1 **The association between undetected heteroresistance and antibiotic treatment failure in**

2 **complicated urinary tract infection**

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21

22  **Abstract**

23  Background: Antibiotic resistance is a worsening public health threat. One poorly understood

24  aspect of this problem is unexpected antibiotic treatment failure; when an infecting isolate is

25  deemed susceptible to a given antibiotic, yet treatment with that drug fails. It has been proposed

26  that heteroresistance may be an explanation for at least some unexplained treatment failures.

27  Heteroresistance occurs when a bacterial isolate harbors a minor subpopulation of resistant cells

28  which coexists with a majority susceptible population. The clinical relevance of heteroresistance

29  is not clear.

30

31  Methods: We obtained 291 index isolates from 288 unique patients in the piperacillin/tazobactam

32  arm of the ALLIUM phase 3 clinical trial for the treatment of Gram-negative pathogens causing

33  complicated urinary tract infections. The MIC for all isolates was below the

34  piperacillin/tazobactam resistance breakpoint according to standard antimicrobial susceptibility

35  testing. We performed population analysis profiles on these isolates to detect

36  piperacillin/tazobactam heteroresistance and conducted a post hoc analysis to examine the

37  impact of heteroresistance on clinical outcomes.

38

39  Findings: We observed that 33/288 (11.5%) of the patients were infected with isolates that were

40  heteroresistant to piperacillin/tazobactam and that patients infected with heteroresistant isolates

41  had an increased rate of treatment failure when compared to patients infected with a non-

42  heteroresistant isolate (odds ratio [OR] 2.13, 95% CI 1.02, 4.41; adjusted OR 1.74, 95% CI 0.82,

43  3.71). Further, patients without a removable catheter were at particular risk of treatment failure

44  from infection with heteroresistant isolates.

45

46  Interpretation: These data demonstrate that patients infected with a piperacillin/tazobactam

47  heteroresistant isolate are at an increased risk for piperacillin/tazobactam treatment failure. The

48  results help contextualize commonly observed unexpected antibiotic treatment failure and

49  highlight heteroresistance as a potential cause.

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60

61  Competing Interests: PV is an employee of Allecra Therapeutics. AB was a paid consultant for

62  Allecra at the time of the study. NN reports grants/contracts from Merck and Shionogi;

63  consulting/speaker fees from Astellas. KSK has received consulting fees from Merck, Shionogi,

64  GlaxoSmithKline, MicuRx Pharmaceuticals, AbbVie, Venatorx Pharmaceuticals, and Allecra

65  Therapeutics, and owns stock options in Merck. DSW has pending patents related to

66  heteroresistance.

67

68    **Introduction**

69    Antimicrobial resistance poses a significant threat to public health. In 2019 alone, approximately

70    1.27 million deaths were attributed to antimicrobial resistance and this global crisis is expected

71    to worsen (1). Projections indicate that by 2050, antimicrobial resistance could be responsible for

72    10 million worldwide deaths annually (2). To combat this threat, gaining a better understanding

73    of mechanisms of antibiotic resistance and their contributions to treatment failure could help

74    optimize treatment regimens.

75

76    One clinical issue that currently lacks adequate attention is unexpected treatment failure.

77    Unexpected treatment failure occurs when a bacterial isolate infecting a patient is not eradicated

78    despite the isolate appearing susceptible to the given antibiotic based on *in vitro* laboratory

79    testing. One possible cause of treatment failure is heteroresistance, a form of antibiotic resistance

80    where a minor subpopulation of resistant cells co-exists with a majority susceptible population.

81    Notably, these resistant subpopulations are often undetected by standard susceptibility testing

82    methods yet rapidly replicate in the presence of a given antibiotic.

83

84    The clinical significance of heteroresistance remains uncertain. Studies employing murine

85    infection models have demonstrated that heteroresistance can mediate treatment failure (3-5).

86    Additionally, there have been documented case studies wherein antibiotic therapy failed to

87    eliminate an infection with an isolate heteroresistant to the antibiotic utilized for treatment (6, 7).

88

89    Given the limited clinical data regarding the impact of heteroresistance on treatment outcomes,

90    there remains a critical gap in understanding. Accordingly, we performed a post hoc analysis of

91     the ALLIUM trial to examine the effect of heteroresistance on clinical outcomes amongst

92     complicated urinary tract infection (cUTI) subjects treated with piperacillin/tazobactam.

93     Particular focus was given to cUTI without a removeable source of infection, due the higher

94     treatment failure rate in this type of UTI.  We hypothesized that heteroresistance might

95     associated with treatment failure in cUTI without a removeable source of infection.

96

97     **Materials and Methods**

98     Study Design and Population

99     We obtained clinical isolates from the ALLIUM clinical trial (NCT03687255), a phase 3,

100    randomized, double blinded trial to compare the efficacy of piperacillin/tazobactam versus

101    cefepime/enmetazobactam in treating hospitalized patients with ongoing cUTI, including acute

102    pyelonephritis (AP) (8). In this study, we performed secondary nested cohort analyses, in concert

103    with population analysis profiles, to identify heteroresistant isolates and assess heteroresistance

104    as a potential contributor to clinical outcome. The 291 isolates we obtained from this clinical trial

105    originated from patients assigned to the piperacillin/tazobactam arm, specifically from the

106    microbiologically evaluable subset, and were collected prior to any patient exposure to

107    piperacillin/tazobactam. Each patient received intravenous treatment with

108    piperacillin/tazobactam and was clinically evaluated at day 3 of treatment, end of treatment

109    (EOT), test of cure (TOC; EOT +7 days [+/- 2 days]), and late follow up (LFU; EOT + 14 days

110    [+/- 2 days]). Treatment outcome was determined by a composite of clinical and microbiological

111    outcomes with microbiological cure being defined as a reduction in urine bacterial load to $<10^3$

112    CFU/mL. Additional information pertaining to the trial protocol is available

113    https://classic.clinicaltrials.gov/ct2/show/NCT03687255 (8).

114

115    Clinical Isolates

116    The study population consisted of 288 patients from whom 291 Gram-negative isolates were

117    obtained. The isolates consisted of 216 *Escherichia coli*, 30 *Klebsiella* spp., 20 *Proteus* spp., 11

118    *Pseudomonas aeruginosa*, 4 *Enterobacter* spp., 3 *Citrobacter* spp., 3 *Serratia marcescens*, 2

119    *Acinetobacter ursingii*, 1 *Morganella morganii*, and 1 *Providencia rettgeri* (Supplementary

120    Table 1). The MIC of all isolates included in this study was below the piperacillin/tazobactam

121    resistance breakpoint according to the 2024 CLSI susceptibility interpretive criteria for broth

122    microdilution testing (9). Enterobacterales isolates with MICs at the susceptible-dose dependent

123    breakpoint of 16/4 µg/mL were included due to the extended infusion utilized in the trial (4 g

124    piperacillin, 0.5 g tazobactam, by 2-hour infusion every 8 hours) and because

125    piperacillin/tazobactam is concentrated at high levels in the urine (8). Breakpoints of <64/4

126    µg/mL and <128/4 ug/mL were used for *P. aeruginosa* and *A. ursingii*, respectively. All isolates

127    were part of the microbiologically evaluable population of the piperacillin/tazobactam arm of the

128    trial (8). The bacterial isolates were acquired from IHMA and stored at -80°C.

129

130    Population Analysis Profile

131    Population analysis profile (PAP) was performed by inoculating 1.5 mL of Mueller-Hinton Broth

132    (MHB) with a single colony streaked from a frozen stock of a given isolate. The culture was

133    incubated overnight for 16-18 hours in a 37°C shaking incubator at 200 RPM. The culture was

134    then serially diluted in PBS 1:10 over a series of 6 dilutions. The undiluted sample and the

135    dilution series were then plated together on Mueller-Hinton Agar (MHA) plates containing no

136    drug, 4/4, 8/4, 16/4, 32/4, or 64/4 ug/mL of piperacillin/tazobactam (MedChemExpress).

137    Colonies were enumerated after a 15-18 hour incubation at 37°C. Isolates were classified

138    resistant if they exhibited at least 50% survival on the 16/4 ug/mL piperacillin/tazobactam MHA

139    plate as compared to the number of colonies surviving on the MHA plate without drug. Isolates

140    were classified heteroresistant if they had less than 50% survival, but more than 0.0001%

141    survival, on two out of the three piperacillin/tazobactam concentrations (8/4 ug/mL, 16/4 ug/mL,

142    and 32/4 ug/mL) when compared to growth on the MHA plate without drug. Isolates that were

143    not classified resistant or heteroresistant were classified susceptible by PAP, and from this point

144    forward, will be referred to as non-heteroresistant (Supplementary Figure 1). Single biological

145    replicates were used, and each strain was assessed by PAP ≥3 times. In instances where patients

146    had more than one pathogen, if one or more of the pathogens is heteroresistant then the patient's

147    infection was categorized as heteroresistant; if none were heteroresistant then the infection was

148    categorized as non-heteroresistant.

149

150    Genome analysis

151    DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, cat. # A1120)

152    and susceptible isolates by PAP were sequenced using 400 Mbp long reads Illumina whole

153    genome sequencing and heteroresistant isolates by PAP were sequenced with a combination of

154    600 Mbp long reads of nanopore sequencing and 400 Mbp long reads of Illumina whole genome

155    sequencing. Raw paired-end sequence reads were analyzed using Bactopia pipeline version 3.0.1

156    (10)[1]. Briefly, the paired-end sequencing reads were fed into Bactopia pipeline with assigned

157    quality checks, and reads below the quality requirements were filtered out. The remaining reads

158    were assembled using Shovill version 1.1.0 (https://github.com/tseemann/shovill.git). Assembled

159    contigs were annotated using prokka version 1.14.6 (11). To construct phylogenetic tree,

160  Bactopia output files and raw reads were subjected to mashtree workflow on Bactopia. Mashtree

161  workflow uses mash version 2.3 to create mash distance and estimate mutation rates between

162  sequences and generates a newick format phylogenetic tree based on mash distance (12, 13). The

163  phylogenetic tree was visualized and annotated using Interactive Tree of Life (iTOL) version 6

164  (14).

165

166  Statistical Analyses

167  Statistical analyses were performed to determine associations between heteroresistance and

168  overall, clinical, and microbiological outcomes. Descriptive and inferential statistics were

169  performed for all variables. All categorical data were reported as percentages. Univariate

170  analyses were conducted using Fisher's Exact Test or Chi-square test for dichotomous variables

171  and using Student's t-test or Wilcoxon's Rank Sum test for continuous variables. Multivariable

172  analyses were conducted using logistic regression to evaluate the relationship between

173  heteroresistance and treatment outcomes while addressing potential confounding. All p values

174  were two-sided and a p value of $< 0.05$ was considered significant. A propensity score for the

175  likelihood of a pathogen to be heteroresistant was developed by assessing univariate predictors

176  of heteroresistance ($p<0.20$). These predictors were included in a logistic regression model

177  predicting heteroresistance. The propensity score was calculated by adding together the β-

178  coefficients of the model covariates. This propensity score was then included in logistic

179  regression models assessing the association between heteroresistance and outcomes. Data

180  analysis was performed using SAS software, version 9.4 (SAS Institute, Cary, NC).

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184 **Results**
185 **Bacterial Isolates and Patient Characteristics**

186 Two-hundred and eighty-eight subjects with 291 isolates were included in the study. The 291

187 bacterial isolates that had an MIC below the CLSI resistance breakpoint for

188 piperacillin/tazobactam as determined by BMD were tested for piperacillin/tazobactam

189 heteroresistance by population analysis profile (PAP). Three subjects each had 2 UTI pathogens

190 – in two of these instances both isolates were non-heteroresistant (and the subjects were

191 categorized as non-heteroresistant) and in the other instance, one of the pathogens was

192 heteroresistant and therefore the infection was categorized as heteroresistant. 33/288 (11.5%) of

193 the patients were infected with piperacillin/tazobactam heteroresistant isolate and the remaining

194 255/288 (88.5%) were non-heteroresistant (Table 1). Overall, the patients infected by

195 heteroresistant isolates had similar baseline characteristics as the patients infected by non-

196 heteroresistant isolates except for patients with diabetes (Table 1). Interestingly, patients with

197 diabetes had an increased likelihood of infection with a heteroresistant isolate (P-value 0.046)

198 (Table 1).

199

200 Extended spectrum beta-lactamase (ESBL)-encoding Enterobacterales were more likely to be

201 heteroresistant when compared to non-ESBL-encoding Enterobacterales (P-value < 0.001)

202 (Table 1). Although only 13/288 (4.5%) of patients were infected with non-Enterobacterales

203 isolates, we observed that those isolates were more likely to be heteroresistant (4/13; 30.8%) than

204 the Enterobacterales isolates (29/275; 10.5%) (P-value 0.049) (Table 1). The most common

205 pathogen was *E. coli*, consisting of 216/291 (74.2%) of all isolates and 25/33 (75.8%) of the

206 heteroresistant isolates, with the second most prevalent being *Klebsiella* spp. (30/291; 10.3%),

207 consisting of 2/33 (6.1%) of the heteroresistant isolates (Supplementary Table 1).

208

209 **Heteroresistance and Treatment Outcome**

210 The primary outcome of treatment failure at TOC occurred in 17/33 (51.5%) of the patients

211 infected with heteroresistant isolates and 85/255 (33.3%) of those infected with non-

212 heteroresistant isolates (Table 2), demonstrating that patients infected with a

213 piperacillin/tazobactam heteroresistant isolate had a significantly increased failure rate (2.13 OR,

214 95% CI 1.02, 4.41) (Table 2). In adjusted analysis, after controlling for the propensity for

215 heteroresistance (propensity score includes acute pyelonephritis infection, BMI, and diabetes),

216 heteroresistance was associated with treatment failure with a similar effect size, although this did

217 not reach statistical significance (OR 1.74, 95% CI 0.82, 3.71) (Table 2). However, subgroup

218 analysis revealed that patients with a cUTI without a removable source had a significantly higher

219 rate of treatment failure (OR 4.68, 95% CI 1.15, 18.97), and that this remained significant in

220 adjusted analysis controlling for the propensity for heteroresistance (OR 4.56, 1.12, 18.62)

221 (Table 3, Supplementary Table 3).

222

223 In addition to heteroresistance being associated with an increase in overall treatment failure, we

224 observed that heteroresistance was associated with a significant increase in microbiological

225 failure (OR 2.10, 95% CI 1.01, 4.36) (Table 2). After controlling for the propensity for HR,

226 while the association of heteroresistance with microbiologic failure had a similar effect size, the

227 association no longer reached statistical significance (OR 1.70, 95% CI 0.80, 3.64) (Table 2).

228 Patients infected with heteroresistant isolates had a higher frequency of clinical failure compared

229 to those infected with non-heteroresistant isolates, although this did not reach statistical

230    significance in the unadjusted (OR 2.45, 95% CI 0.64, 9.40) or adjusted analysis controlling for

231    the propensity for HR (OR 1.18, 95% CI 0.35, 3.99) (Table 2).

232

233    Interestingly, heteroresistant isolates were more likely to have an elevated MIC compared to the

234    non-heteroresistant isolates, with 36.4% of heteroresistant isolates having an MIC of 16/4 ug/mL

235    or 32/4 ug/mL compared to 1.9% of non-heteroresistant isolates (OR 28.9, 95% CI 9.3, 89.9,

236    Supplementary Figure 2). Furthermore, there was an observable trend that treatment failure

237    increased as MIC increased amongst susceptible isolates. Interestingly, this trend was not

238    observed amongst the heteroresistant isolates (Supplementary Table 2).

239

240

241    **Discussion**

242    Our study aimed to assess the potential contribution of heteroresistance to antibiotic treatment

243    failure. The clinical trial data and isolates used allowed us to examine this question due to the

244    large size of the patient cohort, regimented monotherapy treatment protocol, and uniform

245    assessment of treatment outcomes (8). We observed that patients infected with

246    piperacillin/tazobactam heteroresistant isolates had a significant, greater than two-fold higher

247    rate of treatment failure when compared to patients infected with non-heteroresistant isolates

248    (Table 2) and greater than four-fold higher rate of treatment failure among patients without a

249    removable source of infection (Table 3).

250

251    Previous research revealed that the resistant subpopulations in heteroresistant isolates can rapidly

252    replicate during antibiotic exposure, leading to their enrichment (3, 15). These findings

253    suggested that heteroresistant isolates might drive treatment failure. Following this hypothesis,

254    results from murine infection models demonstrated that heteroresistance can indeed cause

255    treatment failure (3-5). This convergence of evidence, combined with the results presented here,

256    demonstrates that the presence of heteroresistance increases the likelihood of treatment failure.

257

258    The MIC for every isolate included in this study was below the resistance breakpoint for

259    piperacillin/tazobactam when tested by broth microdilution (BMD) in accordance with 2024

260    CLSI standards. The increase in treatment failure rates exhibited by heteroresistant isolates,

261    juxtaposed with their apparent susceptibility according to BMD, emphasizes the capacity of

262    heteroresistant isolates to remain undetected by conventional susceptibility testing while

263    concurrently causing a higher rate of treatment failure. The most likely reason that BMD is

264    unable to detect the piperacillin/tazobactam resistant subpopulation in heteroresistant isolates is

265    that these cells are too infrequent in the overall population (16). BMD utilizes an inoculum of

266    $\sim5\times10^4$ CFU per well. If a heteroresistant isolate harbors 1 in 100,000 resistant cells, a resistant

267    cell may not be present in the BMD inoculum. On average, the heteroresistant isolates in this

268    study had less than 1 in 100,000 resistant cells surviving at the breakpoint concentration of

269    piperacillin/tazobactam (Supplementary Figure 3).

270

271    These population dynamics may also explain the higher rate of piperacillin/tazobactam treatment

272    failure among patients without a removable catheter and infected with heteroresistant isolates.

273    Given that the average frequency of resistant cells was 1 in 100,000 among heteroresistant

274    isolates in this study, it follows that a high burden of total infecting bacterial cells would be

275    necessary for sufficient resistant cells to be present to withstand piperacillin/tazobactam

276    treatment and remain to cause treatment failure. Having a non-removable source provides a

277    surface on which total bacterial load can persist, and in the presence of active therapy, a resistant

278    subpopulation is present in sufficient number to ultimately mediate treatment failure.

279

280    We noted and adjusted for (via a propensity score) three patient characteristics that were

281    associated with the patient being infected by a heteroresistant isolate: acute pyelonephritis, BMI,

282    and diabetes status. Potential reasons why these variables were associated with heteroresistance

283    are described in Supplementary Figure 4. These three variables were also confounders of the

284    association between heteroresistance and treatment failure (Supplementary Figure 4).

285    Furthermore, we observed a strong association between ESBL-encoding Enterobacterales

286    isolates and heteroresistance (Table 1). Previous studies have demonstrated that heteroresistant

287    strains often harbor resistant subpopulations generated via increased copy number of specific

288    genomic regions encoding antibiotic resistance genes such as beta-lactamases (17-20). The

289    higher prevalence of ESBL-encoding isolates within the heteroresistant cohort could therefore

290    signify their tendency to amplify ESBL genes and thus generate resistant subpopulations. This

291    puts ESBL production on the causal pathway between heteroresistance and treatment failure and

292    accordingly was not adjusted for in the propensity score (Supplementary Figure 4). To further

293    analyze this correlation, non-heteroresistant and heteroresistant *E. coli* isolates were sequenced

294    and a phylogenetic tree was generated (Supplementary Figure 5). We found that ESBL-encoding

295    *E. coli* were often clustered together. Additionally, we found a strong clustering of *E. coli*

296    heteroresistant isolates within the ESBL-encoding cluster (Supplementary Figure 5). Further

297    studies will need to be conducted to better understand these observed phylogenetic clusters. We

298    also observed an association between increased prevalence of heteroresistance in non-

299 Enterobacterales compared to Enterobacterales, although the explanation for this finding is

300 unclear. To further meaningfully explore this association, a much larger sample size of non-

301 Enterobacterales isolates would be required.

302

303 When treating a patient for a serious infection, there are multiple concurrent factors that

304 influence the ultimate treatment outcome – treatment factors (e.g., antibiotic potency, dosing,

305 penetration to the site of infection, etc.), host factors (e.g., immune status, baseline

306 comorbidities, etc.), and pathogen/infection factors (e.g., virulence, extent of organism load, etc).

307 Antibiotic susceptibility testing provides crucial information to determine if an organism is

308 susceptible to various antibiotics. While 'susceptible' generally equates to clinical efficacy of an

309 antibiotic against the isolated bacterium, treatment failure can nonetheless occur. Understanding

310 factors that mediate treatment failure despite apparent antibiotic susceptibility as measured by

311 current clinical diagnostic tests is important to improve patient outcomes. The data presented

312 here demonstrate that heteroresistance is a contributor to treatment failure, at least in the setting

313 evaluated here. Further quantifying the prevalence of this phenotype across bacterial species and

314 infection types will be necessary in future studies. Not only do the current findings highlight the

315 need for better understanding of antibiotic treatment strategies to overcome heteroresistance, but

316 advancement of novel susceptibility testing methods capable of detecting heteroresistance should

317 be a focus as well.

318

319 This study has several limitations to note. First, the sample size for patients infected with

320 heteroresistant isolates was relatively small, which in multivariable analysis, limited the ability to

321 demonstrate a significant association between heteroresistance and treatment failure, and also

322     limited the power to detect associations between heteroresistance and outcomes in sub-group

323     analyses. Future studies with larger cohorts of patients infected with heteroresistant isolates can

324     address this limitation. Second, as these isolates originated from a previous clinical trial with

325     strict treatment guidelines, our findings do not necessarily reflect the impact of heteroresistance

326     during "real world" use of antibiotics. Third, these isolates were from patients with cUTI and AP

327     and therefore may not be reflective of other bacterial infection types. Despite these limitations,

328     the strengths of this study include the uniform antibiotic treatment and patient outcome

329     assessment as well as the strong association between heteroresistance and treatment failure.

330

331     In conclusion, we show that patients infected with piperacillin/tazobactam heteroresistant

332     isolates have an increased likelihood of treatment failure when compared to those infected with

333     non-heteroresistant isolates. Heteroresistance, particularly among Gram-negative pathogens, is

334     an important and often overlooked phenomenon that may play a crucial role in antibiotic

335     treatment failure.

336

| Characteristic | Patients with a Non-heteroresistant Pathogen* (n=255) | Patients with a Heteroresistant Pathogen (n=33) | P Value |
|---|---|---|---|
| Age, Y | | | |
| <65 | 170/255 (66.7%) | 21/33 (63.6%) | |
| 65 to <75 | 52/255 (20.4%) | 9/33 (27.3%) | |
| >=75 | 33/255 (12.9%) | 3/33 (9.1%) | |
| Sex | | | 0.25 |
| Female | 161/255 (63.1%) | 17/33 (51.5%) | |
| Race | | | 0.85 |
| Asian | 1/255 (.4%) | 0/33 (0%) | |
| White | 242/255 (94.9%) | 32/33 (97%) | |
| Other | 12/255 (4.7%) | 1/33 (3%) | |
| Ethnicity[a] | | | 0.29 |
| Hispanic or Latino | 18/255 (7.1%) | 4/32 (12.5%) | |
| Not Hispanic or Latino | 237/255 (92.9%) | 28/32 (87.5%) | |
| BMI, mean (SD) | 26.0 (5.3) | 27.9 (6.6) | 0.12 |
| Baseline eGFR group, median (IQR), mL/min/1.732[b] | 62 (26) | 75 (34) | 0.34 |
| Infection Type | | | 0.16 |
| Acute pyelonephritis | 145/255 (58.9%) | 13/33 (39.4%) | |

| | | | |
|---|---|---|---|
| Complicated UTI without removable source of infection | 64/255 (25.1%) | 12/33 (36.4%) | |
| Complicated UTI with removable source of infection | 46/255 (18%) | 8/33 (24.2%) | |
| Prior Antibiotic exposure | | | 0.10 |
| Short-acting antibiotic up to 24 h | 19/255 (7.5%) | 0/33 (0%) | |
| Region | | | 0.76 |
| Eastern Europe | 193/255 (75.7%) | 25/33 (75.8%) | |
| Americas | 15/255 (5.9%) | 1/33 (3%) | |
| Other countries | 47/255 (18.4%) | 7/33 (21.2%) | |
| Baseline Charlson Comorbidity Index Score[c] | | | |
| CCINDEX <3 | 158/253 (62.5%) | 19/33 (57.6%) | 0.57 |
| Presence of concurrent bacteremia at baseline | 21/255 (8.2%) | 1/33 (3%) | 0.49 |
| Diabetes at baseline | 28/255 (11%) | 8/33 (24.2%) | **0.046** |
| Enterobacterales baseline pathogen | 246/255 (96.5%) | 29/33 (87.9%) | **0.049** |
| Enterobacterales baseline pathogen, extended-spectrum beta-lactamase encoding[d] | 37/246 (15%) | 18/29 (62.1%) | **<.0001** |

337

338 **Table 1. Characteristics of patients with non-heteroresistant and heteroresistant isolates.**

339 *Isolates that are susceptible by population analysis profile.

340    [a] Ethnicity information is not available for a single patient in the heteroresistant category.

341    [b] Baseline EGFRGR information is not available for 15 patients in the non-heteroresistant

342    category and 2 in the heteroresistant.

343    [c] Baseline CCINDEX information is not available for 2 patients in the non-heteroresistant

344    category.

345    [d] Enterobacterales baseline pathogens were genotyped for extended-spectrum beta-lactamases if

346    their minimum inhibitory concentration was equal to or greater than 1 ug/mL or more for

347    ceftazidime, ceftriaxone, cefepime, meropenem, or cefepime/enmetazobactam (8).

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| Outcome | Non-heteroresistant * n=255 | Heteroresistant n=33 | Crude Odds Ratio (95% CI) | Adjusted Odds Ratio [†] (95% CI) |
|---|---|---|---|---|
| Overall Failure | 85 (33.3%) | 17 (51.5%) | 2.13 (1.02, 4.41) | 1.74 (0.82, 3.70) |
| Microbiological Failure | 79 (31%) | 16 (48.5%) | 2.10 (1.01, 4.36) | 1.70 (0.80, 3.64) |
| Clinical Failure | 10 (3.9%) | 3 (9.1%) | 2.45 (0.64, 9.40) | 1.18 (0.35, 3.99) |

**Table 2. Treatment outcomes of patients infected with non-heteroresistant or heteroresistant isolates.**

*Isolates that are susceptible by population analysis profile.

† Adjusted for propensity score based on acute pyelonephritis infection, BMI, and diabetes

| Infection Type | Non-heteroresistant * Isolates Failure/Total | Heteroresistant Isolates Failure/Total | Crude Odds Ratio (95% CI) | Adjusted Odds Ratio [†] (95% CI) |
|---|---|---|---|---|
| Complicated UTI without Removable Source of Infection (n=76) | 25/64 (39.1%) | 9/12 (75%) | 4.68 (1.15, 18.97) | 4.56 (1.12, 18.62) |
| Complicated UTI with Removable Source of Infection (n=54) | 20/46 (43.5%) | 2/8 (25%) | 0.43 (0.079, 2.38) | 0.42 (0.073, 2.38) |
| Acute Pyelonephritis (n=158) | 40/145 (27.6%) | 6/13 (46.2%) | 2.25 (0.71, 7.10) | 1.69 (0.5, 5.70) |

377

378 **Table 3. Association of heteroresistance and treatment failure stratified by infection type.**

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380 *Isolates that are susceptible by population analysis profiles.

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382 †Adjusted for propensity score based on acute pyelonephritis infection, BMI, and diabetes

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455

**Supplementary Materials**

**Supplementary Table 1**

| Species | Non-heteroresistant* isolates/Total Isolates | Heteroresistant isolates/Total isolates | Non-heteroresistant* Failure Rate | Heteroresistant Failure Rate |
|---|---|---|---|---|
| TOTAL | 258/291 (88.7%) | 33/291 (11.3%) | 86/258 (33.3%) | 17/33 (51.5%) |
| Enterobacterales | 249/278 (89.6%) | 29/278 (10.4%) | 80/249 (32.1%) | 15/29 (51.7%) |
| *Escherichia* | 191/216 (88.4%) | 25/216 (11.6%) | 59/191 (30.9%) | 12/25 (48%) |
| *Enterobacter* | 3/4 (75%) | 1/4 (25%) | 1/3 (33.3%) | 1/1 (100%) |
| *Citrobacter* | 3/3 (100%) | 0/3 (0%) | 0/3 (0%) | N/A |
| *Klebsiella* | 28/30 (93.3%) | 2/30 (6.7%) | 8/28  (28.6%) | 2/2 (100%) |
| *Morganella* | 1/1 (100%) | 0/1 (0%) | 0/1 (0%) | N/A |
| *Proteus* | 19/20 (95%) | 1/20 (5%) | 8/19 (42.1%) | 0/1 (0%) |
| *Providencia* | 1/1 (100%) | 0/1 (0%) | 1/1 (100%) | N/A |
| *Serratia* | 3/3 (100%) | 0/3 (0%) | 3/3 (100%) | N/A |
| Non-Enterobacterales | 9/13 (69.2%) | 4/13 (30.8%) | 6/9 (66.7%) | 2/4 (50%) |
| *Acinetobacter* | 2/2 (100%) | 0/2 (0%) | 1/2 (50%) | N/A |
| *Pseudomonas* | 7/11 (63.6%) | 4/11 (36.4%) | 5/7 (71.4%) | 2/4 (50%) |

**Supplementary Table 1. Treatment failure rate as a function of heteroresistance for each pathogen in the study.**

*Isolates that are susceptible according to population analysis profiles.

**Supplementary Table 2**

| Piperacillin/tazobactam MIC (ug/mL) | Enterobacterales (n=278) | | Non-Enterobacterales (n=13) | |
| --- | --- | --- | --- | --- |
| | Non-heteroresistant* Failure/total | Heteroresistant Failure/total | Non-heteroresistant* Failure/total | Heteroresistant Failure/total |
| 0.25/4 | 3/12 (25%) | 1/1 (100%) | 0/1 (0%) | 0/0 |
| 0.5/4 | 8/25 (32%) | 0/0 | 0/0 | 0/0 |
| 1/4 | 36/127 (28.3%) | 0/1 (0%) | 0/0 | 0/0 |
| 2/4 | 17/55 (30.9%) | 2/5 (40%) | 0/1 (0%) | 0/0 |
| 4/4 | 10/20 (50%) | 3/6 (50%) | 2/2 (100%) | 0/0 |
| 8/4 | 4/7 (57.1%) | 3/8 (37.5%) | 3/3 (100%) | 0/0 |
| 16/4 | 2/3 (66.7%) | 6/8 (75%) | 1/2 (50%) | 1/2 (50%) |
| 32/4 | N/A | N/A | 0/0 | 1/2 (50%) |

**Supplementary Table 2. Treatment failure rates by piperacillin/tazobactam MIC for Enterobacterales and non-Enterobacterales.**

*Isolates that are susceptible by population analysis profiles.

**Supplementary Table 3.**

| Characteristic | Non-heteroresistant* (n=64) | Heteroresistant (n=12) | P Value |
|---|---|---|---|
| Age, Y | | | 0.62 |
|   <65 | 19/64 (29.7%) | 4/12 (33.3%) | |
|   65 to <75 | 32/64 (50%) | 7/12 (58.3%) | |
|   >=75 | 13/64 (20.3%) | 1/12 (8.3%) | |
| Sex | | | 0.53 |
|   Female | 23/64 (35.9%) | 3/12 (25%) | |
| Race | | | 0.63 |
|   White | 62/64 (96.9%) | 12/12 (100%) | |
|   Other | 2/64 (3.1%) | 0/12 (0%) | |
| Ethnicity[a] | | | 1.00 |
|   Hispanic or Latino | 2/64 (3.1%) | 0/11 (0%) | |
|   Not Hispanic or Latino | 62/64 (96.9%) | 11/11 (91.7%) | |
| BMI, mean (SD) | 28.6 (4.9) | 29.8 (6.9) | 0.60 |
| Baseline eGFR group, median (IQR), mL/min/1.732[b] | 63 (23) | 72.5 (32.5) | 0.33 |
| Prior Antibiotic exposure | | | 0.53 |
|   Short-acting antibiotic up to 24h | 2/64 (3.1%) | 0/12 (0%) | |
| Region | | | 0.82 |

| | | | |
|---|---|---|---|
| Eastern Europe | 46/64 (71.9%) | 9/12 (75%) | |
| Americas | 2/64 (3.1%) | 0/12 (%) | |
| Other countries | 16/64 (25%) | 3/12 (25%) | |
| Baseline Charlson Comorbidity Index Score[c] | | | 0.76 |
| CCINDEX <3 | 28/64 (44.4%) | 6/12 (50%) | |
| Presence of concurrent bacteremia at baseline | 4/64 (6.3%) | 0/12 (0%) | 1.00 |
| Diabetes at baseline | 12/64 (18.8%) | 3/12 (25%) | 0.69 |
| Enterobacterales baseline pathogen | 62/64 (96.9%) | 10/12 (83.3%) | 0.12 |
| Enterobacterales baseline pathogen, extended-spectrum beta-lactamase encoding[d] | 9/62 (14.5%) | 4/12 (40%) | 0.07 |

**Supplementary Table 3. Characteristics of patients without a removable source and either non-heteroresistant or heteroresistant isolates.**

*Isolates that are susceptible by population analysis profile.

[a] Ethnicity information is not available for a single patient in the heteroresistant category.

[b] Baseline EGFRGR information is not available for 15 patients in the non-heteroresistant category and 2 in the heteroresistant.

[c] Baseline CCINDEX information is not available for 2 patients in the non-heteroresistant category.

[d] Enterobacterales baseline pathogens were genotyped for extended-spectrum beta-lactamases if their minimum inhibitory concentration was equal to or greater than 1 ug/mL or more for ceftazidime, ceftriaxone, cefepime, meropenem, or cefepime/enmetazobactam.
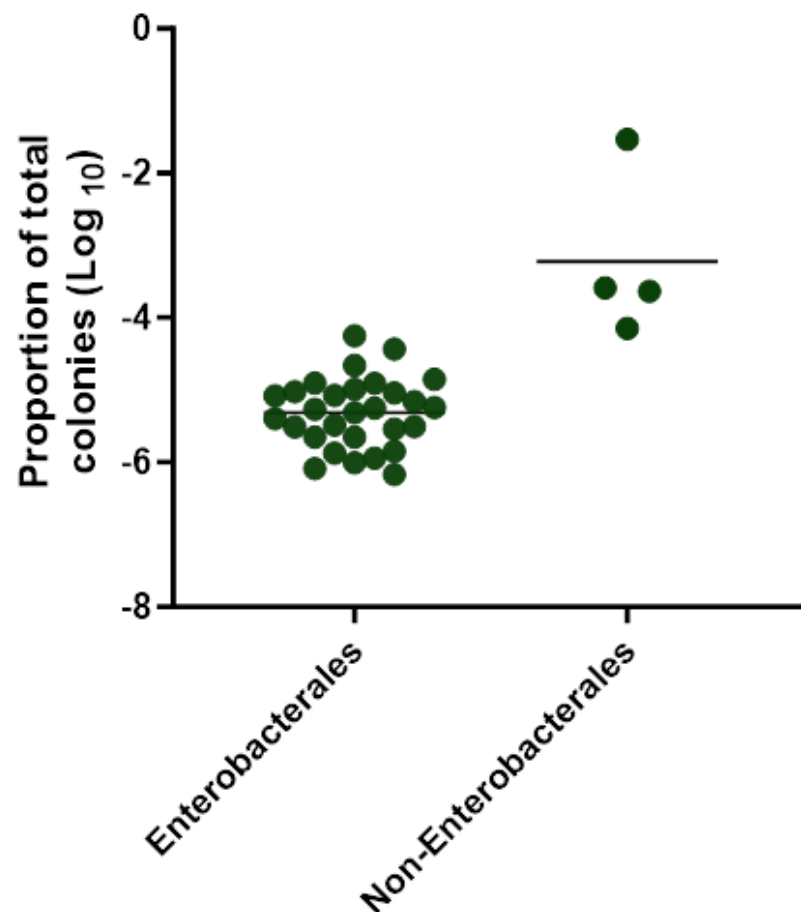
**Supplementary Figure 1**
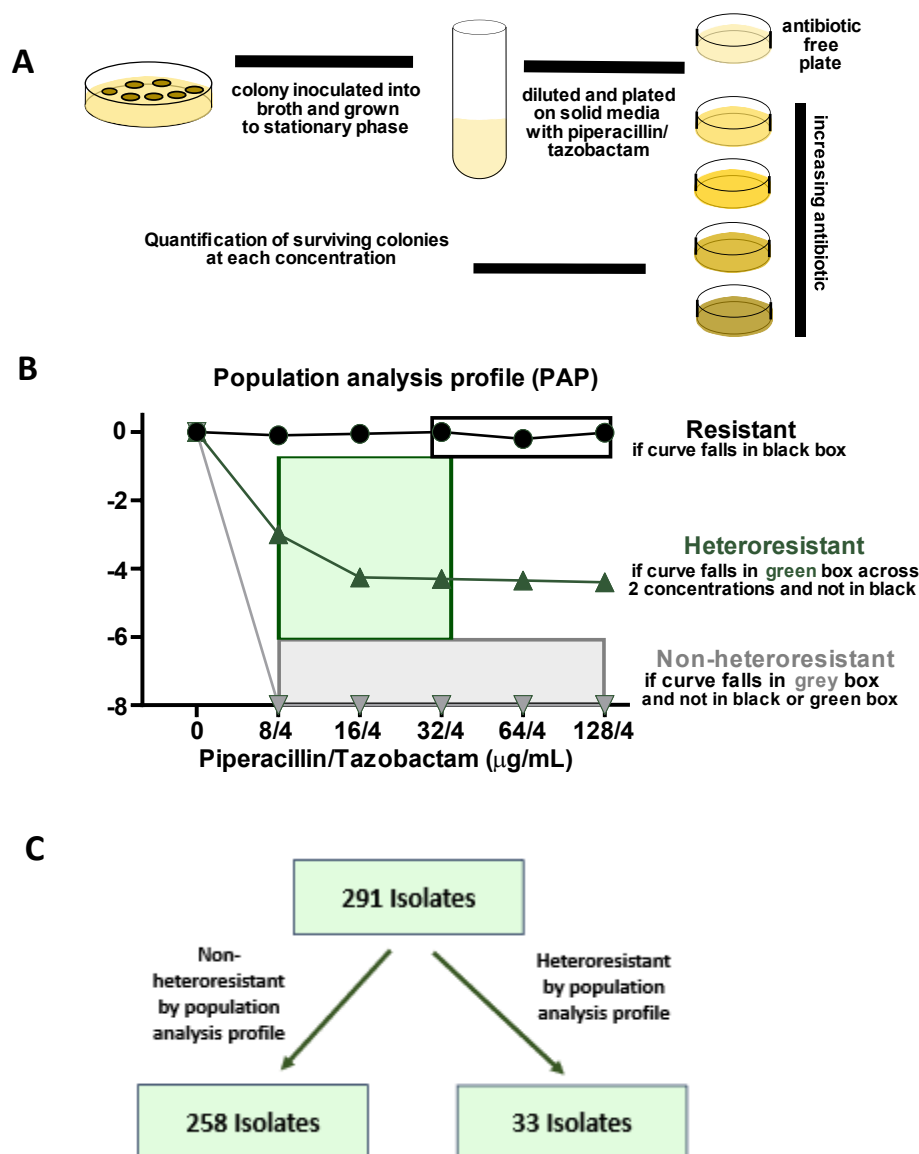


**Supplementary Figure 1. Population Analysis Profile.** (A) A clinical isolate is streaked for isolation, inoculated into broth, and incubated overnight. The following day the bacterial culture is serially diluted and plated on plates with either no antibiotic or increasing concentrations of piperacillin/tazobactam. (B) After overnight incubation, the colonies are enumerated and the number of surviving colonies on the various drug concentrations are compared to the number on the plate not containing drug. (C) Isolates were designated either non-heteroresistant (susceptible) or heteroresistant by population analysis profiles. Adopted from Choby et al, Lancet Infect Dis. 2021;21(5):597-8.

**Supplementary Figure 2**
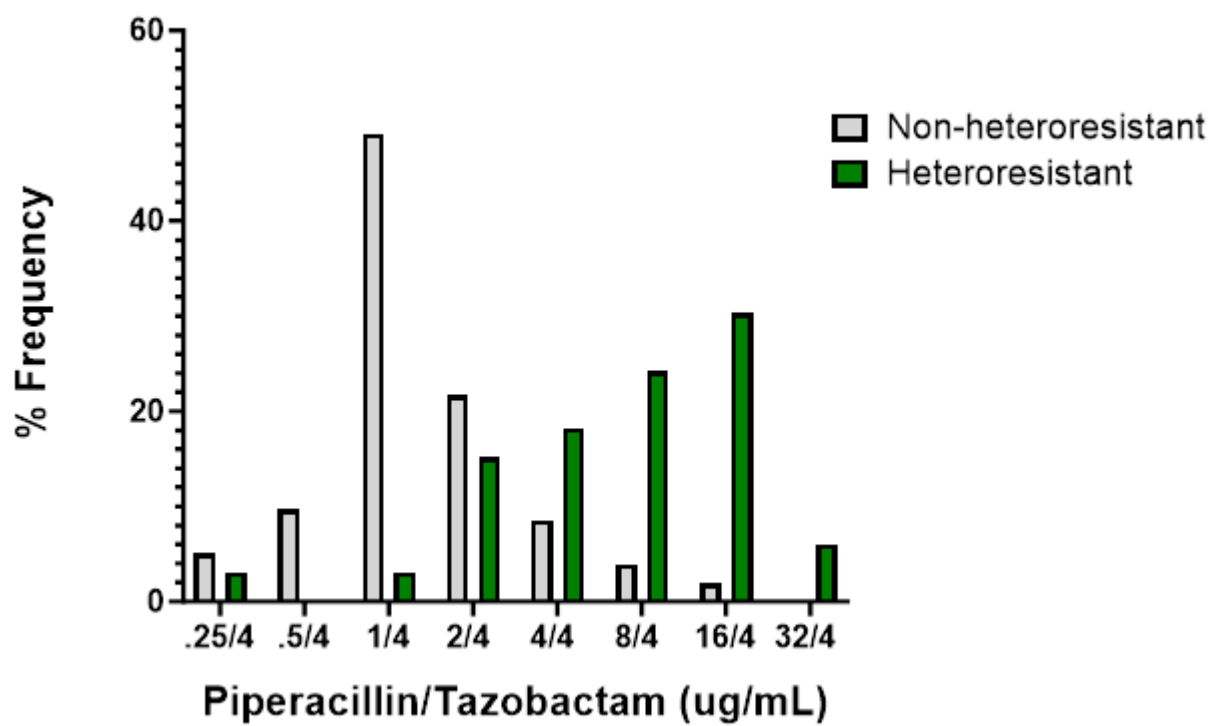


**Supplementary Figure 2. Frequency of isolates with each piperacillin/tazobactam MIC based on broth microdilution testing for non-heteroresistant and heteroresistant isolates.**
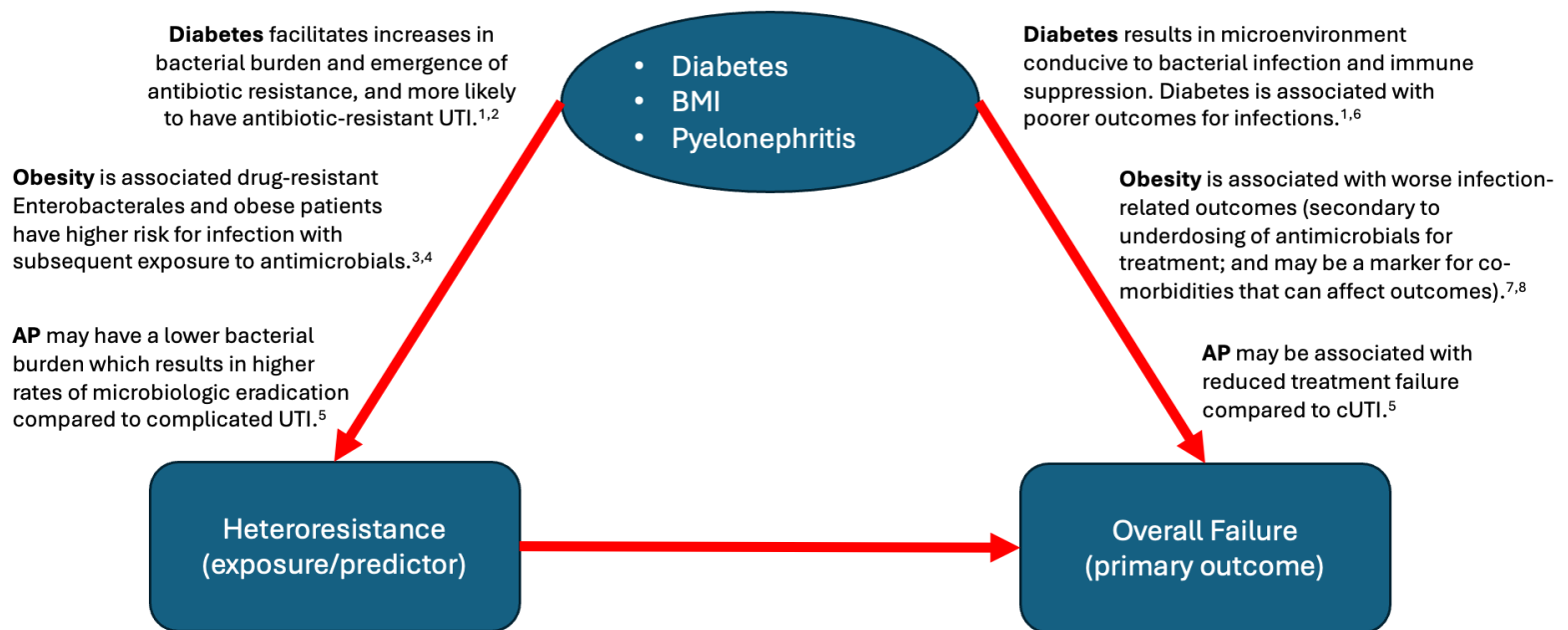
**Supplementary Figure 3**



**Supplementary Figure 3. Frequency of the resistant subpopulation in heteroresistant isolates.**
Piperacillin/tazobactam heteroresistant Enterobacterales and non-Enterobacterales isolates were plated on 32/4ug/mL piperacillin/tazobactam and the proportion of surviving cells is shown relative to agar without antibiotic.
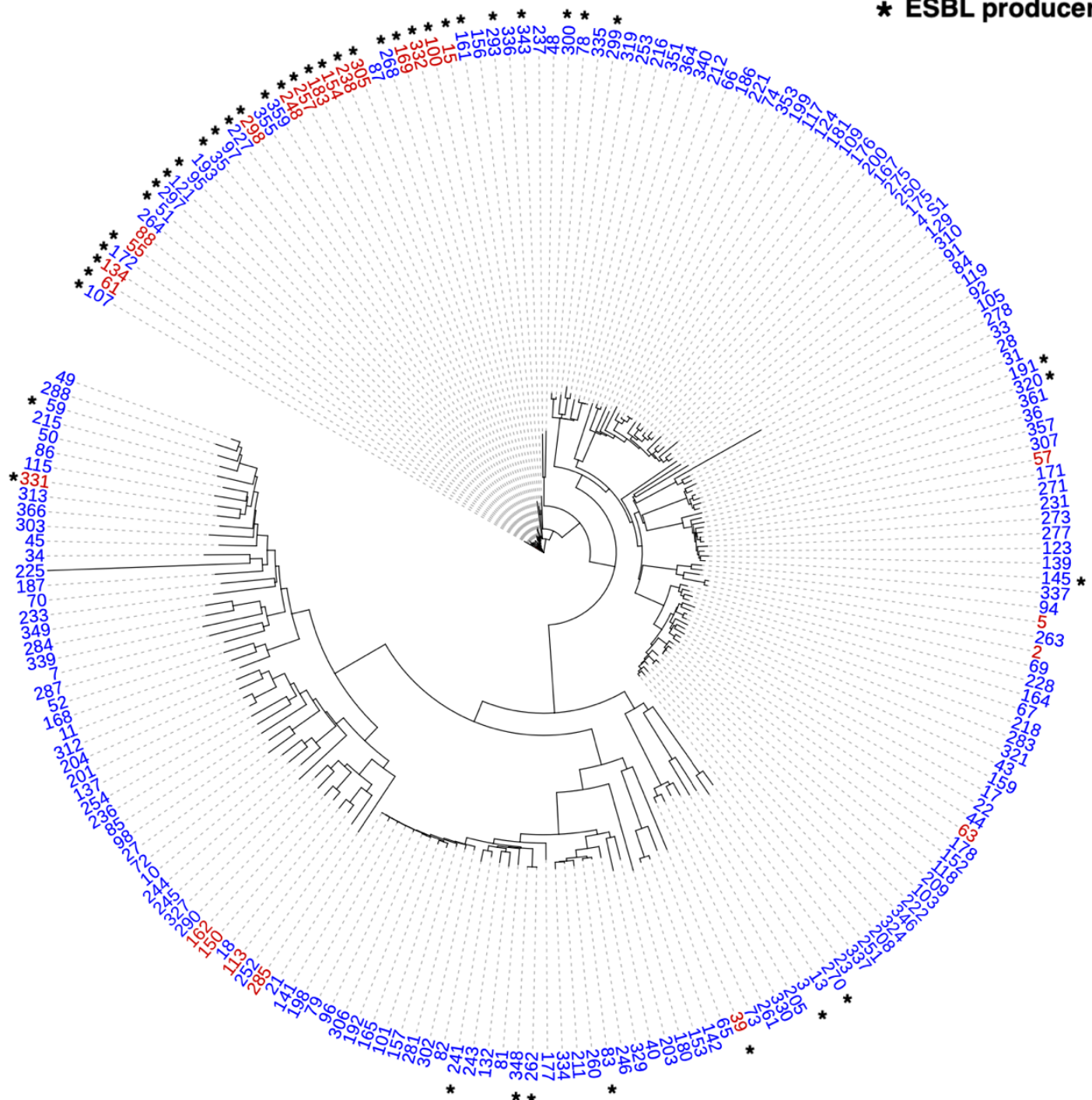
**Supplementary Figure 4**

Diabetes facilitates increases in bacterial burden and emergence of antibiotic resistance, and more likely to have antibiotic-resistant UTI.[1,2]

Obesity is associated drug-resistant Enterobacterales and obese patients have higher risk for infection with subsequent exposure to antimicrobials.[3,4]

AP may have a lower bacterial burden which results in higher rates of microbiologic eradication compared to complicated UTI.[5]

- Diabetes
- BMI
- Pyelonephritis

Diabetes results in microenvironment conducive to bacterial infection and immune suppression. Diabetes is associated with poorer outcomes for infections.[1,6]

Obesity is associated with worse infection-related outcomes (secondary to underdosing of antimicrobials for treatment; and may be a marker for co-morbidities that can affect outcomes).[7,8]

AP may be associated with reduced treatment failure compared to cUTI.[5]

Heteroresistance (exposure/predictor)

Overall Failure (primary outcome)

**Supplementary Figure 4. Directed acyclic graph (DAG) indicating potential reasons why variables were associated with heteroresistance.**

**Supplementary Figure 5**

**Supplementary Figure 5. Phylogenetic clustering of heteroresistant, ESBL producers among *E. coli* in the study.** Genome sequences for *E. coli* isolates in the study were phylogenetically clustered. Genomes for non-heteroresistant isolates (susceptible by population analysis profile testing; blue) and heteroresistant isolates (red) are indicated. ESBL isolates are indicated with an asterisk.

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