Genome-wide association studies reveal distinct genetic correlates and increased heritability of antimicrobial resistance in *Vibrio cholerae* under anaerobic conditions

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Abstract

The antibiotic formulary is threatened by high rates of antimicrobial resistance (AMR) among enteropathogens. Enteric bacteria are exposed to anaerobic conditions within the gastrointestinal tract, yet little is known about how oxygen exposure influences AMR. The facultative anaerobe *Vibrio cholerae* was chosen as a model to address this knowledge gap. We obtained *V. cholerae* isolates from 66 cholera patients, sequenced their genomes, and grew them under anaerobic and aerobic conditions with and without three clinically relevant antibiotics (ciprofloxacin, azithromycin, doxycycline). For ciprofloxacin and azithromycin, the minimal inhibitory concentration (MIC) increased under anaerobic conditions compared to aerobic conditions. Using standard resistance breakpoints, 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 were sensitive under both conditions. Using genome-wide association studies (GWAS), we found associations between genetic elements and AMR phenotypes that varied by oxygen exposure and antibiotic concentrations. These AMR phenotypes were more heritable, and the AMR-associated genetic elements were more often discovered, under anaerobic conditions. These AMR-associated genetic elements are promising targets for future mechanistic research. Our findings provide a rationale to determine if increased MICs under anaerobic conditions are associated with therapeutic failures and/or microbial escape in cholera patients. If so, there may be a need to determine new AMR breakpoints for anaerobic conditions.

Impact statement

Many bacterial pathogens experience anaerobic conditions in the gut, but antimicrobial resistance (AMR) phenotypes are generally tested under ambient aerobic conditions in the laboratory. To better understand AMR under conditions more similar to natural infections, we used *Vibrio cholerae* as a model enteric pathogen. We sequenced the genomes and assessed the growth of *V. cholerae* isolates with different concentrations of three antibiotics, under anaerobic and aerobic conditions. In support of the hypothesis that AMR varies according to oxygen exposure, *V. cholerae* was more resistant to antibiotics under anaerobic conditions. We found many previously known genes associated with resistance; however, some of these genes were only resistance-associated under aerobic conditions. Resistance to azithromycin and doxycycline only had a detectable genetic component under anaerobic conditions. Together, our results point to distinct genetic mechanisms of resistance under anaerobic conditions and suggest several candidate genes for experimental follow-up.

Data summary

All sequencing data generated in this study are available in NCBI under BioProject PRJNA818081.

41 **INTRODUCTION**

42 Clinically relevant laboratory methods are essential to gauge the extent to which the
43 antibiotic formulary is threatened by antimicrobial resistance (AMR). Knowledge gaps
44 remain on the degree to which *in vitro* AMR assays reflect *in vivo* AMR physiology.
45 Facultative anaerobic pathogens experience hypoxia and anoxia within the
46 gastrointestinal tract, yet AMR assays rely on aerobic conditions [1]. How oxygen
47 exposure effects AMR is poorly understood. To investigate this question, we chose the
48 facultative anaerobe *Vibrio cholerae* as a model system. In *V. cholerae,* classic
49 mechanisms for AMR, and physiologic pathways for anaerobic respiration and
50 fermentation, are well characterized [2-9]. The disease cholera is also one of the few
51 non-invasive diarrheal diseases for which antibiotics are indicated, albeit conditionally
52 [10-12].

53 Rehydration is the definitive intervention for acute diarrheal disease [11]; antibiotics are
54 supportive and indicated for only a few diarrheal diseases, including cholera. The World
55 Health Organization (WHO) recommends ciprofloxacin, azithromycin or doxycycline for
56 cholera patients with severe dehydration [10-12]; antibiotics shorten the frequency and
57 duration of diarrhea. In practice, guideline adherence in cholera endemic regions may
58 be low out of clinical concern that a patient 'might' have cholera and may develop
59 severe dehydration, contributing to rates of inappropriate antibiotic usage that can rise
60 above 90% [13, 14]. Strong regional associations between antibiotic use and rise of
61 AMR have been observed across enteric taxa [15, 16]. Given that AMR genes
62 frequently co-localize on mobile elements [17], inappropriate single-agent therapy poses
63 a risk of multidrug-resistance (MDR) selection.

64 Associations between AMR phenotypes and genotypes are known for the three
65 antibiotics recommended to treat cholera; the cognate AMR mechanisms share
66 commonality across Gram negative taxa. Ciprofloxacin (a fluoroquinolone) resistance
67 mechanisms include mutations in genes encoding type II topoisomerases:
68 heterotetrameric DNA gyrase ($GyrA_2GyrB_2$) and DNA topoisomerase IV ($ParC_2ParE_2$).
69 Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* can
70 yield additive resistance phenotypes [18]. Fluoroquinolone resistance can also arise by
71 efflux pump upregulation, by downregulation of outer membrane porins that permit
72 quinolone entry, and by the expression of the quinolone resistance protein (Qnr, a
73 pentapeptide repeat protein) that protects the target gyrase protein [18]. Resistance can
74 increase over 30-fold compared to wild-type when strains harbor *qnr* family genes.

75 In *V. cholerae*, diverse AMR genes, including *qnr,* often reside on an integrative and
76 conjugative element (ICE; 'SXT' in *V. cholerae*) [17, 19, 20]. Azithromycin (a macrolide)
77 resistance mechanisms are similarly diverse and include mutations in the 23S ribosomal
78 RNA (rRNA) target genes and ribosomal protein genes. Macrolide resistance is
79 conveyed by carriage of rRNA methyltransferase genes (*erm*) and associated induction
80 mechanisms, *cis*-acting peptides, efflux systems (e.g. *mef, msr)*, macrolide esterases
81 (e.g. *ere*), and macrolide phosphotransferases such as *mphA* which can reside on the
82 *V. cholerae* SXT element [21]. Doxycycline (a tetracycline) resistance is conferred by
83 mutations in the 16S rRNA component of the 30S ribosomal subunit [22]. Additional
84 mechanisms include tetracycline-specific ribosomal protection proteins (RPPs),
85 tetracycline specific efflux pumps (e.g. *tet*(59)) which can reside on SXT element

intrinsic efflux pumps, AraC-family transcriptional activators (e.g. MarA), and cytoplasmic ATP-dependent serine proteases [22].

Associations between AMR phenotypes and genotypes have been studied by random mutagenesis, phenotypic screening, and network analyses [23-28], and applied in *V. cholerae* [29]. These approaches uncover how the effect of an antibiotic is shaped by a large number of often more subtle physiologic perturbations, including altered DNA synthesis/repair, central metabolism/growth, and SOS response [30, 31]. AMR assays conducted under aerobic conditions alone may not reflect these physiologic perturbations experienced in the host. Within bacteria, aerobic oxidative phosphorylation generates reactive oxygen species (ROS) that are lethal unless a sufficient defense is mounted by factors like superoxide dismutase, catalase, and glutathione systems [32, 33]. Under anaerobic conditions, growth rate typically slows and proton motive force is reduced [34, 35], which can have both synergistic and antagonistic effects on antibiotics [31, 36]. In *Escherichia coli,* ROS are generated after fluroquinolone treatment under aerobic conditions [37] and fluoroquinolone resistance increases under anaerobic conditions [38, 39]. The extent to which tetracyclines and macrolides induce ROS and how anaerobiosis influences resistance and susceptibility is less known [30, 40].

The objective of this study was to compare AMR phenotypes, with underlying genotypes, under aerobic and anaerobic conditions among isolates obtained from cholera patients. The study rationale assumes that the lower gastrointestinal tract of cholera patients is hypoxic/anaerobic, despite animal experiments that suggest aerobic respiration in the upper gastrointestinal tract is important for infection [41, 42]. Using

minimal inhibitory concentration assays (MICs), we found that AMR increased under anaerobic conditions for select antibiotics, and novel genetic targets for AMR were discovered under anaerobic conditions.

METHODS

Clinical sample collection. The two sample collections analyzed were part of previously published IRB approved studies [13, 43]. In the primary collection, stool samples were obtained during the spring cholera outbreak period of 2006 at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) in Dhaka, Bangladesh. Samples were collected prior to hospital administration of antibiotics; patient histories were negative for known antibiotic exposure. The library consisted of 67 *V. cholerae* isolates (Supplementary Table 1); paired stool supernatant for mass spectrometry was available for 50 isolates. In the secondary collection, samples were obtained in 2018 as part of a cholera surveillance study conducted across Bangladesh [13]. Samples were collected at hospital admission independent of reported antibiotic exposure; 277 out of 282 isolates cultured and were analyzed to assess generalizability of the AMR profiles identified in the primary collection.

Antimicrobial resistance testing. Growth kinetics and the MIC determinations for ciprofloxacin, azithromycin, and doxycycline were performed on isolates from the primary collection in LB broth with twelve two-fold serial dilutions with concentrations spanning the CLSI MIC breakpoints [1] for *V. cholerae* (ciprofloxacin = 2 μg/ml; azithromycin = 8 μg/ml; doxycycline = 8 μg/ml) [1]. Isolates were prepared and grown aerobically at 37˚C in 15-ml tubes containing 5-ml LB broth at 220 rpm. Bacteria were back-diluted to a final optical density (OD) 600 nm of 0.01 (200 μl/well) in LB with or

132 without the respective antibiotic dilution-series in black Corning CoStar clear-bottom 96-
133 well plates. Plates were placed in a BioTek Synergy H1 reader pre-warmed to 37˚C with
134 the lid on. Anaerobic conditions were generated using a continuous chamber flow (5%
135 CO_2, 95% N_2) and a BioTek CO_2/O_2 gas controller; anaerobic growth plates were given
136 a 10-minute equilibration period. OD 600 nm was measured every 2 minutes for 8 hours
137 at 37˚C with orbital shaking at 220 rpm. A standard logistic equation was fit to growth
138 curve data using the R package *growthcurver* version 0.3.0 [44]. Outcome measures
139 were intrinsic growth velocity (growth rate that would occur if there were no restrictions
140 on total population size), carrying capacity (K; maximum possible population size), and
141 area under the curve (AUC). The MIC was determined using a logistic fit for growth over
142 the twelve, two-fold serial dilutions of the test antibiotic. Binary phenotypic
143 sensitive/resistance categories were set in concordance with Clinical and Laboratory
144 Standards Institute (CLSI; M45 2018 3rd edition) [45]. In general, CLSI breakpoints are
145 set by clinical and bacteriological response data, pharmacokinetic and
146 pharmacodynamic simulations, and expert working group experience. The breakpoints
147 for *V. cholerae* are based on aerobic assays. Susceptible (sensitive) is defined by CLSI
148 as the "category that implies that isolates are inhibited by the usually achievable
149 concentrations of antimicrobial agent when the dosage recommended to treat the site of
150 infection is used". Those not sensitive were scored as resistant (combines
151 indeterminate and resistant). Three isolates and one reference strain (E7946) were
152 used to assess media acidification during anaerobic and aerobic growth; the pH was
153 measured using dipsticks (pH range 5.2-7.2). The assays were conducted with and

154 without the addition of 20mM fumarate as an alternative electron acceptor for anaerobic
155 respiration.

156 **Use of catalase to determine if ROS contribute to antibiotic sensitivity.** To test if
157 the reduction of ROS was associated with increased resistance to antibiotics under
158 aerobic conditions, MICs were determined for two select strains (E7946, EN160) with
159 and without catalase (10 U/ml; final concentration) added to the media. Growth curves
160 were performed with viable counts as endpoints to determine the minimum dose for
161 lethality by H_2O_2 or protection by catalase.

162 **Whole-genome sequencing.** Genomic DNA was extracted from *V. cholerae* isolates
163 from the primary collection using the Qiagen DNeasy Blood and Tissue Kit. Library
164 construction was completed using the Illumina Nextera XT v.2 DNA Library Preparation
165 Kit. Libraries were sequenced in three illumina MiSeq runs. Two batches of twenty-four
166 genomes and one batch of nineteen were pooled and sequenced on a MiSeq for 500
167 cycles per run. Using CLC Genomics Workbench v20, raw reads were filtered by length,
168 trimmed, and mapped to the reference genome (*V. cholerae* O1 El Tor E7946) to
169 identify single-nucleotide variants. Of the 67 isolates, 66 yielded sufficient coverage
170 (>50X) of the *V. cholerae* genome. We proceeded with these 66 genomes for further
171 analysis. To identify genes not present in the reference genome, contigs were
172 assembled *de novo* using CLC Genomics Workbench v20.

173 **Genome-wide association studies (GWAS).** To extract genomic variants capturing all
174 sources of variation in the genome (i.e. single nucleotide variants, indels and gene
175 presence/absence) without *a priori* assumption about the underlying gene content of

176 each sample (e.g. accessory genes or plasmids), unitigs were generated from the 66
177 genomes assembled using GATB [46]. Unitigs are sequences of variable length (unlike
178 k-mers of fixed length *k*) which represent the variations in the population of genomes
179 under study in high-resolution. GWAS were performed using linear mixed models
180 implemented in pyseer v.1.3.6 and adjusted for population stratification using the
181 kinship matrix estimated from the phylogenetic tree[47].

182 To generate the phylogenetic tree, genome alignments consisting entirely of variable
183 nucleotides were produced from whole genome SNP data generated by CLC Genomics
184 Workbench v20 using VCF-kit 0.1.6 [48]. The tree was then inferred by RaxML under
185 the general time reversible (GTR) model with rate variation across sites following a
186 GAMMA distribution[49]. We used the linear-mixed model approach to adjust for
187 population stratification and linkage disequilibrium in microbial GWAS [50]. Heritability
188 (h^2), an estimate of the proportion of the phenotype variance that can be explained by
189 total genomic variation represented in the unitigs, was also calculated using pyseer
190 v.1.3.6. Likelihood-ratio test *p*-values for the association tests were adjusted for
191 multiple-testing by Bonferroni correction (at a genome-wide false discovery rate of 0.05)
192 for the number of unique unitig patterns (i.e. only giving one count to a unitig with an
193 identical presence/absence profile across genomes). We also removed unitigs tagged
194 with the errors 'bad-chisq', 'pre-filtering-failed', 'lrt-filtering-failed', 'firth-fail' and 'matrix-
195 inversionerror' after the analysis. To further remove false positive GWAS hits, we
196 removed any considerable clusters of unitigs (> 20) with identical *p*-values, as these are
197 likely to be lineage-specific markers or markers with strong linkage disequilibrium
198 comprised of mostly non-causal variants linked on the same clonal frame. GWAS hits

199 were annotated by mapping the unitigs to two reference genomes of *V. cholerae*,
200 namely, E7946 (NCBI assembly accession number: GCA_002749635.1) and O1
201 biotype El Tor strain N16961 (NCBI assembly accession number: GCA_003063785.1)
202 using BWA. Statistically significant GWAS hits were further annotated with the CARD
203 resistance gene identifier (RGI) after filtering the 'loose' hits and hits with identity <0.90.

204 **Antibiotic detection by liquid chromatography mass spectrometry (LC-MS/MS).**
205 The approach was based on a prior study [51]. Stool supernatant from the primary
206 collection were obtained by centrifugation and filtration (0.2 μM surfactant-free cellulose
207 acetate; Thermo Scientific Nalgene). Proteins were precipitated (1:7 ratio (v/v) of
208 water:methanol). Supernatants were diluted with methanol and water (1:1 v/v) in 0.1%
209 formic acid for liquid chromatography, and 5 μl of supernatant was injected for analysis.
210 LC/MSMS was performed on a 2.1 x 150-mm Hypersil Gold aQ column (particle size, 3
211 μm) using a high-performance liquid chromatography system (Thermo UltiMate 3000
212 series) with an LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific). Mobile
213 phases were 1% formic acid in water (A) and 1% formic acid in methanol (B) and held at
214 a constant 5%B for 2min before ramping to 95%B at 15 min where it was held for an
215 additional minute before returning to starting conditions for a total run time of 25 min.

216 Eluent was ionized using electrospray ionization (ESI) in positive mode at a spray
217 voltage of 5 kV, a nitrogen sheath gas flow rate of 8 L min^{-1}, and capillary temperature
218 of 300°C. Two scan events were programmed to perform an initial scan from *m/z* 100 to
219 1000, which was followed by targeted collision induced dissociation based on a
220 retention time and mass list. Retention time windows ranged from 0.35 minutes to 6.50
221 min, depending on the elution range of the standards at high and low concentrations.

222 Masses were targeted for the most abundant adduct or ion associated with each
223 antibiotic (typically the $[M+H]^+$ ion) with a *m/z* 1 window. Data analysis for amoxicillin,
224 sulfamethoxazole/trimethoprim, azithromycin, tetracycline, doxycycline, metronidazole,
225 nalidixic acid, and ciprofloxacin was performed manually using extracted ion
226 chromatograms and MSMS matching with an in-house antibiotic MSMS library using
227 Xcalibur 2.2 SP 1.48 (Thermo Fisher Scientific).

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

231 **RESULTS**

232 **Comparison of antimicrobial resistance under aerobic and anaerobic conditions.**
233 We measured baseline growth parameters for each isolate under anaerobic and aerobic
234 conditions and found that carrying capacity, area under the growth curve (AUC), and
235 growth velocity were all significantly lower under anaerobic conditions (Supplementary
236 Table 2). We tested an assumption that under anaerobic conditions mixed fermentation
237 and anaerobic respiration would occur in LB media. Analyzing a subset of three isolates
238 and the reference strain E7946, we monitored for acidification as a sign of fermentation
239 and assessed the impact of the addition of an alternative electron acceptor (20 mM
240 fumarate) for anaerobic respiration. Under anaerobic conditions, one out of four strains
241 acidified the LB media to 6.0 (Supplementary Table 3), suggesting fermentation in at
242 least some isolates. The addition of fumarate as an alternative electron acceptor under
243 anaerobic conditions resulted in a small increase in AUC of 25-27% (Supplementary

244 Table 3), suggesting that in LB media anaerobic respiration likely occurs but is limited
245 for alternative electron acceptors. These results are consistent with mixed fermentation
246 and anerobic respiration in our experimental conditions.

247 In this physiologic context and using standard antibiotic breakpoints established for
248 aerobic conditions, AMR differed between anaerobic versus aerobic conditions (Fig. 1);
249 distributions of single and multi-agent AMR phenotypes are shown (Fig. 2A). The MIC
250 modes for ciprofloxacin were 8 μg/ml (min=0.016 μg/ml; max=32 μg/ml) and 2 μg/ml
251 (min=0.004 μg/ml; max=8 μg/ml) under anaerobic and aerobic conditions, respectively
252 (Supplementary Table 4); the rates of resistance under anaerobic (93%; N=62/67) and
253 aerobic (54%; N=36/67) conditions were significantly different (Fig. 1A; McNemar's test
254 $p<0.001$; Supplementary Table 5). For azithromycin, the MIC modes were 32 μg/ml
255 (min= 8 μg/ml; max=124 μg/ml) and 4 μg/ml (min=1 μg/ml; max=32 μg/ml) under
256 anaerobic and aerobic conditions respectively (Supplementary Table 4). The rates of
257 resistance under anaerobic (n=67/67; 100%) and aerobic (n=15/67; 22%) conditions
258 were significantly different (Fig. 1B; McNemar's test $p<0.001$; Supplementary Table 5).
259 For doxycycline, the MIC modes were 1 μg/ml under both aerobic and anaerobic
260 conditions, respectively (Supplementary Table 4); one isolate was resistant under
261 anaerobic conditions alone, and one isolate was resistant under both anaerobic and
262 aerobic conditions. The odds of classifying isolates as resistant increased over 10 times
263 for ciprofloxacin (OR= 10.5; 95% CI= 3.61-37.7) and over 200 times for azithromycin
264 (OR = 213; 95% CI= 31.9->5000) under anaerobic compared to aerobic conditions.

265 To evaluate the generalizability of these findings from the primary sample collection, we
266 also compared aerobic and anaerobic growth curves of 277 isolates from the secondary

267 sample collection. For ciprofloxacin, the rates of resistance were significantly different
268 under anaerobic conditions (21%; n= 58/277) compared to aerobic conditions (1.1%; n=
269 3/277; McNemar's test p<0.001; Supplementary Table 6). For azithromycin, the rates of
270 resistance were significantly different under anaerobic conditions (100%; N= 277/277)
271 compared to aerobic conditions (57%; N= 159/277; McNemar's test p<0.001;
272 Supplementary Table 6). For doxycycline, only two isolates were resistant under
273 anaerobic conditions alone and one under both anaerobic and aerobic conditions
274 (Supplementary Table 6). The odds of classifying isolates as resistant increased over
275 25 times for ciprofloxacin (OR= 25.7; 95% CI= 18.8-34.6) and 119 times for
276 azithromycin (OR = 119; 95% CI= 20.95 – 4739) under anaerobic compared to aerobic
277 conditions.

278 **Addition of catalase to test if reactive oxygen species effect antibiotic**
279 **resistance/sensitivity under aerobic conditions.** In this experiment, catalase was
280 added to the media to quench hydrogen peroxide with the objective of testing the
281 hypothesis that susceptibility under aerobic conditions was associated with ROS (e.g.
282 hydrogen peroxide). For ciprofloxacin, the MICs for the sensitive reference strain E7946
283 (Cip^S, Azi^S, Dox^S) and the resistant clinical isolate EN160 (Cip^R, Azi^R, Dox^S) remained
284 unchanged when catalase was added to the media under aerobic conditions. The
285 addition of catalase was not associated with differences in AUCs for both E7946 and
286 EN160 in media containing ciprofloxacin, azithromycin, or doxycycline at 2-fold below
287 the MIC. The AUCs in LB media with and without catalase alone for E7946 and EN160
288 were not statistically different (Supplementary Table 7).

289 **Molecular AMR correlates under aerobic and anaerobic conditions**. The distribution
290 of known AMR genetic elements is shown (Fig. 2B). AMR-associated point mutations
291 (likely transmitted vertically, not on an established mobilizable element), and genes on
292 known horizontally transferred mobilizable elements, are provided (Supplementary
293 Material). The integrative conjugative element (ICE) SXT/R391 was found in 90%
294 (60/67) of isolates. The ICE elements contained the pentapeptide repeat protein that
295 confers fluoroquinolone resistance (qnr_{Vc}), the macrolide-inactivating
296 phosphotransferase (*mphA*), and the major facilitator superfamily (MFS) efflux pump
297 conferring tetracycline resistance (*tet*(59)) [52-55]. The genes qnr_{Vc}, *mphA*, and *tet*(59)
298 were found in 78% (52/66), 33% (22/66), and 78% (52/66) of isolates, respectively.
299 Ciprofloxacin resistance under both anaerobic and aerobic conditions was significantly
300 associated with qnr_{VC}, *gyrA* and *parC* (Supplementary Table 8). Identification of the
301 known azithromycin AMR gene *mphA* was significantly associated with resistance under
302 aerobic conditions alone (P<0.001). The gene *tet*(59) was not associated with
303 doxycycline resistance under aerobic or aerobic conditions (both p=0.566).

304 We next used GWAS to comprehensively explore the genetic basis of AMR. This
305 approach used the phenotype of AUC to represent 'growth' with or without exposure to
306 the three test antibiotics at five concentrations under either aerobic or anaerobic
307 conditions. Growth phenotypes (analyzed by AUCs) at similar antibiotic concentrations
308 were positively correlated within aerobic and anaerobic conditions for all three
309 antibiotics (Fig. 3). Growth phenotypes were also positively correlated between aerobic
310 and anaerobic conditions for ciprofloxacin (Fig. 3A). However, phenotypes were weakly,
311 or even negatively, correlated between aerobic and anaerobic conditions for

312 azithromycin and doxycycline (Fig. 3B,C). These results support the hypothesis that
313 anaerobic and aerobic growth under antibiotic pressure can differ and be distinct to
314 antibiotic type.

315 The heritability of the AMR phenotypes (AUCs) was estimated prior to the GWAS.
316 Heritability (h^2) is defined as the proportion of phenotypic variation explained by genetic
317 variation, measured as unique contiguous tracts of the assembled genomes (unitigs)
318 that tag both single nucleotide variants, indels, and gene content changes (Methods).
319 We found relatively high heritability (h^2 in the range 0.60-0.99) of growth across
320 concentrations of ciprofloxacin under both aerobic and anaerobic conditions, yielding
321 statistically significant GWAS hits (Table 1; Supplementary Data Files S1 and S2): 20
322 under aerobic conditions and 16 under anaerobic conditions. In contrast, heritability
323 tended to be much lower under aerobic compared to anaerobic conditions for both
324 azithromycin and doxycycline, yielding significant GWAS hits only under anaerobic
325 conditions (Table 1; Supplementary Data Files S3 and S4, respectively): 3 for
326 azithromycin under anaerobic conditions alone and 57 for doxycycline under anaerobic
327 conditions alone.

328 AMR genes identified by GWAS were diverse (Fig. 4; Supplementary Data Files S1-S4).
329 These candidates included known AMR genes, such as qnr_{Vc} and *dfrA,* which were
330 associated with ciprofloxacin resistance under both aerobic and anaerobic conditions.
331 We identified seven genes associated with ciprofloxacin resistance under anaerobic
332 conditions alone (including the stress response gene *barA* and a *radC* homolog involved
333 in DNA repair; Supplementary Data File S2), and ten genes under aerobic conditions
334 alone (including *rtxB*; Supplementary Data File S1). Under anaerobic conditions, most

335 genes were identified at ciprofloxacin concentrations at, or above, 0.25 µg/ml; however,
336 four genes, including *barA*, were identified under one of the lowest tested ciprofloxacin
337 concentrations (0.13 µg/ml; Supplementary Data File S2). GWAS hits for azithromycin
338 and doxycycline resistance were found only under anaerobic conditions. For
339 azithromycin, two genetic elements were identified: *mphA* and a region between *ompT*
340 and *dinG* (*ompT-dinG*; Supplementary Data File S3*)*. For doxycycline, 23 genes were
341 shared across concentrations; however, the gene discovery rate was highest at the
342 lower concentrations (n=53 at 0.13 µg/ml; n =26 µg/ml; Supplementary Data File S4).
343 GWAS hits included the major facilitator superfamily antibiotic efflux pump *tet*(59) (Fig.
344 4, Supplementary Data Files). Most genetic elements identified have unknown function.
345 The identification of known AMR genes by GWAS serves as positive control and
346 suggests that the genes of unknown function may indeed play a role in AMR.

347 **Antibiotics detected in stool by LC-MS/MS.** Finally, we sought to test the hypothesis
348 that AMR genotypes and phenotypes would be associated with measured
349 concentrations of antibiotics in stool. A combined total of 196 antibiotics were detected
350 in the 51 stool supernatants tested by mass spectrometry using a targeted technique for
351 9 common antibiotics (Fig. 5). At least one antibiotic was detected in 98% (n=50/51), at
352 least two antibiotics were detected in 94% (n=48/51), and three or more antibiotics were
353 detected in 90% (n=46/51) of stool supernatants (Fig. 5). Antibiotics detected were
354 ciprofloxacin (n=48/51; 94%), tetracycline / doxycycline (n=46/51; 90%), nalidixic acid
355 (n=41/51; 80%), metronidazole (n=37/51; 73%), sulfamethoxazole/trimethoprim
356 (n=22/51; 43%), and amoxicillin (n=2/51; 4%); azithromycin was not detected. Detection
357 of quinolone/fluoroquinolone and tetracycline/doxycycline in stool by LC-MS/MS was not

358 associated with AMR genotypes or phenotypes (Supplementary Table 9). Associations
359 for azithromycin could not be tested because azithromycin was not detected in any stool
360 supernatant.

361 **DISCUSSION**

362 In this study, *V. cholerae* isolates from cholera patients were found to be more resistant
363 to antibiotic exposure under anaerobic conditions compared to aerobic conditions (Fig.
364 1). This phenotype differed by antibiotic class. Novel genetic elements were found to
365 associate with AMR under anaerobic conditions which also differed by antibiotic class
366 (Fig. 4). The approach of using a continuous variable (area under the growth curve
367 (AUC)) for the AMR phenotype within the framework of GWAS may provide a new
368 approach to identify putative AMR genetic targets for future mechanistic research.

369 CLSI breakpoints for enteropathogens like *V. cholerae* were developed under aerobic
370 conditions [45, 56]. CLSI and other clinical reference bodies set breakpoints to have
371 clinical relevance despite limited data from clinical studies [56-58]. In this context, the
372 odds of classifying isolates in the primary collection as resistant under anaerobic
373 conditions compared to aerobic conditions increased over 10 times for ciprofloxacin and
374 over 200 times for azithromycin. These results are likely general across *V. cholerae*
375 because in the secondary collection, which is separated by more than 10 years, we
376 found that the odds of classifying isolates as resistant under anaerobic conditions
377 compared to aerobic conditions increased over 20 times for ciprofloxacin and over 100
378 times for azithromycin.

379 There are several physiologic explanations for increased antibiotic resistance under
380 anaerobic conditions. ROS induce both intracellular and cell-wall stress and are at
381 higher concentrations under aerobic conditions [59]. ROS may have acted
382 synergistically to potentiate antibiotic lethality [31, 60]. The assays that utilized catalase
383 to quench hydrogen peroxide under aerobic conditions were conducted to evaluate this
384 possibility. The MICs for all three antibiotics did not increase with the addition of
385 catalase suggesting that the reduction of ROS alone cannot account for increased MICs
386 observed under anaerobic conditions (Supplementary Table 7). We hypothesize that
387 reduced growth under anaerobic conditions might decrease the effectiveness of
388 antimicrobial agents that directly or indirectly disrupt cell envelope integrity [61]. While
389 reduced growth was observed under anaerobic conditions, the antibiotics tested are not
390 known to directly disrupt the cell envelope. However, off target effects (e.g. cell
391 envelope stress) by CIP, AZI, and DOX may have occurred.

392 To further investigate AMR phenotypes under anaerobic conditions, future studies will
393 benefit from the use of a defined medium (such as M9) where the carbon source and an
394 alternative electron acceptor (e.g. fumarate, nitrate, dimethylsulfoxide, or trimethylamine
395 N-oxide) can be supplemented to strictly control anaerobic respiration versus
396 fermentation. Buffering the medium (e.g. with PBS or bicarbonate) to a neutral pH is
397 important because *V. cholerae* can acidify the medium over time, with toxic effects. In
398 prior research, acidification occurred after 10 hours [9]. In our shorter 8-hour assay,
399 acidification was observed under anaerobic conditions among 1 of 4 strains tested
400 (Supplementary Table 3). Furthermore, buffering to the alkalinic pH found in cholera
401 stool (pH 8.5-9) will provide important insight given that *V. cholerae* utilizes nitrate for

402 anaerobic respiration only at alkaline pH [2, 9]. Here we used an undefined medium
403 (LB) without a defined, saturating concentration of an alternative electron acceptor. This
404 approach likely resulted in mixed anaerobic respiration and fermentation. To address
405 these limitations in future studies, robotic automation and 384-well formatted assays
406 would enable a scalable system for multiple defined media across broad gradients of
407 antibiotics. Ideally, we would simulate the conditions of the human gut, but in practice
408 these conditions can only be approximated.

409 There are many knowledge gaps on the genetic basis of the AMR phenotypes under
410 varying environmental conditions. This study prioritized the factor of oxygen exposure
411 as a determinant of AMR phenotypes because facultative anaerobic enteropathogens
412 experience hypoxia and anoxia in the animal gut [9]. The first phase of the analysis
413 focused on previously known AMR genotypes with known AMR phenotypes. For
414 ciprofloxacin, mutations in *parC* and carriage of qnr_{Vc} were significantly associated with
415 phenotypic resistance under aerobic and anaerobic conditions; mutations in *gyrA* were
416 significantly associated with resistance under anaerobic conditions alone
417 (Supplementary Table 8). For azithromycin, *mphA* was identified and significantly
418 associated with AMR under aerobic conditions alone. While *tet*(59) was identified, very
419 few isolates were identified as resistant to doxycycline under aerobic (n=1) or anaerobic
420 conditions (n=2). These associative data begin to reveal a difference between AMR
421 genotypes and phenotypes under aerobic and anaerobic conditions.

422 The second phase of analysis sought to use GWAS to identify previously unknown
423 genetic targets associated with AMR. The continuous variable of AUC, as opposed to
424 the binary variable of growth/ no growth, was used in assays with and without antibiotic

425 exposure under aerobic and anaerobic conditions. Antibiotics at the breakpoint and sub-
426 breakpoint concentrations were chosen based on a rationale that different genetic
427 elements might contribute differently to AMR phenotypes at different antibiotic
428 concentrations. As expected, GWAS identified qnr_{Vc} for ciprofloxacin exposure under
429 both aerobic and anaerobic conditions (Fig. 4). GWAS identified *mphA* for azithromycin
430 exposure under anaerobic conditions alone and *tet*(59) for doxycycline exposure under
431 anaerobic conditions alone. These results of known AMR genes served as 'positive
432 controls' for the GWAS. We also note that genes known to be important for anaerobic
433 respiration (e.g. *tatA1, tatC, ccmA-F, ccmH, moaA-D, moeA-B, napA-D, napF, fnr, narP-*
434 *Q, nqrF, dsbA, dsbD, hemN*) in *V. cholerae* were not identified as GWAS hits [9]. This
435 suggests that our GWAS was specific to AMR phenotypes and was not liable to detect
436 genes related to anaerobic conditions alone. We therefore expect novel GWAS hits to
437 be likely candidates for involvement in AMR phenotypes.

438 For ciprofloxacin, seven genes were associated with AMR in anaerobic conditions
439 alone; these included a gene involved in DNA repair (*radC*), 2-component histidine
440 kinase involved in stress response (*barA*), and an ATP-dependent zinc protease. The
441 gene *dfrA31* encodes a trimethoprim-resistant dihydrofolate reductase and APH(6)-Id
442 encodes a streptomycin phosphotransferase enzyme; both genes were identified for
443 aerobic and anaerobic conditions. These two genes are located on the SXT element
444 along with qnr_{Vc} and may therefore be associated due to genetic linkage rather than due
445 to causal roles in ciprofloxacin resistance. For azithromycin, one additional genetic
446 element under anaerobic conditions was discovered to associate with AMR: an
447 intergenic region between *ompT* (porin; known to be associated with AMR) [62, 63] and

448 *dinG* (ATP-dependent DNA helicase). For doxycycline, a diverse set of 57 genetic
449 elements under anaerobic conditions alone were discovered to associate with AMR.
450 These include *vexK* (efflux RND transporter permease associated with AMR) [64-66],
451 and *zorA* (anti-phage defense system ZorAB subunit A; a putative proton channel that
452 may respond to membrane perturbation by depolarization) [67].

453 In addition to the SXT element, genes associated with an AMR phenotype were also
454 discovered on the Vibrio Pathogenicity Island II (VSPII; N16961 VC0506-VC0512 /
455 E7946 loci RS02705-RS02745); these loci are genetically diverse in Bangladesh [68].
456 The GWAS hits in VSPII encode both biofilm/ auto-aggregation associated factors as
457 well as an aerotaxis protein (AerB; VC0512) [69]; findings consistent with roles in AMR
458 and aerobic/anaerobic conditions. These GWAS analyses were of limited power due to
459 the modest sample size, and could be sensitive to false positives at AMR 'hot-spots' like
460 SXT. Despite these limitations, GWAS enabled the discovery of an intriguing list of
461 genetic targets that were associated with AMR and require future mechanistic molecular
462 analysis to test for causal relationships.

463 LC-MS/MS analysis on the stools from the primary collection, stools from which the
464 isolates were obtained, was conducted to test the hypothesis that the rates of AMR
465 genotypes and phenotypes were higher when the stool samples contained the cognate
466 antibiotic. Nearly all patients shed at least one antibiotic, making it difficult to identify
467 AMR correlates to exposure (Fig. 5). This finding is important because studies that
468 leverage natural infection to set clinically meaningful AMR breakpoints under aerobic
469 conditions, and now anaerobic conditions, cannot readily be performed because of the
470 degree of antibiotic exposure among diarrheal patients. Therefore, future interventional

clinical studies with known antibiotic exposure determined *a priori* may be required. Given that the primary collection is from patients that self-reported not taking antibiotics, the detection of a combined total of 196 antibiotics further highlights the ubiquity of antibiotics and the limited value of self-reported antibiotic exposure.

Conclusions

Facultative enteropathogens are exposed to antibiotics under aerobic and anaerobic conditions in both the human gut and in the environment. We used the facultative anaerobic enteropathogen *V. cholerae* as a model to test for differences in AMR phenotypes under aerobic and anaerobic conditions. Increased resistance was found under anaerobic conditions compared to aerobic conditions. Using AMR breakpoints established for aerobic conditions, the odds of classifying isolates as resistant under anaerobic compared to aerobic conditions increased over 10 times for two of the three antibiotics tested. While several known resistance genes were associated with AMR under both conditions, many genes were only associated with AMR under one condition. Heritability tended to be higher, and more genes associated with resistance, under anaerobic conditions. This suggests that key genetic determinants of resistance may be missed when experiments are only performed aerobically. Our findings provide a rationale to determine if increased MICs under anaerobic conditions are associated with therapeutic failures and/or microbial escape in cholera patients, and if true, there may be a need to determine AMR breakpoints for anaerobic conditions.

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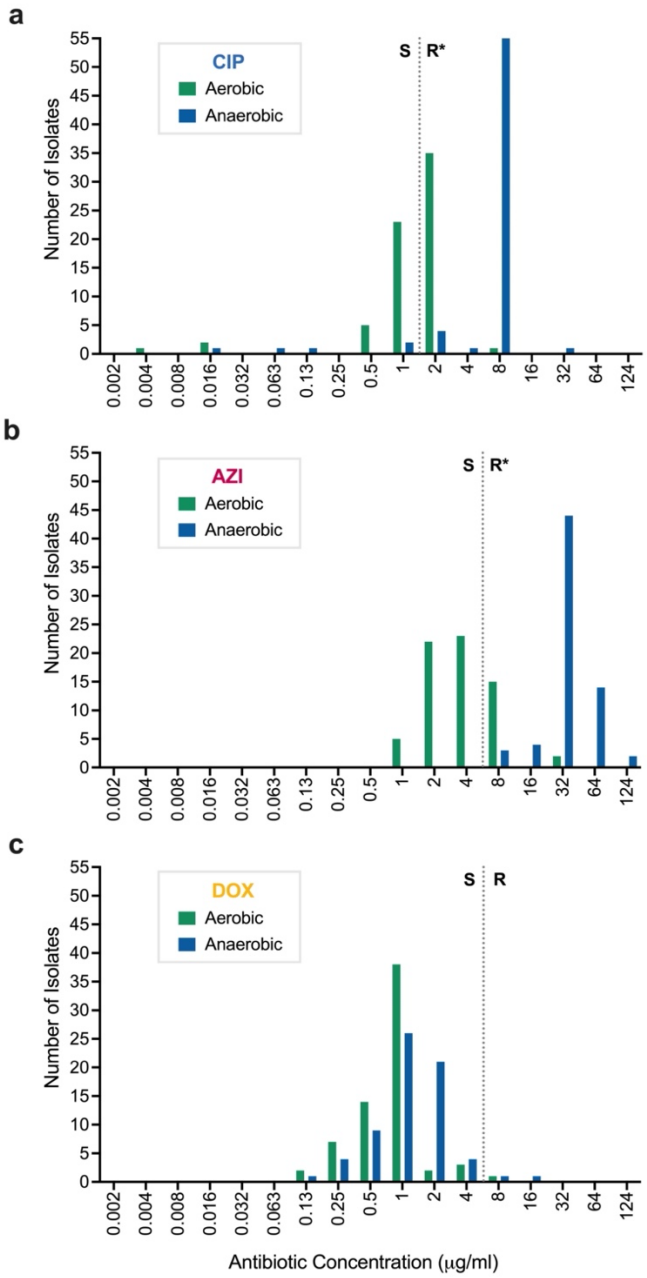
517 **Potential conflicts of interest.**

518 All authors: No reported conflicts.

519 **FIGURES**

520

521 **FIGURE 1**

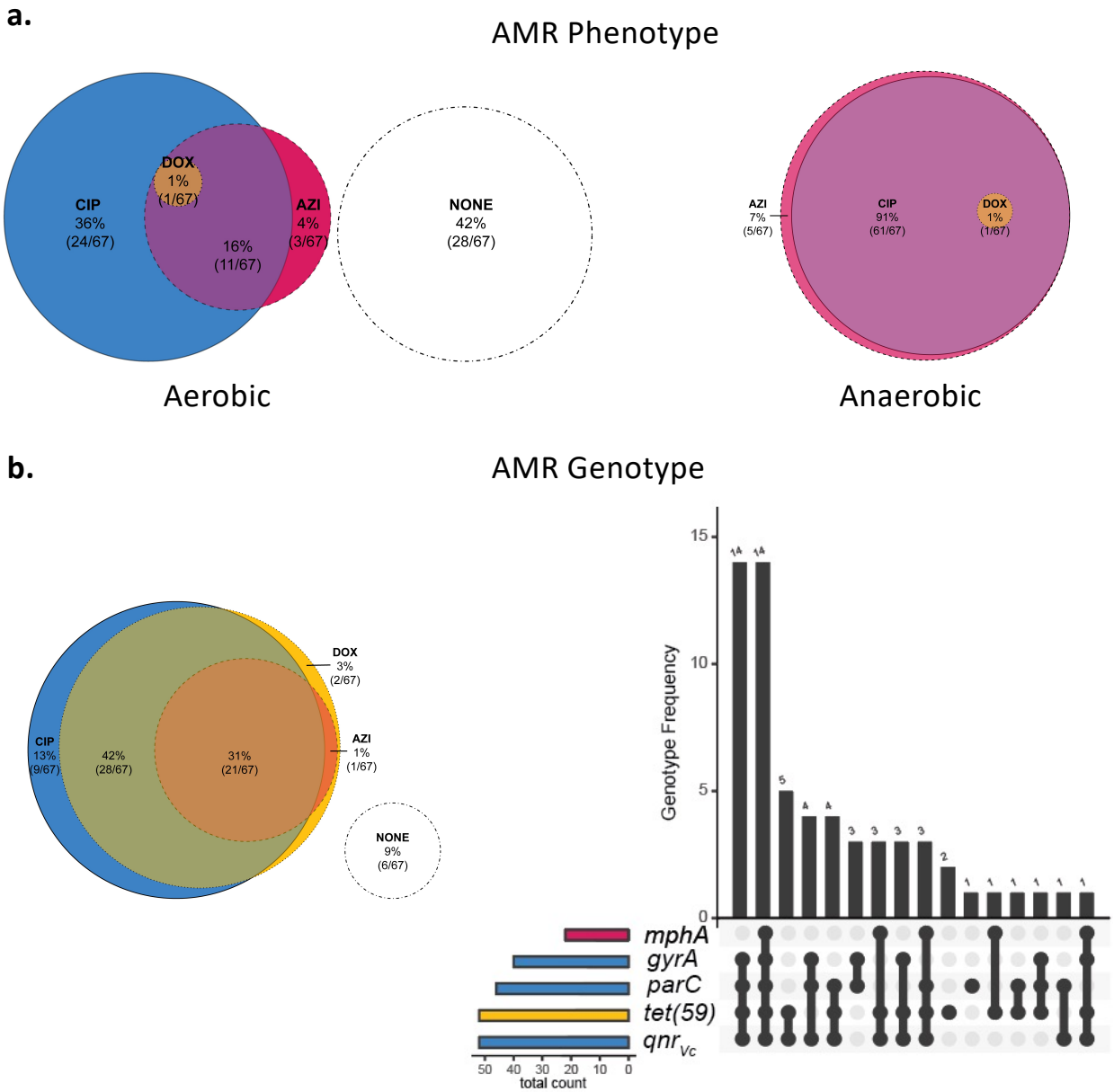


522

523 **FIG 1** Distribution of minimal inhibitory concentrations (MICs) under aerobic and anaerobic conditions
524 among clinical isolates from the primary collection. Ciprofloxacin (CIP; **a**), Azithromycin (AZI; **b**), and
525 Doxycycline (DOX; **c**). Data are from 67 human-shed *V. cholerae* isolates. The MICs for each isolate
526 under aerobic (green) and anaerobic (blue) conditions were enumerated, and the number of isolates with
527 a given MIC (μg/ml) are represented as bars. Dotted lines are the breakpoint for resistance per CLSI
528 standards which are based on assays under aerobic conditions (CIP = 2 μg/ml; AZI = 8 μg/ml; DOX = 8
529 μg/ml). S=sensitive. R = Resistant. "*" represents a significant difference in the frequency of isolates
530 identified as resistant to ciprofloxacin and azithromycin by McNemar's test (both $p<0.001$).

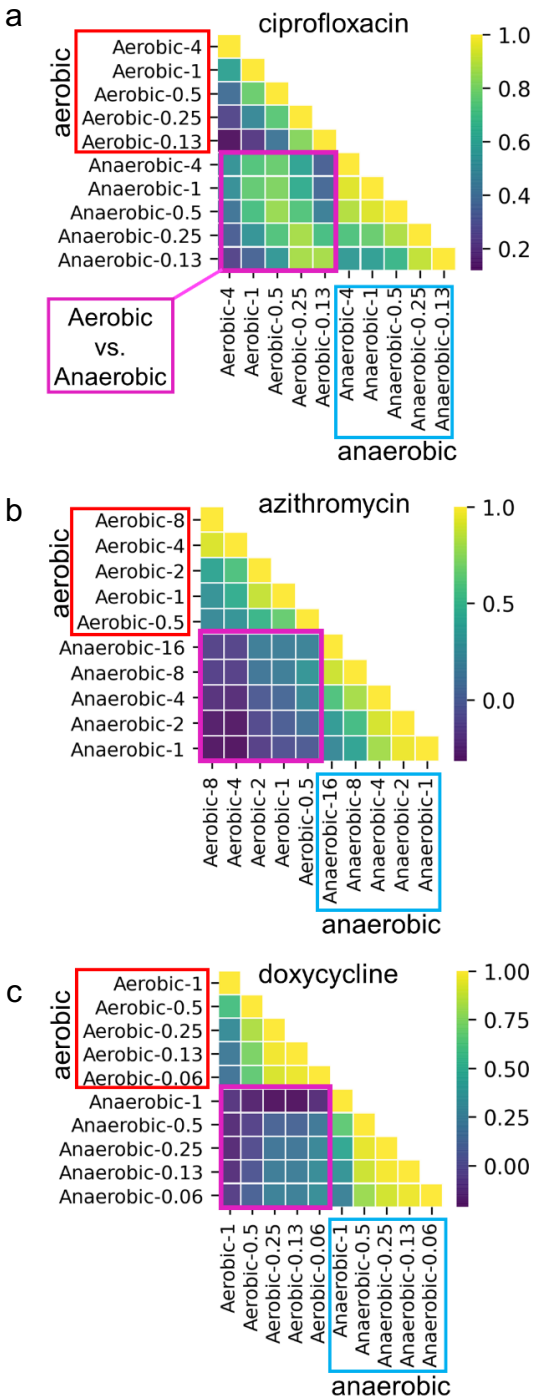
531 **FIGURE 2**

532



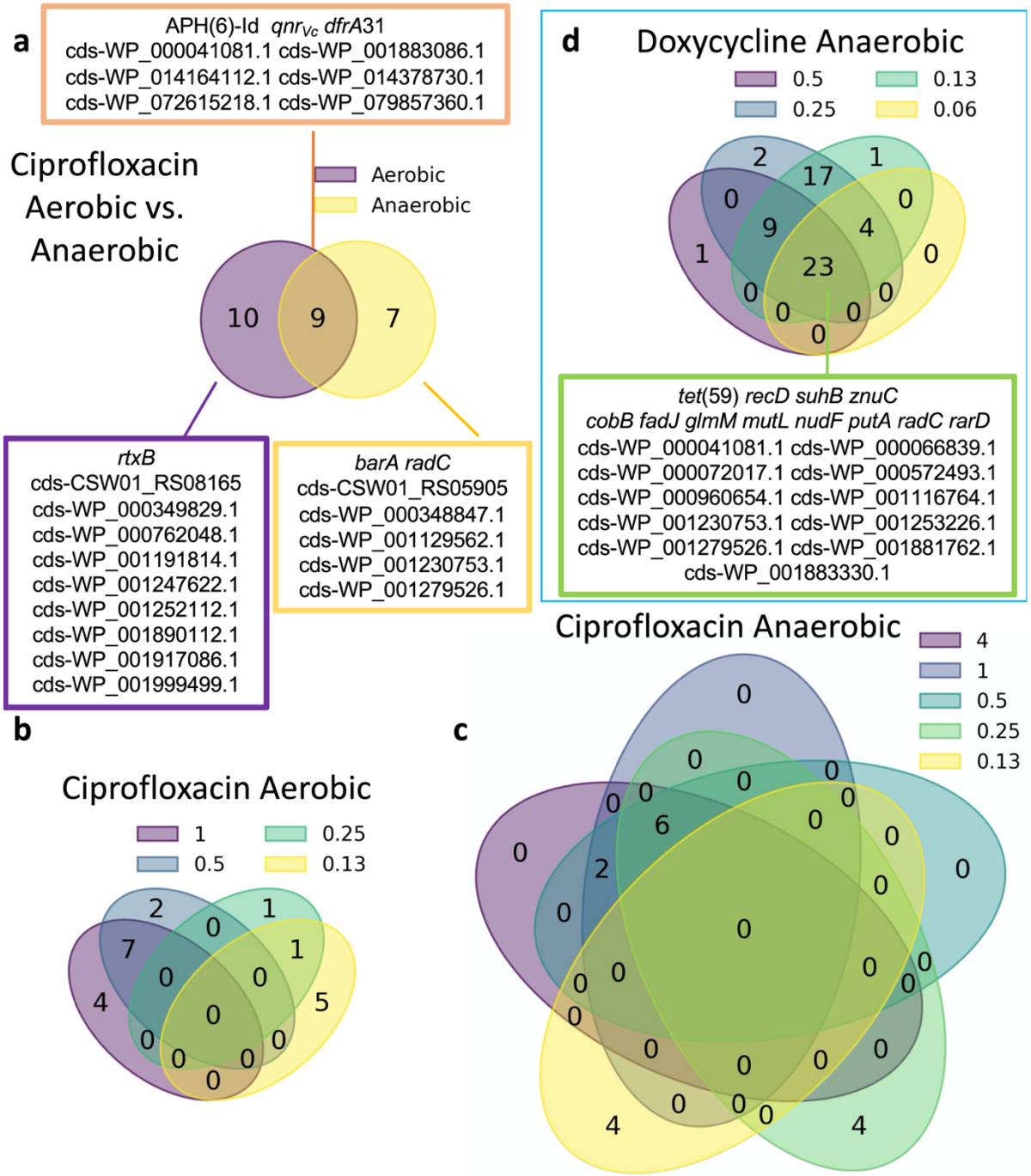
533
534 **FIG 2** AMR phenotypes and known AMR genetic elements in human-shed *V. cholerae* isolates from the
535 primary collection. Pink, yellow, and blue color coding is used to respectively indicate AMR phenotypes
536 and genotypes to azithromycin (AZI), doxycycline (DOX) and ciprofloxacin (CIP); colors blend when
537 overlapped. Isolates with no resistance (sensitive) are shown in white circles. (**a**) Proportional Venn
538 diagram (Euler) of AMR phenotypes to AZI, DOX, and/or CIP under aerobic (left) and anaerobic
539 conditions (right). Counts indicate the number of isolates with the corresponding phenotype. (**b**) AMR
540 genotypes from known resistance genes in whole genome sequences. Shown is the distribution of known
541 AMR genetic elements by proportional Venn diagram (Euler; left) and bar chart (right). On right, the X axis
542 of the bar chart depicts the presence (black points) of known AMR genes (*mphA, gyrA, parC*, *tet*(59),
543 qnr_{Vc}) in a given genome and the Y axis depicts the number of isolates that share the given combination
544 of AMR genes. Coloured bars to the left indicate the number of isolate genomes encoding resistance
545 genes to CIP (blue), AZI (pink), or DOX (yellow). AMR genetic elements to other antibiotics are not
546 shown.

547 **FIGURE 3**



548
549 **FIG 3** Correlation analysis of growth phenotypes at different concentrations of antibiotics under aerobic
550 and anaerobic conditions among *V. cholerae* clinical isolates from the primary collection. Antibiotic
551 exposures were ciprofloxacin (**a**), azithromycin (**b**), and doxycycline (**c**). AUC was analyzed as the growth
552 parameter. Aerobic/anaerobic conditions are labeled horizontally and vertically with the antibiotic
553 concentration in µg/ml (e.g., "Anaerobic-0.06"). Analyses are grouped: aerobic vs aerobic = red boxes;
554 anaerobic vs aerobic = purple box; anaerobic vs anaerobic = blue boxes. Heatmaps show correlation
555 coefficients (scale bar is to right) for similar (yellow) vs dissimilar (purple) growth at two given conditions.

556 **FIGURE 4**



557
558 **FIG 4** Distribution of AMR genes associated with AMR growth phenotypes at different concentrations of
559 antibiotics under aerobic and anaerobic conditions among isolates from the primary collection. Venn
560 diagrams show the overlap between genes associated with (**a**) ciprofloxacin resistance under aerobic vs.
561 anaerobic conditions, (**b**) ciprofloxacin at different concentrations (µg/ml) under aerobic conditions, (**c**)
562 ciprofloxacin at different concentrations (µg/ml) under anaerobic conditions, and (**d**) doxycycline at
563 different concentrations (µg/ml) under anaerobic conditions. Genes shown in boxes had statistically
564 significant associations.

565 **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)

566
567
568 **FIG 5** Antibiotic detection in stool supernatants by mass spectrometry (LC-MS/MS) among cholera
569 samples from the primary collection. Green with "+" = Detected. White with "-" = not detected. CIP=
570 ciprofloxacin, TET/DOX= tetracycline and/or doxycycline, NAL = nalidixic acid, MET = metronidazole,
571 BAC = sulfamethoxazole and/or trimethoprim, AMO = amoxicillin, ERY = erythromycin, CEF =
572 ceftriaxone. Stool supernatants were not available for EN80, 86, 88, 92, 100, 103, 109, 116-120, 126,
573 124, 130, 131.

574 **TABLE**

575

576

577 **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

578 [a] Ciprofloxacin = CIP; Azithromycin = AZI; Doxycycline = DOX
579 [b] Heritability is the proportion of phenotypic variation that is explained by genetic variation. Associated
580 genes are all significant GWAS hits after correction for multiple hypothesis testing ($P < 0.05$ after
581 Bonferroni correction).
582

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