Anaerobic conditions unmask antimicrobial resistance in *Vibrio cholerae*

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Abstract

The antibiotic formulary is threatened by high rates of antimicrobial resistance (AMR) among enteropathogens. Enteric bacteria often undergo anaerobic respiration within the gastrointestinal tract, yet little is known about how anaerobic conditions influence AMR. The facultative enteropathogen *Vibrio cholerae* was chosen as a model to address this knowledge gap. *V. cholerae* isolates were tested for resistance under aerobic and anaerobic conditions against clinically relevant antibiotics. Using conventional breakpoints established for aerobic conditions, the odds of classifying isolates as resistant increased over 10 times for ciprofloxacin and 100 times for azithromycin under anaerobic conditions compared to aerobic conditions; for doxycycline, nearly all isolates remained sensitive. Genome-wide association studies found associations between genetic elements and AMR phenotypes that varied by oxygen exposure and antibiotic concentrations; AMR phenotypes were more heritable – and more AMR genes were discovered – under anaerobic conditions. These findings challenge the paradigm of testing facultative enteropathogens for AMR under aerobic conditions alone.

INTRODUCTION

Clinically relevant laboratory methods are essential to gauge the extent to which the antibiotic formulary is threatened by antimicrobial resistance (AMR). Knowledge gaps remain on the degree to which *in vitro* AMR assays reflect *in vivo* AMR physiology. Facultative anaerobic pathogens experience hypoxia and anoxia within the gastrointestinal tract, yet AMR assays rely on aerobic conditions [1]. How 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* [2-8], and cholera is one of the few non-invasive diarrheal diseases for which antibiotics are indicated, albeit conditionally [9-11].

Rehydration is the definitive intervention for acute diarrheal disease [9]; antibiotics are supportive and indicated for only a few diarrheal diseases, including cholera. The World Health Organization (WHO) recommends ciprofloxacin, azithromycin or doxycycline for cholera patients with severe dehydration [9-11]; antibiotics shorten the frequency and duration of diarrhea. In practice, guideline adherence in cholera endemic regions may be low out of clinical concern that a patient ‘might’ have cholera and may develop severe dehydration, contributing to rates of inappropriate antibiotic usage that can rise above 90% [12, 13]. Strong regional associations between antibiotic use and rise of AMR have been observed across enteric taxa [14, 15]. Given that AMR genetic elements frequently co-localize on mobile elements [16], inappropriate single-agent therapy also poses a risk of multidrug-resistance (MDR) selection.

38 Associations between AMR phenotypes and genotypes are known for the three
39 antibiotics recommended to treat cholera; the cognate AMR mechanisms share
40 commonality across Gram negative taxa. Ciprofloxacin (a fluoroquinolone) resistance
41 mechanisms include mutations in genes encoding type II topoisomerases:
42 heterotetrameric DNA gyrase ($GyrA_2GyrB_2$) and DNA topoisomerase IV ($ParC_2ParE_2$).
43 Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* can
44 yield additive resistance phenotypes [17]. Efflux pump upregulation, downregulation of
45 outer membrane porins that permit quinolone entry and Qnr expression that protects the
46 target gyrase also convey fluoroquinolone resistance [17]. Resistance can increase over
47 30-fold compared to wild-type when strains harbor *qnr* family genes.

48 In *V. cholerae*, diverse AMR genes, including *qnr,* often reside on an integrative and
49 conjugative element (ICE; 'SXT' in *V. cholerae*) [16, 18, 19]. Azithromycin (a macrolide)
50 resistance mechanisms are similarly diverse and include mutations in the 23S ribosomal
51 RNA (rRNA) target genes and ribosomal protein genes. Macrolide resistance is also
52 conveyed by carriage of rRNA methyltransferase genes (*erm*) and associated induction
53 mechanisms, *cis*-acting peptides, efflux systems (e.g. *mef, msr)*, macrolide esterases
54 (e.g. *ere*), and macrolide phosphotransferases (e.g. *mphA); mphA* often resides in the
55 *V. cholerae* SXT element [20]. Doxycycline (a tetracycline) resistance is conferred by
56 mutations in the 16S rRNA component of the 30S ribosomal subunit [21]. Additional
57 mechanisms include tetracycline-specific ribosomal protection proteins (RPPs),
58 tetracycline specific efflux pumps (e.g. *tet(59)*) that can reside on SXT element intrinsic
59 efflux pumps, AraC-family transcriptional activators (e.g. MarA), and cytoplasmic ATP-
60 dependent serine proteases [21].

61 Associations between AMR phenotypes and genotypes have been studied by random
62 mutagenesis, phenotypic screening, and network analyses, and applied in *V. cholerae*
63 [22]. These approaches uncovered how the effect of an antibiotic is shaped by a large
64 number of often more subtle physiologic perturbations, including altered DNA
65 synthesis/repair, central metabolism/growth, and SOS response [23, 24]. A limitation of
66 AMR assays conducted under aerobic conditions alone may not reflect these
67 physiologic perturbations experienced in the host. Aerobic oxidative phosphorylation
68 generates reactive oxygen species (ROS) that are lethal unless a sufficient defense is
69 mounted by factors like superoxide dismutase, catalase, and glutathione systems [25,
70 26]. Under anaerobic conditions, growth rate typically slows and proton motive force is
71 reduced [27, 28], which can have both synergistic and antagonistic effects on antibiotics
72 [24, 29]. In *Escherichia coli,* ROS are generated after fluroquinolone treatment under
73 aerobic conditions[30] and fluoroquinolone resistance increases under anaerobic
74 conditions [31, 32]. The extent to which tetracyclines and macrolides induce ROS and
75 how anaerobiosis influences resistance is less known [23, 33].

76 The objective of this study was to compare AMR phenotypes, with underlying
77 genotypes, under aerobic and anaerobic conditions. We found that resistance increased
78 significantly under anaerobic conditions for select antibiotics, and distinct genetic
79 elements associated with AMR with and without oxygen exposure.

80 **METHODS**

81 **Clinical sample collection.** The two sample collections analyzed were part of
82 previously published IRB approved studies [12, 34]. In the primary collection, stool

83 samples were obtained during the spring cholera outbreak period of 2006 at the
84 International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka,
85 Bangladesh. Samples were collected prior to hospital administration of antibiotics;
86 patient histories were negative for known antibiotic exposure. The library consisted of
87 67 *V. cholerae* isolates (Supplementary Table 1); paired stool supernatant for mass
88 spectrometry was available for 50 isolates. In the secondary collection, samples were
89 obtained in 2018 as part of a cholera surveillance study conducted across Bangladesh
90 [12]. Samples were collected at hospital admission independent of reported antibiotic
91 exposure; 120 isolates were analyzed to assess generalizability of the AMR profiles
92 identified in the primary collection.

93 **Antimicrobial resistance testing.** Growth kinetics and the minimal inhibitory
94 concentration (MIC) determinations for ciprofloxacin, azithromycin, and doxycycline
95 were performed on isolates from the primary collection in LB broth with twelve two-fold
96 serial dilutions with concentrations approximated for CLSI MIC breakpoints[1] for *V.*
97 *cholerae* (ciprofloxacin = 2 μg/ml; azithromycin = 8 μg/ml; doxycycline = 8 μg/ml)[1].
98 Isolates were prepared and grown aerobically at 37˚C in 15-ml tubes containing 5-ml LB
99 broth at 220 rpm Bacteria were back-diluted to a final OD_{600} of 0.01 (200 μl/well) in LB
100 with or without the respective antibiotic dilution-series in black Corning CoStar clear-
101 bottom 96-well plates. Plates were placed in a BioTek Synergy H1 reader pre-warmed
102 to 37˚C with the lid on. Anaerobic conditions were generated using a continuous
103 chamber flow (5% CO_2, 95% N_2) and a BioTek CO_2/O_2 gas controller; anaerobic growth
104 plates were given a 10-minute equilibration period. OD_{600} was measured every 2
105 minutes for 8 hours at 37˚C with orbital shaking at 220 rpm. A standard logistic equation

106 was fit to growth curve data using the R package *growthcurver* version 0.3.0[35].
107 Outcome measures were intrinsic growth velocity (growth rate that would occur if there
108 were no restrictions on total population size), carrying capacity (K; maximum possible
109 population size), and area under the curve (AUC). The MIC was determined using a
110 logistic fit for growth over the twelve, two-fold serial dilutions of the test antibiotic. Binary
111 phenotypic resistance categories were set in concordance with Clinical and Laboratory
112 Standards Institute (CLSI); intermediate and resistant isolates were scored as resistant.
113 CLSI standards are based on assays conducted aerobically and break points are set by
114 clinical correlates of disease.

115 **Use of catalase to determine if ROS contribute to antibiotic sensitivity.** To test if
116 the reduction of ROS was associated with increased resistance to antibiotics under
117 aerobic conditions, MICs were determined for two select strains (E7946, EN160)
118 with/without catalase (10 U/ml) added to the media. Growth curves were performed with
119 viable counts as endpoints to determine the minimum dose for lethality by H_2O_2 or
120 protection by catalase.

121 **Molecular procedures.** Whole-genome sequencing was performed with conventional
122 methods (Supplementary Material). Known resistance genes were identified using the
123 comprehensive antibiotic resistance database (CARD) [36] and ResFinder [37]. A series
124 of genome-wide association studies (GWAS) were conducted on the primary collection
125 to detect genomic variants associated with antibiotic resistance phenotypes under both
126 aerobic and anaerobic conditions; a total of 30 GWAS were generated by 3 antibiotics x
127 5 antibiotic concentrations x 2 oxygen conditions. The sample size for analysis was 67
128 genomes. GWAS bioinformatic methods are provided (Supplementary Material).

129 **Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS).**
130 The approach was based on a former study [38]. Preparation of stool supernatants for
131 analysis using high-performance liquid chromatography (Thermo UltiMate 3000 series)
132 and an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific) are provided
133 (Supplementary Material).

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

137 **RESULTS**

138 **Comparison of antimicrobial resistance under aerobic and anaerobic conditions.**
139 Pairwise analyses for baseline growth parameters found that carrying capacity, AUC,
140 and growth velocity of the population were significantly lower under anaerobic
141 compared to aerobic conditions (Supplementary Table 2). In this physiologic context,
142 AMR differed depending on anaerobic versus aerobic conditions (Figure 1); distributions
143 of single and multi-agent AMR phenotypes are shown (Figure 2A). The MIC modes for
144 ciprofloxacin were 7 μg/ml (min=0.0016 μg/ml; max=32 μg/ml) and 2 μg/ml (min=0.004
145 μg/ml; max=8 μg/ml) under anaerobic and aerobic conditions, respectively
146 (Supplementary Table 3); the rates of resistance under anaerobic (93%; N=62/67) and
147 aerobic (54%; N=36/67) conditions were significantly different (Figure 1a;
148 Supplementary Table 4). For azithromycin, the MIC modes were 32 μg/ml (min= 8
149 μg/ml; max=128 μg/ml) and 4 μg/ml (min=1 μg/ml; max=32 μg/ml) under anaerobic and
150 aerobic conditions respectively (Supplementary Table 3). The rates of resistance under

151 anaerobic (n=67/67; 100%) and aerobic (n=15/67; 22%) conditions were significantly
152 different (Figure 1b; Supplementary Table 4). For doxycycline, the MIC modes were 1
153 µg/ml under both aerobic and anaerobic conditions, respectively (Supplementary Table
154 3); only two isolates were found to be resistant under anaerobic conditions and one
155 under aerobic conditions. The odds of classifying isolates as resistant increased over 10
156 times for ciprofloxacin (OR= 10.5; 95% CI= 3.61-37.7) and over 200 times for
157 azithromycin (OR = 213; 95% CI= 31.9->5000) under anaerobic compared to aerobic
158 conditions.

159 To evaluate the generalizability of these findings from the primary sample collection,
160 pairwise analyses were conducted on 120 isolates from the secondary sample
161 collection (Supplementary Table 5). For ciprofloxacin, the rates of resistance were
162 significantly different under anaerobic (39%; n= 47/120) and aerobic conditions (1.6%;
163 n= 2/120; McNemar's test p<0.001). For azithromycin, the rates of resistance were
164 significantly different under anaerobic (100%; N= 120/120) and aerobic conditions (47%;
165 N= 56/120; McNemar's test p<0.001); only two isolates were resistant under anaerobic
166 conditions and zero under aerobic conditions. The odds of classifying isolates as
167 resistant increased over 37 times for ciprofloxacin (OR= 37.5; 95% CI= 9.34-328) and
168 over 136 times under azithromycin (OR = 136; 95% CI= 22.3 - >5000) for anaerobic
169 compared to aerobic conditions.

170 **Addition of catalase to test if reactive oxygen species effect antibiotic**
171 **resistance/sensitivity under aerobic conditions.** For ciprofloxacin, the MICs for the
172 sensitive reference strain E7946 (Cip^S, Azi^S, Dox^S) and the resistant clinical isolate
173 EN160 (Cip^R, Azi^R, Dox^S) remained unchanged when catalase was added to the media

174 under aerobic conditions. The addition of catalase was not associated with differences
175 in AUCs for both E7946 and EN160 in media containing ciprofloxacin, azithromycin, or
176 doxycycline at 2-fold below the MIC. The AUCs in LB media with and without catalase
177 alone for E7946 and EN160 were not statistically different (Supplementary Table 6).

178 **Molecular AMR correlates under aerobic and anaerobic conditions**. The distribution
179 of known AMR genetic elements are shown (Fig 2B). AMR associated point mutations
180 (likely transmitted vertically, not on an established mobilizable element), and genes on
181 known horizontally transferred mobilizable elements, are provided (Supplementary
182 Material). The integrative conjugative element (ICE) SXT/R391 was found in 90%
183 (60/67) of isolates. The ICE elements contained a pentapeptide repeat protein
184 conferring fluoroquinolone resistance (qnr_{Vc}), a macrolide-inactivating
185 phosphotransferase (*mph*(A)), and a major facilitator superfamily (MFS) efflux pump
186 conferring tetracycline resistance (*tet*(59)) [39-42]. The genes qnr_{Vc}, *mph*(A), and *tet*(59)
187 were found in 78% (52/67), 33% (22/67), and 78% (52/67) of isolates, respectively.
188 Ciprofloxacin resistance under both anaerobic and aerobic conditions was significantly
189 associated with qnr_{VC}, *gyrA* and *parC* (Supplementary Table 7). Identification of the
190 known azithromycin AMR gene *mph(A)* was significantly associated with resistance
191 under aerobic conditions alone ($P<0.001$). The gene *tet(59)* was not associated with
192 doxycycline resistance under aerobic or aerobic conditions (both $p=0.566$).

193 GWAS was conducted to more broadly explore the genetic basis of AMR. This
194 approach used the phenotype of AUC to represent 'growth' with or without exposure to
195 the three antibiotics at five concentrations under either aerobic or anaerobic conditions.
196 Phenotypes (AUCs) at similar antibiotic concentrations were positively correlated within

197 aerobic and anaerobic conditions for all three antibiotics (Figure 3). Phenotypes were
198 also positively correlated between aerobic and anaerobic conditions for ciprofloxacin
199 (Figure 3a). However, AUCs were weakly, or even negatively, correlated between
200 aerobic and anaerobic conditions for azithromycin and doxycycline (Figure 3b,c). These
201 results reinforce the proposal that anaerobic and aerobic growth are distinct under
202 antibiotic pressure.

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

213 AMR genes identified by GWAS were diverse (Figure 4; Supplementary Data Files).
214 These candidates included known AMR genes, such as *qnrVC* and *dfrA,* which were
215 associated with ciprofloxacin resistance under both aerobic and anaerobic conditions.
216 We identified seven genes associated with ciprofloxacin resistance under anaerobic
217 conditions alone (including the stress response gene *barA* and a *radC* homolog involved
218 in DNA repair), and ten genes under aerobic conditions alone (including *rtxB*). Under
219 anaerobic conditions, most genes were identified at ciprofloxacin concentrations at or

220 above 0.25 µg/ml; however, four genes, including *barA*, were identified under one of the
221 lowest tested ciprofloxacin concentrations (0.13 µg/ml). GWAS hits for azithromycin and
222 doxycycline resistance were found only under anaerobic conditions. For azithromycin,
223 two genetic elements were identified: *mphA* and a region between *ompT* and *dinG*
224 (*ompT-dinG)*. For doxycycline, 23 genes were shared across concentrations; however,
225 the gene discovery rate was highest at the lower concentrations (n=53 at 0.13 µg/ml; n
226 =26 µg/ml). GWAS hits included the major facilitator superfamily antibiotic efflux pump
227 *tet(59)* (Figure 4, Supplementary Data Files). The majority of genetic elements identified
228 have unknown function, yet the expected detection of known AMR genes suggests that
229 the genes of unknown function may indeed play a role in AMR.

230 **Antibiotics detected in stool by LC-MS/MS.** A total of 196 antibiotics were detected in
231 the 51 stool supernatants tested (Figure 5). At least one antibiotic was detected in 98%
232 (n=50/51), at least two antibiotics were detected in 94% (n=48/51), and three or more
233 antibiotics were detected in 90% (n=46/51) of stool supernatants (Figure 5). Antibiotics
234 detected were ciprofloxacin (n=48/51; 94%), tetracycline / doxycycline (n=46/51; 90%),
235 nalidixic acid (n=41/51; 80%), metronidazole (n=37/51; 73%), bactrim
236 (sulfamethoxazole/trimethoprim; n=22/51; 43%), and amoxicillin (n=2/51; 4%);
237 azithromycin was not detected. Detection of quinolone/fluoroquinolone and
238 tetracycline/doxycycline in stool by LC-MS/MS was not associated with AMR genotypes
239 or phenotypes (Supplementary Table 8). Associations for azithromycin could not be
240 tested because azithromycin was not detected in any stool supernatant.

241

242 **DISCUSSION**

243 In this study, the rate of resistance among clinical isolates differed significantly when
244 tested under aerobic versus anaerobic conditions. The extent to which isolates were
245 resistant under anaerobic conditions differed by antibiotic class. The genetic basis of
246 resistance was uniquely discoverable under anaerobic conditions, and also differed by
247 antibiotic class. The approach of using AUC growth curve data as a dependent
248 continuous variable for GWAS to identify AMR genetic elements under aerobic and
249 anaerobic conditions may provide a new framework to investigate AMR.

250 The odds of classifying isolates as resistant under anaerobic conditions compared to
251 aerobic conditions increased over 10 times for ciprofloxacin and over 200 times for
252 azithromycin. These results are likely generalizable across *V. cholerae* taxa in that
253 analysis of a second collection separated by more than 10 years found that the odds of
254 classifying isolates as resistant under anaerobic conditions compared to aerobic
255 conditions increased over 30 times for ciprofloxacin and over 100 times for
256 azithromycin. Physiologic mechanisms for increased resistance to antibiotics under
257 anaerobic conditions may include reduced growth velocity limiting the impact of agents
258 that directly or indirectly disrupt cell envelope integrity. Additional mechanisms may
259 include ROS that induce both intracellular and cell wall stress and can act
260 synergistically to potentiate antibiotic lethality. The assays that utilized catalase to
261 quench hydrogen peroxide were conducted to evaluate this possibility. The MIC for all
262 three antibiotics did not increase with the addition of catalase suggesting that the
263 reduction of ROS alone cannot account for increased MICs observed under anaerobic
264 conditions.

265 Multiple knowledge gaps on the genetic basis of AMR remain. This study prioritized the
266 factor of oxygen exposure as a determinant of AMR phenotype because facultative
267 enteropathogens experience hypoxia and anoxia in the animal host. The first phase of
268 the analysis focused on known AMR genotypes with known AMR phenotypes. For
269 ciprofloxacin, mutations in *parC* and carriage of qnr_{Vc} significantly associated with
270 phenotypic resistance under aerobic and anaerobic conditions; mutations in *gyrA*
271 significantly associated with resistant phenotypes under anaerobic conditions alone. For
272 azithromycin, *mphA* was identified and significantly associated with AMR phenotypes
273 under aerobic conditions alone. While *tet*(59) was identified, very few isolates were
274 identified as resistant to doxycycline under aerobic (n=1) or anaerobic conditions (n=2).
275 These data demonstrate that the relationship between AMR genotype and phenotype
276 may differ between aerobic and anaerobic conditions.

277 The second phase of analysis sought to identify unknown genetic elements associated
278 with AMR. Initial attempts to use categorical data (resistant vs sensitive) in the GWAS
279 resulted in modest gene discovery rates (data not shown). To address this problem, the
280 continuous variable of AUC strengthened the GWAS. Breakpoint and sub-breakpoint
281 concentrations were chosen based on a rationale that different genetic elements might
282 contribute differently to AMR phenotypes at different antibiotic concentrations. GWAS
283 expectedly identified qnr_{Vc} for ciprofloxacin exposure under aerobic and anaerobic
284 conditions, *mphA* for azithromycin exposure under anaerobic conditions alone and
285 *tet*(59) for doxycycline exposure under anaerobic conditions alone. The lack of detection
286 of *mphA* under aerobic conditions by GWAS conflicted with the conventional analysis.
287 For ciprofloxacin, seven genes were discovered in anaerobic conditions alone; these

288 included a homolog for DNA repair (*radC*), 2-component histidine kinase involved in
289 stress response (*barA*), and an ATP-dependent zinc protease. The genes *dfrA31* and
290 APH(6)-Id were identified for both aerobic and anaerobic conditions and encode a
291 trimethoprim-resistant dihydrofolate reductase and a streptomycin phosphotransferase
292 enzyme, respectively. These genes are located on SXT with qnr_{Vc} and may therefore be
293 associated due to genetic linkage rather than due to causal roles in ciprofloxacin
294 resistance. For azithromycin, two genetic elements under anaerobic conditions alone
295 were identified: *mphA* (mentioned above) and an intergenic region between *ompT*
296 (porin; known to be associated with AMR)[43, 44] and *dinG* (ATP-dependent DNA
297 helicase). For doxycycline, a diverse set of 57 genetic elements under anaerobic
298 conditions alone were identified that included *tet(59)* (mentioned above), *vexK* (efflux
299 RND transporter permease associated with AMR) [45-47], and *zorA* (anti-phage
300 defense system ZorAB subunit A; a putative proton channel that may respond to
301 membrane perturbation by depolarization)[48].

302 Genes associated with an AMR phenotype were found on SXT (e.g. *tet(59), aph(6)-Id,*
303 *radC, dfrA31*) and discovered on the Vibrio Pathogenicity Island II (VSPII; N16961
304 VC0506-VC0512 / E7946 loci RS02705-RS02745); these loci are genetically diverse in
305 Bangladesh[49]. The GWAS hits in VSPII encode both biofilm/ autoaggregation
306 associated factors as well as an aerotaxis protein (AerB; VC0512)[50]; findings
307 consistent with roles in AMR and aerobic/ anaerobic conditions. The GWAS were
308 limited by sample size and were at risk of increased false positive discovery rates at
309 AMR 'hot-spots' like SXT. Despite these limitations, GWAS enabled the discovery of an

310 intriguing list of genetic targets that were associated with AMR and require future
311 molecular manipulation to test for causal relationships.

312 LC-MS/MS analysis on the stools, from which the isolates were obtained, was
313 conducted to test the hypothesis that the rates of AMR genotypes and phenotypes were
314 higher when the stool samples contained the cognate antibiotic. Nearly all patients shed
315 at least one antibiotic making it difficult to identify AMR correlates to exposure.

316 **Conclusions**

317 Facultative enteropathogens are exposed to antibiotics under aerobic and anaerobic
318 conditions in both the human gut and in the environment. We used the facultative
319 enteropathogen *V. cholerae* as a model to test for differences in AMR phenotypes under
320 aerobic and anaerobic conditions. The odds of classifying isolates as resistant under
321 anaerobic compared to aerobic conditions increased over 10 times for two of the three
322 antibiotics tested. While several known resistance genes were associated with AMR
323 under both conditions, many genes were only associated with AMR under one
324 condition. Heritability tended to be higher and more genes associated with resistance
325 under anaerobic conditions, suggesting that key genetic determinants of resistance may
326 be missed when experiments are only performed aerobically. Taken together, these
327 findings challenge clinical and experimental paradigms of testing facultative
328 enteropathogens for AMR under aerobic conditions alone.

329

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Data availability

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

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Disclaimer

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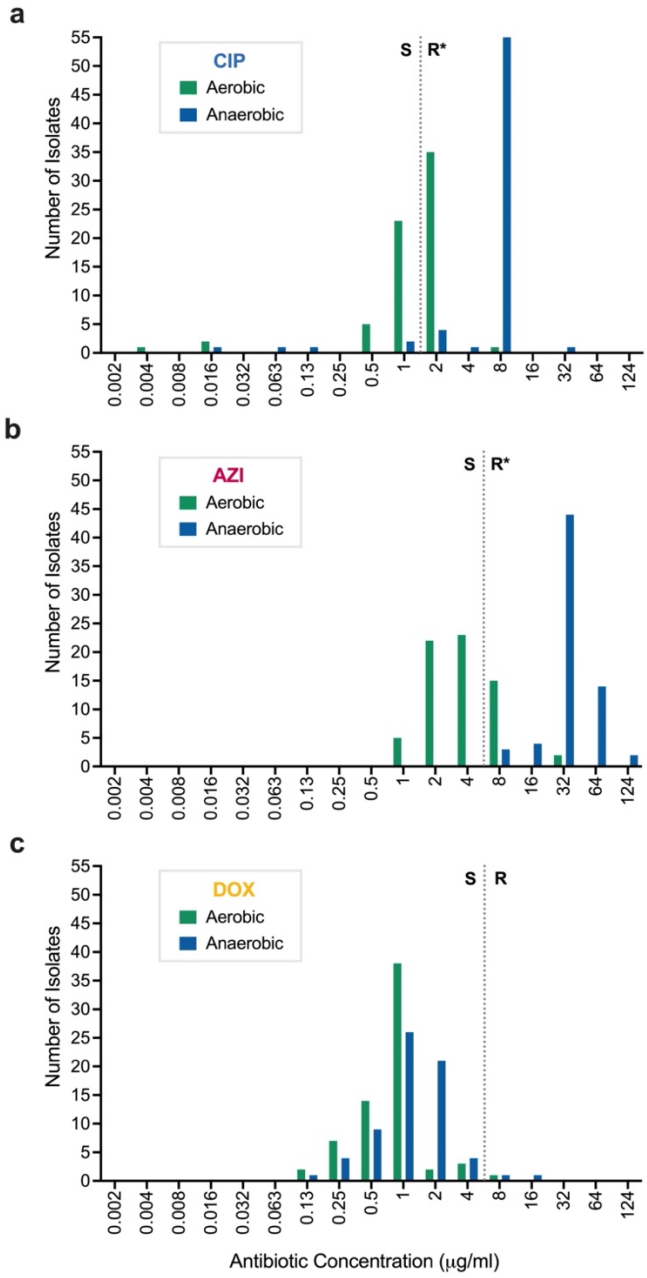
Potential conflicts of interest.

All authors: No reported conflicts.

359 **FIGURES**

360

361 **FIGURE 1**

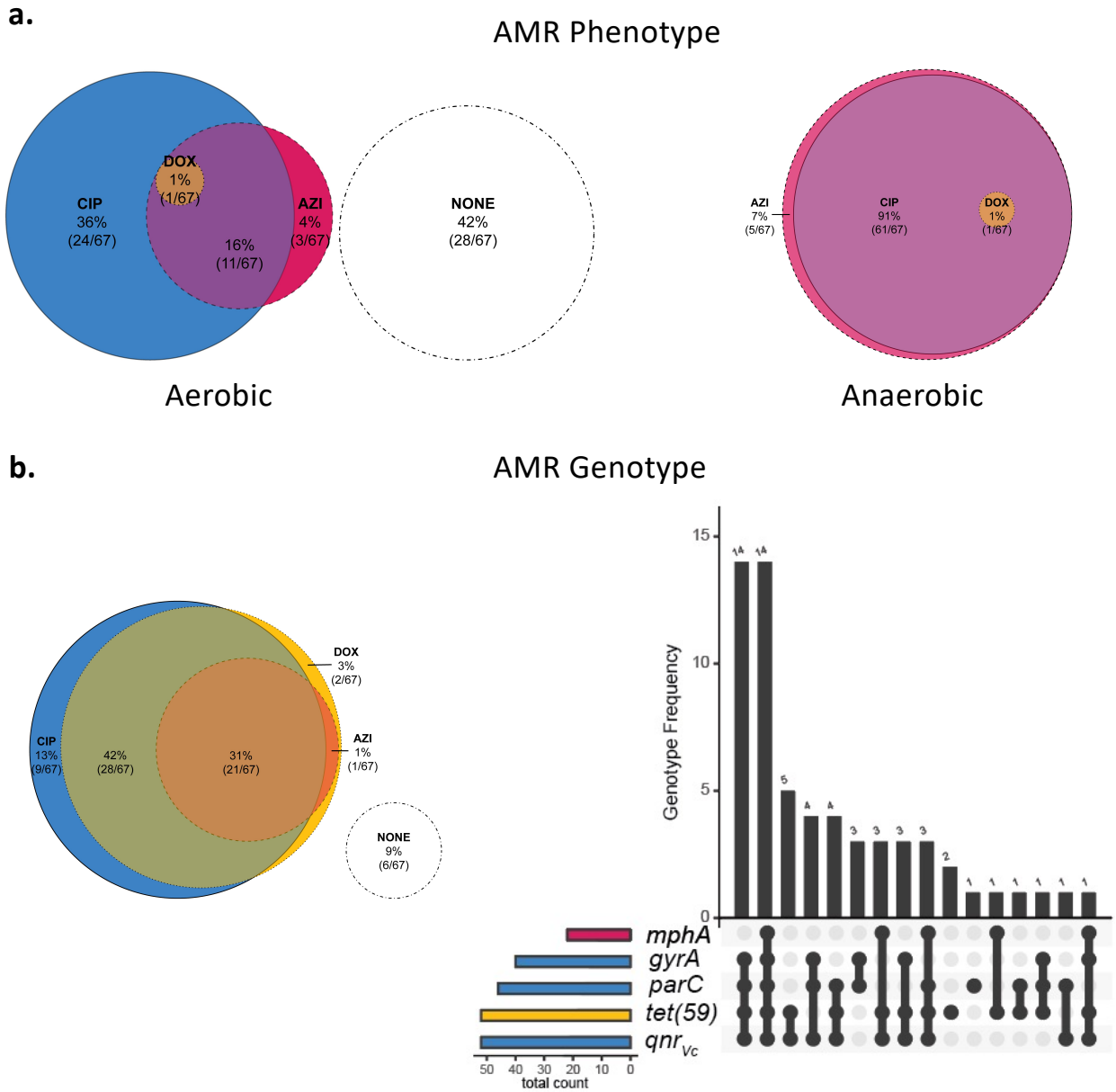


362

363 **Figure 1. Distribution of antibiotic resistance among clinical isolates under aerobic and anaerobic**
364 **conditions.** Ciprofloxacin (CIP; **a**), Azithromycin (AZI; **b**), and Doxycycline (DOX; **c**). Resistance was
365 determined by MIC under aerobic (green) and anaerobic conditions (blue). Data are from 67 human-shed
366 *V. cholerae* isolates. Dotted line is the breakpoint for resistance per CLSI standards which is based on
367 assays under aerobic conditions (CIP = 2 μg/ml; AZI = 8 μg/ml; DOX = 8 μg/ml). "*" represents a
368 significant difference in the frequency of isolates identified as resistant to ciprofloxacin and azithromycin
369 by McNemar's test (both $p<0.001$). S=sensitive. R = Resistant.

370 **FIGURE 2**

371



372
373 **Figure 2. AMR phenotypes and known AMR genetic elements in human-shed *V. cholerae* isolates**.
374 **a,** Proportional Venn diagram (Euler) of AMR phenotypes to azithromycin (AZI), doxycycline (DOX) and/or
375 ciprofloxacin (CIP) under aerobic (left) and anaerobic conditions (right). **b,** Distribution of specific known
376 AMR genetic elements by proportional Venn diagram (Euler; left) and bar chart (right). AMR genetic
377 elements to other antibiotics are not shown.

FIGURE 3

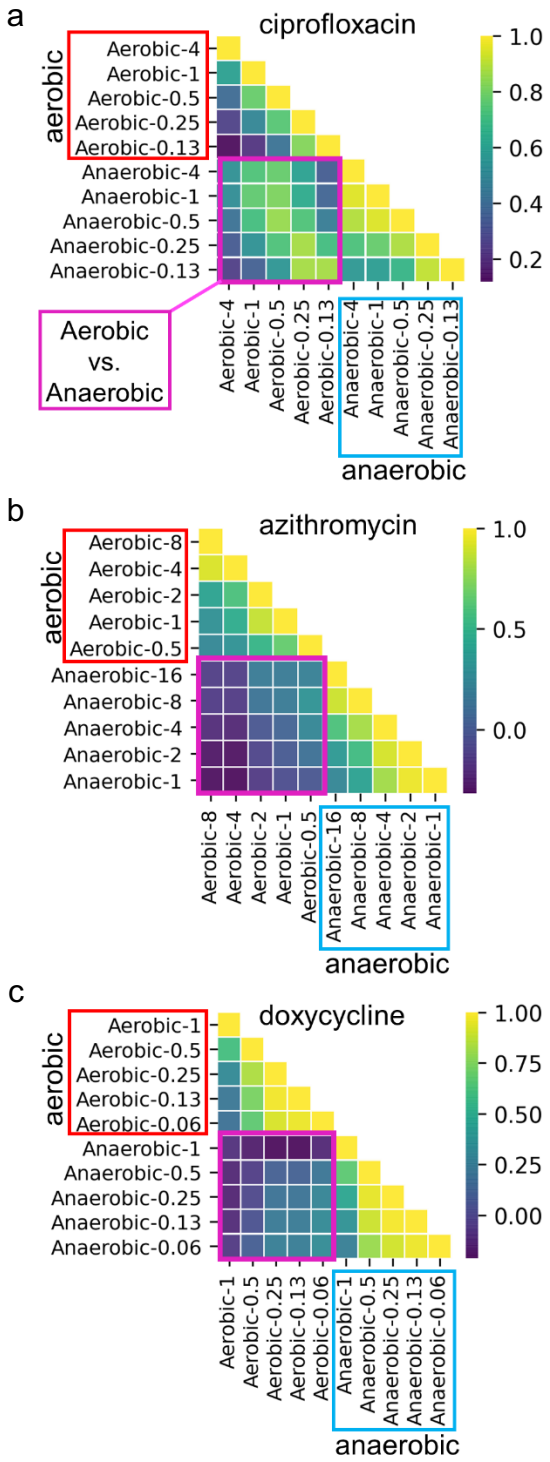
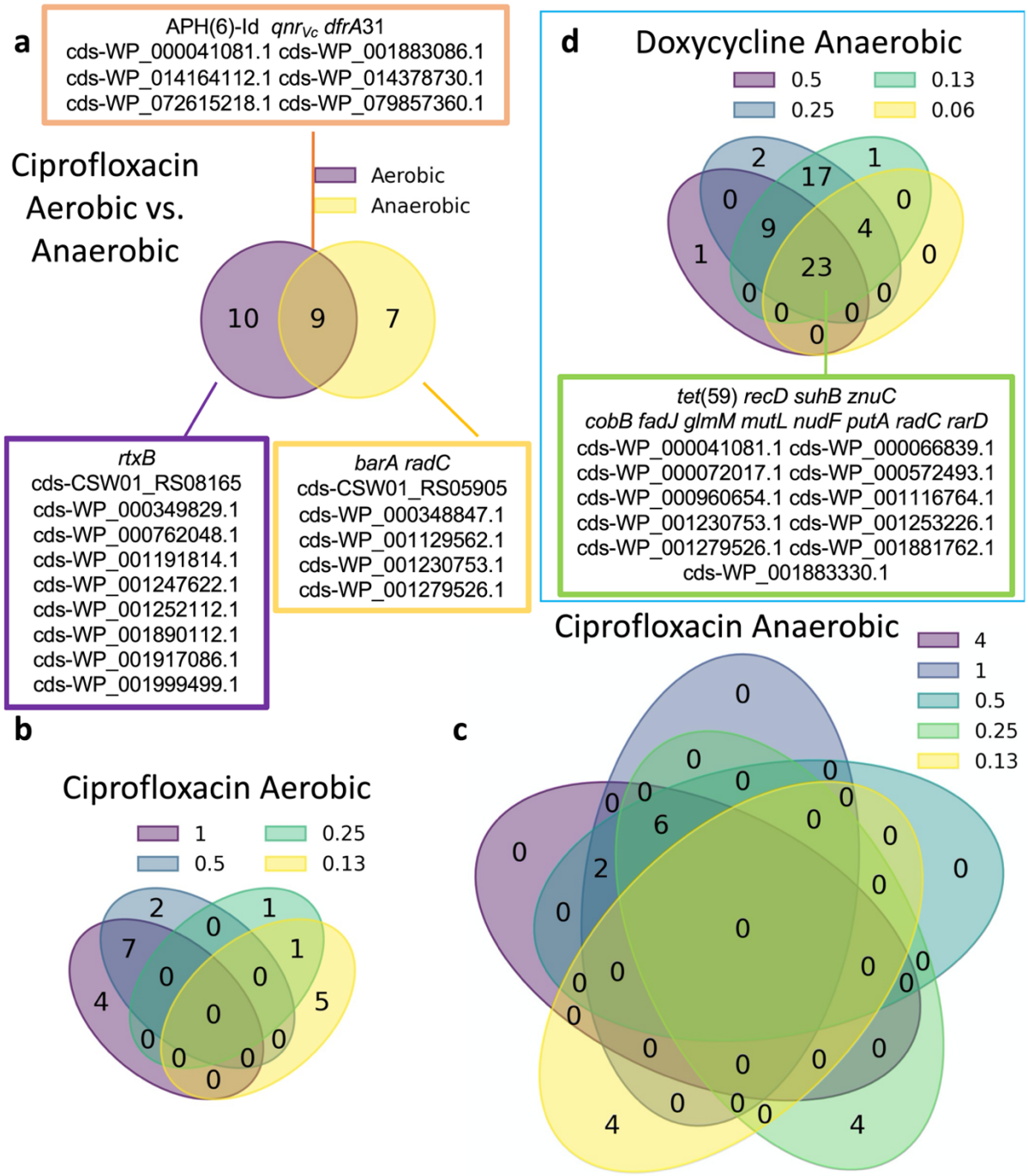


Figure 3. Correlations among *V. cholerae* growth phenotypes at different concentration of antibiotics and respiratory conditions. Correlations of *V. cholerae* growth phenotypes (AUC) to ciprofloxacin (CIP; **a**), azithromycin (AZI; **b**), and doxycycline (DOX; **c**) at different antibiotic concentrations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

386 **FIGURE 4**



387
388 **Figure 4. Distribution of AMR genes associated with AMR growth phenotypes at different**
389 **concentrations of antibiotics under aerobic and anaerobic conditions.** Venn diagrams show the
390 overlap between genes associated with **(a)** ciprofloxacin resistance under aerobic vs. anaerobic
391 conditions, **(b)** ciprofloxacin at different concentrations (µg/ml) under aerobic conditions, **(c)** ciprofloxacin
392 at different concentrations (µg/ml) under anaerobic conditions, and (**d)** doxycycline at different
393 concentrations (µg/ml) under anaerobic conditions. Genes shown in boxes had statistically significant
394 associations.

395 **FIGURE 5.**

	CIP	TET/DOX	NAL	MET	BAC	AMO	AZI	ERY	CEF	Detected (N)
EN123	+	+	+	+	+	+	-	-	-	6
EN160	+	+	+	+	+	+	-	-	-	6
EN127	+	+	+	+	+	-	-	-	-	5
EN129	+	+	+	+	+	-	-	-	-	5
EN135	+	+	+	+	-	-	-	-	-	4
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
EN079	+	+	+	+	-	-	-	-	-	4
EN125	+	+	+	-	+	-	-	-	-	4
EN143	+	+	+	-	-	-	-	-	-	3
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	46	41	37	22	2	0	0	0	196 total detected
Positive (%)	94	90	80	73	43	4	0	0	0	98% with antibiotic(s)

396
397
398 **Figure 5. Antibiotic detection in stool supernatants by mass spectrometry (LC-MS/MS)**. Green with
399 "+" = Detected. White with "-" = not detected. CIP= ciprofloxacin, TET/DOX= tetracycline and/or
400 doxycycline, NAL = nalidixic acid, MET = metronidazole, BAC = sulfamethoxazole and/or trimethoprim,
401 AMO = amoxicillin, ERY = erythromycin, CEF = ceftriaxone. Stool supernatants were not available for
402 EN80, 86, 88, 92, 100, 103, 109, 116-120, 126, 124, 130, 131.

403 **TABLE**

404

405

406 **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

407 [a] Ciprofloxacin = CIP; Azithromycin = AZI; Doxycycline = DOX
408 [b] Heritability is the proportion of phenotypic variation that is explained by genetic variation. Associated
409 genes are all significant GWAS hits after correction for multiple hypothesis testing ($P < 0.05$ after
410 Bonferroni correction).
411

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540

SUPPLEMENTARY MATERIAL

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Supplementary Data Files (excel file with four datafile tabs)

Date File S1. GWAS Hits. Ciprofloxacin resistance phenotype. Aerobic.
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Data File S3. GWAS Hits. Azithromycin resistance phenotype. Anaerobic.
Data File S4. GWAS Hits. Doxycycline resistance phenotype. Anaerobic.

33 **Supplementary Methods.**
34
35 **Whole-genome sequencing.** Genomic DNA was extracted from *V. cholerae* isolates
36 from the primary collection using the Qiagen DNeasy Blood and Tissue Kit. Library
37 construction was completed using the Illumina Nextera XT v.2 DNA Library Preparation
38 Kit. Twenty-four genomes were pooled and sequenced on an Illumina MiSeq for 500
39 cycles per run. Using CLC Genomics Workbench v20, raw reads were filtered by length,
40 trimmed, and mapped to the reference genome (*V. cholerae* O1 El Tor E7946) to
41 identify single-nucleotide variants. To identify genes not present in the reference
42 genome, contigs were assembled *de novo* using CLC Genomics Workbench v20.

43 **Genome-wide association studies (GWAS).** To extract genomic variants, which can
44 potentially capture all sources of variation in the genome (i.e. single nucleotide variants,
45 indels and gene presence/absence) without *a priori* assumption about the underlying
46 gene content of each sample (e.g. accessory genes or plasmids), unitigs were
47 generated from genomes assembled using GATB[1]. Unitigs are sequences of variable
48 length (unlike k-mers of fixed length *k*) which represent the variations in the population
49 of genomes under study in high-resolution. GWAS were performed using linear mixed
50 models implemented in pyseer v.1.3.6 and adjusted for population stratification using
51 the kinship matrix estimated from the phylogenetic tree[2].

52 To generate the phylogenetic tree, genome alignments consisting entirely of variable
53 nucleotides were produced from whole genome SNP data generated by CLC Genomics
54 Workbench v20 using VCF-kit 0.1.6[3]. The tree was then inferred by RaxML under the
55 general time reversible (GTR) model with rate variation across sites following a GAMMA
56 distribution[4]. We used the linear-mixed model approach to adjust for population

57 stratification and linkage disequilibrium in microbial GWAS[5]. Heritability (h^2), an
58 estimate of the proportion of the phenotype variance that can be explained by total
59 genomic variation represented in the unitigs, was also calculated using pyseer v.1.3.6.
60 Likelihood-ratio test *p*-values for the association tests were adjusted for multiple-testing
61 by Bonferroni correction (at a genome-wide false discovery rate of 0.05) for the number
62 of unique unitig patterns (i.e. only giving one count to a unitig with an identical
63 presence/absence profile across genomes). We also removed unitigs tagged with the
64 errors 'bad-chisq', 'pre-filtering-failed', 'lrt-filtering-failed', 'firth-fail' and 'matrix-
65 inversionerror' after the analysis. To further remove false positive GWAS hits, we
66 removed any considerable clusters of unitigs (> 20) with identical *p*-values, as these are
67 likely to be lineage-specific markers or markers with strong linkage disequilibrium
68 comprised of mostly non-causal variants linked on the same clonal frame. GWAS hits
69 were annotated by mapping the unitigs to two reference genomes of *V. cholerae*,
70 namely, E7946 (NCBI assembly accession number: GCA_002749635.1) and O1
71 biotype El Tor strain N16961 (NCBI assembly accession number: GCA_003063785.1)
72 using BWA. Statistically significant GWAS hits were further annotated with the CARD
73 resistance gene identifier (RGI) after filtering the 'loose' hits and hits with identity <0.90.

74 **Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS).**
75 Stool supernatant from the primary collection were obtained by centrifugation and
76 filtration (0.2 µM surfactant-free cellulose acetate; Thermo Scientific Nalgene). Proteins
77 were precipitated (1:7 ratio (v/v) of water::methanol). Supernatants were diluted with
78 methanol and water (1:1 v/v) in 0.1% formic acid for liquid chromatography, and 5 µl of
79 supernatant was injected for analysis. LC/MSMS was performed on a 2.1 x 150-mm

80 Hypersil Gold aQ column (particle size, 3 μm) using a high-performance liquid
81 chromatography system (Thermo UltiMate 3000 series) with an LTQ XL ion trap mass
82 spectrometer (Thermo Fisher Scientific). Mobile phases were 1% formic acid in water
83 (A) and 1% formic acid in methanol (B) and held at a constant 5%B for 2min before
84 ramping to 95%B at 15 min where it was held for an additional minute before returning
85 to starting conditions for a total run time of 25 min.

86 Eluent was ionized using electrospray ionization (ESI) in positive mode at a spray
87 voltage of 5 kV, a nitrogen sheath gas flow rate of 8 L min^{-1}, and capillary temperature
88 of 300°C. Two scan events were programmed to perform an initial scan from *m/z* 100 to
89 1000, which was followed by targeted collision induced dissociation based on a
90 retention time and mass list. Retention time windows ranged from 0.35 minutes to 6.50
91 min, depending on the elution range of the standards at high and low concentrations.
92 Masses were targeted for the most abundant adduct or ion associated with each
93 antibiotic (typically the $[M+H]^+$ ion) with a *m/z* 1 window. Data analysis for amoxicillin,
94 sulfamethoxazole/trimethoprim, azithromycin, tetracycline, doxycycline, metronidazole,
95 nalidixic acid, and ciprofloxacin was performed manually using extracted ion
96 chromatograms and MSMS matching with an in-house antibiotic MSMS library using
97 Xcalibur 2.2 SP 1.48 (Thermo Fisher Scientific).

98

99 **Supplementary Results.**

100

101 **Antibiotic resistance phenotypes of known AMR genetic elements under aerobic**

102 **and anaerobic conditions**. The distribution of known AMR genetic elements (Figure 2)

103 was grouped by point mutations (likely transmitted vertically, not on an established

104 mobilizable element) and horizontal transmission (on an established mobilizable

105 element).

106 *Point mutations in known AMR genes.* For ciprofloxacin, the most identified known

107 resistance mutations were those in the topoisomerase encoding genes *gyrA*

108 (VC1258/RS06370) (Ser83Ile) and *parC* (VC2430/RS12340) (Ser85Leu). These were

109 present in 60% (40/67) and 70% (47/67) of all isolates, while 54% (36/57) contained

110 both. For azithromycin, we found no mutations in known resistance genes encoding

111 ribosomal proteins L4 (*rplD;*VC2595/RS13175) and L22 (*rplV;*VC2591/RS13155) [6, 7].

112 For tetracycline (proxy for doxycycline), one of the 9 total 16S rRNA genes (VCr001)

113 was found to have a single nucleotide insertion of a G nucleotide at position 327 within

114 the sequences of 15% (10/67) of isolates; the significance is unknown. No mutations

115 were detected in the 30S ribosomal protein encoded by genes *rpsJ* (VC2597/RS13185)

116 or *rpsC* (VC2590/RS13150), whose mutations have been associated with tetracycline

117 class resistance in other Gram negative organisms [8-10].

118 *Known AMR genes on mobile genetic elements.* The integrative conjugative element

119 (ICE) SXT/R391 was found in 90% (60/67) of isolates. The ICE contained a

120 pentapeptide repeat protein conferring fluoroquinolone resistance (qnr_{Vc}), a macrolide-

121 inactivating phosphotransferase (*mph*(A)), and a major facilitator superfamily (MFS)

122 efflux pump conferring tetracycline resistance (*tet*(59)) [11-14]. The genes qnr_{Vc},

123 *mph*(A), and *tet*(59) were also found in 78% (52/67), 33% (22/67), and 78% (52/67) of

124 isolates, respectively.

125

126

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

128

		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)

[a] Prefix of "L_" was used to distinguish the strain number in the library from the clinical isolate number, which is maintained to be consistent with prior publications.

[b] CIP^R = ciprofloxacin resistance. AZI^R = azithromycin resistance. DOX^R = doxycycline resistance.

132 [c] Sources of strains: (1) Mekalanos, J. J. Duplication and amplification of toxin genes in Vibrio cholerae. Cell 35,
133 253-263, (1983) ; (2) Nelson, E. J. et al. Complexity of rice-water stool from patients with Vibrio cholerae plays a
134 role in the transmission of infectious diarrhea. Proc Natl Acad Sci U S A 104, 19091-19096, (2007).

135 **Supplementary Table 2.** Baseline growth parameters of *V. cholerae* clinical isolates
136 under aerobic and anaerobic conditions
137

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**

138 [a] AUC = area under the curve. K = carrying capacity. Media was LB alone without antibiotics. Velocity =
139 growth rate in percent increase per minute at half the carrying capacity.
140 [b] Wilcoxon signed-rank test for growth. Bold = statistically significant (P<0.05).
141

142 **Supplementary Table 3.** Minimal inhibitory concentrations (MICs) for
143 ciprofloxacin, azithromycin, and doxycycline among *V. cholerae* clinical
144 isolates
145

	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

146 [a] Concentration of antibiotic.
147 [b] Distribution of MICs for clinical isolates grown under aerobic and anaerobic conditions. Bold text
148 signifies the concentration at which the MIC mode was determined among the clinical isolates.
149

150 **Supplementary Table 4.** Comparison of rates of resistance detected under aerobic
151 versus anaerobic conditions among *V. cholerae* clinical isolates in the primary collection
152

	N	R^{Ae}/R^{An}	R^{Ae}/S^{An}	S^{Ae}/R^{An}	S^{Ae}/S^{An}	p[b]
Ciprofloxacin[a]	67	36	0	26	5	**<0.001**
Azithromycin[a]	67	15	0	52	0	**<0.001**
Doxycycline[a]	67	0	1	1	65	1

153 [a] Distribution of paired resistant ('R') and sensitive ('S') phenotypes for isolates under aerobic ('Ae') and
154 anaerobic ('An') conditions.
155 [b] McNemar's Exact Test. Bold = statistically significant (P<0.05)
156

157 **Supplementary Table 5.** Comparison of rates of resistance detected under aerobic
158 versus anaerobic conditions among *V. cholerae* clinical isolates in the secondary
159 collection
160

	N	R^{Ae}/R^{An}	R^{Ae}/S^{An}	S^{Ae}/R^{An}	S^{Ae}/S^{An}	p[b]
Ciprofloxacin[a]	120	1	1	46	72	**<0.001**
Azithromycin[a]	120	56	0	64	0	**<0.001**
Doxycycline[a]	120	0	0	2	118	0.480

161 [a] Distribution of paired resistant ('R') and sensitive ('S') phenotypes for isolates under aerobic ('Ae') and
162 anaerobic ('An') conditions.
163 [b] McNemar's Exact Test. Bold = statistically significant (P<0.05)
164
165

166 **Supplementary Table 6.** Effect of catalase on growth parameters for *V. cholerae*
167 E7946 and EN160 under aerobic conditions
168

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	

169 [a] E7946 (Cip^S, Azi^S,Dox^S) is the reference strain and EN160 (Cip^R, Azi^R, Dox^S) is a clinical isolate.
170 Biological replicates in experiments 1-3 and 5-7 were 3, each with 4 technical replicates. Biological
171 replicates for experiments 4 and 8 were 9, each with 4 technical replicates.
172 [b] CIP = ciprofloxacin. AZI = azithromycin. DOX = doxycycline. Assays were run at CIP = 0.5, AZI = 2, and
173 DOX = 0.25 µg/ml for EN160; E7946 was run at CIP = 0.002, AZI = 1, and DOX = 0.013
174 µg/ml.
175 [c] CIP and AZI were tested with 3 biological replicates; DOX with 2 biological replications; LB controls with
176 9 biological replicates.
177 [d] AUC = area under the curve. IQR = interquartile range.
178 [e] Student's t-test. Bold = statistically significant (P<0.05)

179 **Supplementary Table 7.** Comparison of antibiotic resistance phenotypes and known
180 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

181 [a] R = resistant phenotype, S = sensitive phenotype, P = gene present, NP = gene not present.
182 Enumerations include isolates with at least the specific gene named; isolates may have more than one
183 resistance gene (e.g. *qnrVC, gyrA* and *parC*)
184 [b] Fisher's Exact Test. Bold = statistically significant (P<0.05)
185
186

187 **Supplementary Table 8.** Test of association between antibiotic detection by
188 mass spectrometry and AMR genotypes and phenotypes among *V. cholerae*
189 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

190 [a] D=detected, ND=not detected, R=resistant by MIC, S=sensitive by MIC, P = Present, NP
191 = Not present.
192 [b] Fisher's Exact Test. Bold = statistically significant (P<0.05)
193
194

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