-the-treatment-of-
633 cholera.pdf?ua=1> (2018).

634 15 Nelson, E. J., Nelson, D. S., Salam, M. A. & Sack, D. A. Antibiotics for both
635 moderate and severe cholera. *N Engl J Med* **364**, 5-7,
636 doi:10.1056/NEJMp1013771.
637 16 Leibovici-Weissman, Y. *et al.* Antimicrobial drugs for treating cholera. *Cochrane*
638 *Database Syst Rev*, CD008625, doi:10.1002/14651858.CD008625.pub2 (2014).
639 17 Khan, A. I. *et al.* Electronic decision-support improves diarrhoeal disease
640 guideline adherence (mHealth Diarrhoea Management, mHDM, Trial): a cluster
641 randomized controlled trial. *Lancet DH* **2**, e250-258,
642 doi:https://doi.org/10.1016/S2589-7500(20)30062-5 (2020).
643 18 Biswas, D. *et al.* An ethnographic exploration of diarrheal disease management
644 in public hospitals in Bangladesh: From problems to solutions. *Soc Sci Med* **260**,
645 113185, doi:10.1016/j.socscimed.2020.113185 (2020).
646 19 Ingle, D. J., Levine, M. M., Kotloff, K. L., Holt, K. E. & Robins-Browne, R. M.
647 Dynamics of antimicrobial resistance in intestinal Escherichia coli from children in
648 community settings in South Asia and sub-Saharan Africa. *Nat Microbiol* **3**, 1063-
649 1073, doi:10.1038/s41564-018-0217-4 (2018).
650 20 Weill, F. X. *et al.* Genomic history of the seventh pandemic of cholera in Africa.
651 *Science* **358**, 785-789, doi:10.1126/science.aad5901 (2017).
652 21 Towner, K. J., Pearson, N. J., Mhalu, F. S. & O'Grady, F. Resistance to
653 antimicrobial agents of *Vibrio cholerae* E1 Tor strains isolated during the fourth
654 cholera epidemic in the United Republic of Tanzania. *Bull World Health Organ*
655 **58**, 747-751 (1980).

656 22 Burrus, V., Marrero, J. & Waldor, M. K. The current ICE age: biology and
657 evolution of SXT-related integrating conjugative elements. *Plasmid* **55**, 173-183,
658 doi:S0147-619X(06)00003-5 [pii]
659 10.1016/j.plasmid.2006.01.001 (2006).
660 23 Redgrave, L. S., Sutton, S. B., Webber, M. A. & Piddock, L. J. Fluoroquinolone
661 resistance: mechanisms, impact on bacteria, and role in evolutionary success.
662 *Trends Microbiol* **22**, 438-445, doi:10.1016/j.tim.2014.04.007 (2014).
663 24 Hooper, D. C. & Jacoby, G. A. Topoisomerase Inhibitors: Fluoroquinolone
664 Mechanisms of Action and Resistance. *Cold Spring Harb Perspect Med* **6**,
665 doi:10.1101/cshperspect.a025320 (2016).
666 25 Piddock, L. J. Mechanisms of fluoroquinolone resistance: an update 1994-1998.
667 *Drugs* **58 Suppl 2**, 11-18, doi:10.2165/00003495-199958002-00003 (1999).
668 26 Everett, M. J., Jin, Y. F., Ricci, V. & Piddock, L. J. Contributions of individual
669 mechanisms to fluoroquinolone resistance in 36 Escherichia coli strains isolated
670 from humans and animals. *Antimicrob Agents Chemother* **40**, 2380-2386,
671 doi:10.1128/AAC.40.10.2380 (1996).
672 27 Komp Lindgren, P., Marcusson, L. L., Sandvang, D., Frimodt-Moller, N. &
673 Hughes, D. Biological cost of single and multiple norfloxacin resistance mutations
674 in Escherichia coli implicated in urinary tract infections. *Antimicrob Agents*
675 *Chemother* **49**, 2343-2351, doi:10.1128/AAC.49.6.2343-2351.2005 (2005).
676 28 Morgan-Linnell, S. K. & Zechiedrich, L. Contributions of the combined effects of
677 topoisomerase mutations toward fluoroquinolone resistance in Escherichia coli.

678 *Antimicrob Agents Chemother* **51**, 4205-4208, doi:10.1128/AAC.00647-07
679 (2007).
680 29 Martinez-Martinez, L., Pascual, A. & Jacoby, G. A. Quinolone resistance from a
681 transferable plasmid. *Lancet* **351**, 797-799, doi:10.1016/S0140-6736(97)07322-4
682 (1998).
683 30 Briales, A. *et al.* Prevalence of plasmid-mediated quinolone resistance
684 determinants qnr and aac(6')-Ib-cr in Escherichia coli and Klebsiella pneumoniae
685 producing extended-spectrum beta-lactamases in Spain. *Int J Antimicrob Agents*
686 **39**, 431-434, doi:10.1016/j.ijantimicag.2011.12.009 (2012).
687 31 Wozniak, R. A. *et al.* Comparative ICE genomics: insights into the evolution of
688 the SXT/R391 family of ICEs. *PLoS Genet* **5**, e1000786,
689 doi:10.1371/journal.pgen.1000786 (2009).
690 32 Beaber, J. W., Hochhut, B. & Waldor, M. K. Genomic and functional analyses of
691 SXT, an integrating antibiotic resistance gene transfer element derived from
692 *Vibrio cholerae*. *J Bacteriol* **184**, 4259-4269, doi:10.1128/jb.184.15.4259-
693 4269.2002 (2002).
694 33 Garriss, G., Waldor, M. K. & Burrus, V. Mobile antibiotic resistance encoding
695 elements promote their own diversity. *PLoS Genet* **5**, e1000775,
696 doi:10.1371/journal.pgen.1000775 (2009).
697 34 Fonseca, E. L., Dos Santos Freitas, F., Vieira, V. V. & Vicente, A. C. New qnr
698 gene cassettes associated with superintegron repeats in *Vibrio cholerae* O1.
699 *Emerg Infect Dis* **14**, 1129-1131, doi:10.3201/eid1407.080132 (2008).

700 35 Fyfe, C., Grossman, T. H., Kerstein, K. & Sutcliffe, J. Resistance to Macrolide
701 Antibiotics in Public Health Pathogens. *Cold Spring Harb Perspect Med* **6**,
702 doi:10.1101/cshperspect.a025395 (2016).
703 36 Grossman, T. H. Tetracycline Antibiotics and Resistance. *Cold Spring Harb*
704 *Perspect Med* **6**, a025387, doi:10.1101/cshperspect.a025387 (2016).
705 37 Zhu, Z. *et al.* Entropy of a bacterial stress response is a generalizable predictor
706 for fitness and antibiotic sensitivity. *Nat Commun* **11**, 4365, doi:10.1038/s41467-
707 020-18134-z (2020).
708 38 Wood, S. *et al.* in *The Pangenome: Diversity, Dynamics and Evolution of*
709 *Genomes* (eds H. Tettelin & D. Medini) 169-202 (2020).
710 39 Warrier, I. *et al.* The Transcriptional landscape of Streptococcus pneumoniae
711 TIGR4 reveals a complex operon architecture and abundant riboregulation
712 critical for growth and virulence. *PLoS Pathog* **14**, e1007461,
713 doi:10.1371/journal.ppat.1007461 (2018).
714 40 Jensen, P. A., Zhu, Z. & van Opijnen, T. Antibiotics Disrupt Coordination between
715 Transcriptional and Phenotypic Stress Responses in Pathogenic Bacteria. *Cell*
716 *Rep* **20**, 1705-1716, doi:10.1016/j.celrep.2017.07.062 (2017).
717 41 van Opijnen, T., Dedrick, S. & Bento, J. Strain Dependent Genetic Networks for
718 Antibiotic-Sensitivity in a Bacterial Pathogen with a Large Pan-Genome. *PLoS*
719 *Pathog* **12**, e1005869, doi:10.1371/journal.ppat.1005869 (2016).
720 42 Cain, A. K. *et al.* A decade of advances in transposon-insertion sequencing. *Nat*
721 *Rev Genet* **21**, 526-540, doi:10.1038/s41576-020-0244-x (2020).

722 43 Dorr, T. *et al.* A Transposon Screen Identifies Genetic Determinants of *Vibrio*
723 *cholerae* Resistance to High-Molecular-Weight Antibiotics. *Antimicrob Agents*
724 *Chemother* **60**, 4757-4763, doi:10.1128/AAC.00576-16 (2016).
725 44 Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. & Collins, J. J. A
726 common mechanism of cellular death induced by bactericidal antibiotics. *Cell*
727 **130**, 797-810, doi:10.1016/j.cell.2007.06.049 (2007).
728 45 Maslowska, K. H., Makiela-Dzbenska, K. & Fijalkowska, I. J. The SOS system: A
729 complex and tightly regulated response to DNA damage. *Environ Mol Mutagen*
730 **60**, 368-384, doi:10.1002/em.22267 (2019).
731 46 Dwyer, D. J. *et al.* Antibiotics induce redox-related physiological alterations as
732 part of their lethality. *Proc Natl Acad Sci U S A* **111**, E2100-2109,
733 doi:10.1073/pnas.1401876111 (2014).
734 47 Staerck, C. *et al.* Microbial antioxidant defense enzymes. *Microb Pathog* **110**, 56-
735 65, doi:10.1016/j.micpath.2017.06.015 (2017).
736 48 Smirnova, G., Muzyka, N. & Oktyabrsky, O. Transmembrane glutathione cycling
737 in growing Escherichia coli cells. *Microbiol Res* **167**, 166-172,
738 doi:10.1016/j.micres.2011.05.005 (2012).
739 49 Bryan, L. E. & Kwan, S. Mechanisms of aminoglycoside resistance of anaerobic
740 bacteria and facultative bacteria grown anaerobically. *J Antimicrob Chemother* **8**
741 **Suppl D**, 1-8, doi:10.1093/jac/8.suppl_d.1 (1981).
742 50 Bryan, L. E. General mechanisms of resistance to antibiotics. *J Antimicrob*
743 *Chemother* **22 Suppl A**, 1-15, doi:10.1093/jac/22.supplement_a.1 (1988).

744 51 Bryant, R. E., Fox, K., Oh, G. & Morthland, V. H. Beta-lactam enhancement of
745 aminoglycoside activity under conditions of reduced pH and oxygen tension that
746 may exist in infected tissues. *J Infect Dis* **165**, 676-682,
747 doi:10.1093/infdis/165.4.676 (1992).
748 52 Dwyer, D. J., Kohanski, M. A., Hayete, B. & Collins, J. J. Gyrase inhibitors induce
749 an oxidative damage cellular death pathway in Escherichia coli. *Mol Syst Biol* **3**,
750 91, doi:10.1038/msb4100135 (2007).
751 53 Drlica, K. & Zhao, X. Bacterial death from treatment with fluoroquinolones and
752 other lethal stressors. *Expert Rev Anti Infect Ther*, 1-18,
753 doi:10.1080/14787210.2021.1840353 (2020).
754 54 Hong, Y. *et al.* Reactive oxygen species play a dominant role in all pathways of
755 rapid quinolone-mediated killing. *J Antimicrob Chemother* **75**, 576-585,
756 doi:10.1093/jac/dkz485 (2020).
757 55 Luan, G., Hong, Y., Drlica, K. & Zhao, X. Suppression of Reactive Oxygen
758 Species Accumulation Accounts for Paradoxical Bacterial Survival at High
759 Quinolone Concentration. *Antimicrob Agents Chemother* **62**,
760 doi:10.1128/AAC.01622-17 (2018).
761 56 Zhao, X. & Drlica, K. Reactive oxygen species and the bacterial response to
762 lethal stress. *Curr Opin Microbiol* **21**, 1-6, doi:10.1016/j.mib.2014.06.008 (2014).
763 57 Zhao, X., Hong, Y. & Drlica, K. Moving forward with reactive oxygen species
764 involvement in antimicrobial lethality. *J Antimicrob Chemother* **70**, 639-642,
765 doi:10.1093/jac/dku463 (2015).

766 58 Nelson, E. J. *et al.* Complexity of rice-water stool from patients with *Vibrio*
767 *cholerae* plays a role in the transmission of infectious diarrhea. *Proc Natl Acad*
768 *Sci U S A* **104**, 19091-19096, doi:0706352104 [pii]
769 10.1073/pnas.0706352104 (2007).
770 59 Sprouffske, K. & Wagner, A. Growthcurver: an R package for obtaining
771 interpretable metrics from microbial growth curves. *BMC Bioinformatics* **17**, 172,
772 doi:10.1186/s12859-016-1016-7 (2016).
773 60 Jia, B. *et al.* CARD 2017: expansion and model-centric curation of the
774 comprehensive antibiotic resistance database. *Nucleic Acids Res* **45**, D566-
775 D573, doi:10.1093/nar/gkw1004 (2017).
776 61 McArthur, A. G. *et al.* The comprehensive antibiotic resistance database.
777 *Antimicrob Agents Chemother* **57**, 3348-3357, doi:10.1128/AAC.00419-13
778 (2013).
779 62 Zankari, E. Comparison of the web tools ARG-ANNOT and ResFinder for
780 detection of resistance genes in bacteria. *Antimicrob Agents Chemother* **58**,
781 4986, doi:10.1128/AAC.02620-14 (2014).
782 63 Kleinheinz, K. A., Joensen, K. G. & Larsen, M. V. Applying the ResFinder and
783 VirulenceFinder web-services for easy identification of acquired antibiotic
784 resistance and E. coli virulence genes in bacteriophage and prophage nucleotide
785 sequences. *Bacteriophage* **4**, e27943, doi:10.4161/bact.27943 (2014).
786 64 Drezen, E. *et al.* GATB: Genome Assembly & Analysis Tool Box. *Bioinformatics*
787 **30**, 2959-2961, doi:10.1093/bioinformatics/btu406 (2014).

788 65 Lees, J. A., Galardini, M., Bentley, S. D., Weiser, J. N. & Corander, J. pyseer: a
789 comprehensive tool for microbial pangenome-wide association studies.
790 *Bioinformatics* **34**, 4310-4312, doi:10.1093/bioinformatics/bty539 (2018).
791 66 Cook, D. E. & Andersen, E. C. VCF-kit: assorted utilities for the variant call
792 format. *Bioinformatics* **33**, 1581-1582, doi:10.1093/bioinformatics/btx011 (2017).
793 67 Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-
794 analysis of large phylogenies. *Bioinformatics* **30**, 1312-1313,
795 doi:10.1093/bioinformatics/btu033 (2014).
796 68 Saber, M. M. & Shapiro, B. J. Benchmarking bacterial genome-wide association
797 study methods using simulated genomes and phenotypes. *Microb Genom* **6**,
798 doi:10.1099/mgen.0.000337 (2020).
799 69 Alexandrova, L. *et al.* Identification of widespread antibiotic exposure in cholera
800 patients correlates with clinically relevant microbiota changes. *J Infect Dis*,
801 doi:10.1093/infdis/jiz299 (2019).
802 70 Davydova, N., Streltsov, V., Wilce, M., Liljas, A. & Garber, M. L22 ribosomal
803 protein and effect of its mutation on ribosome resistance to erythromycin. *J Mol*
804 *Biol* **322**, 635-644, doi:10.1016/s0022-2836(02)00772-6 (2002).
805 71 Hansen, L. H., Mauvais, P. & Douthwaite, S. The macrolide-ketolide antibiotic
806 binding site is formed by structures in domains II and V of 23S ribosomal RNA.
807 *Mol Microbiol* **31**, 623-631, doi:10.1046/j.1365-2958.1999.01202.x (1999).
808 72 Williams, G. & Smith, I. Chromosomal mutations causing resistance to
809 tetracycline in Bacillus subtilis. *Mol Gen Genet* **177**, 23-29,
810 doi:10.1007/BF00267249 (1979).

811 73 Wei, Y. & Bechhofer, D. H. Tetracycline induces stabilization of mRNA in Bacillus
812 subtilis. *J Bacteriol* **184**, 889-894, doi:10.1128/jb.184.4.889-894.2002 (2002).
813 74 Beabout, K. *et al.* The ribosomal S10 protein is a general target for decreased
814 tigecycline susceptibility. *Antimicrob Agents Chemother* **59**, 5561-5566,
815 doi:10.1128/AAC.00547-15 (2015).
816 75 Leclercq, S. O. *et al.* Diversity of the Tetracycline Mobilome within a Chinese Pig
817 Manure Sample. *Appl Environ Microbiol* **82**, 6454-6462, doi:10.1128/AEM.01754-
818 16 (2016).
819 76 Grossman, T. H. *et al.* Target- and resistance-based mechanistic studies with
820 TP-434, a novel fluorocycline antibiotic. *Antimicrob Agents Chemother* **56**, 2559-
821 2564, doi:10.1128/AAC.06187-11 (2012).
822 77 Noguchi, N. *et al.* Nucleotide sequence and characterization of erythromycin
823 resistance determinant that encodes macrolide 2'-phosphotransferase I in
824 Escherichia coli. *Antimicrob Agents Chemother* **39**, 2359-2363,
825 doi:10.1128/AAC.39.10.2359 (1995).
826 78 Chesneau, O., Tsvetkova, K. & Courvalin, P. Resistance phenotypes conferred
827 by macrolide phosphotransferases. *FEMS Microbiol Lett* **269**, 317-322,
828 doi:10.1111/j.1574-6968.2007.00643.x (2007).
829 79 Mathur, J. & Waldor, M. K. The *Vibrio cholerae* ToxR-regulated porin OmpU
830 confers resistance to antimicrobial peptides. *Infect Immun* **72**, 3577-3583,
831 doi:10.1128/IAI.72.6.3577-3583.2004 (2004).

832 80 Li, C. C., Crawford, J. A., DiRita, V. J. & Kaper, J. B. Molecular cloning and
833 transcriptional regulation of ompT, a ToxR-repressed gene in *Vibrio cholerae*.
834 *Mol Microbiol* **35**, 189-203, doi:10.1046/j.1365-2958.2000.01699.x (2000).
835 81 Buckley, A. M. *et al.* The AcrAB-TolC efflux system of Salmonella enterica
836 serovar Typhimurium plays a role in pathogenesis. *Cell Microbiol* **8**, 847-856,
837 doi:10.1111/j.1462-5822.2005.00671.x (2006).
838 82 Taylor, D. L., Bina, X. R. & Bina, J. E. *Vibrio cholerae* VexH encodes a multiple
839 drug efflux pump that contributes to the production of cholera toxin and the toxin
840 co-regulated pilus. *PLoS One* **7**, e38208, doi:10.1371/journal.pone.0038208
841 (2012).
842 83 Bina, X. R., Philippart, J. A. & Bina, J. E. Effect of the efflux inhibitors 1-(1-
843 naphthylmethyl)-piperazine and phenyl-arginine-beta-naphthylamide on
844 antimicrobial susceptibility and virulence factor production in *Vibrio cholerae*. *J*
845 *Antimicrob Chemother* **63**, 103-108, doi:10.1093/jac/dkn466 (2009).
846 84 Doron, S. *et al.* Systematic discovery of antiphage defense systems in the
847 microbial pangenome. *Science* **359**, doi:10.1126/science.aar4120 (2018).
848 85 Baddam, R. *et al.* Genome Dynamics of *Vibrio cholerae* Isolates Linked to
849 Seasonal Outbreaks of Cholera in Dhaka, Bangladesh. *mBio* **11**,
850 doi:10.1128/mBio.03339-19 (2020).
851 86 Murphy, S. G., Johnson, B. A., Ledoux, C. M. & Dörr, T. *Vibrio*
852 *cholerae*'s mysterious Seventh Pandemic island (VSP-II) encodes novel
853 Zur-regulated zinc starvation genes involved in chemotaxis and autoaggregation.
854 *bioRxiv*, 2021.2003.2009.434465, doi:10.1101/2021.03.09.434465 (2021).

855 87 Angeles, L. F. *et al.* Retrospective suspect screening reveals previously ignored
856 antibiotics, antifungal compounds, and metabolites in Bangladesh surface
857 waters. *Sci Total Environ* **712**, 136285, doi:10.1016/j.scitotenv.2019.136285
858 (2020).
859