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Molecular Surveillance and Assessment of Ceftolozane/Tazobactam Resistance with Common β-Lactam Antibiotics and β-Lactamase Genes

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ABSTRACT

Ceftolozane/tazobactam (c/t) is a potent β-lactam antibiotic which combines the fifth generation cephalosporin ceftolozane and tazobactam, a β-lactamase inhibitor. The c/t combination therapy was approved in 2014 for the treatment of multidrug resistant (MDR) Enterobacteriaceae, especially intra-abdominal and urinary tract infections. The aim of this study is to assess c/t activity and to examine the association of c/t resistance with four common β-lactamase resistance genes found in clinical Enterobacteriaceae isolates collected from mainly urinary tract infections in an agricultural region in California (USA) between 2013-2020. We tested 993 Extended Spectrum β-lactamases (ESBL) producing Enterobacteriaceae isolates (885 E. coli, 94 K. pneumoniae, 14 other) for c/t susceptibility by Kirby-Bauer disk diffusion and screened using PCR for four common resistance genes with β-lactamase activity(blaTEM, blaOXA, blaSHV, and blaCTX-M) for 855 of the isolates. We also investigated co-resistance of c/t and nine other β-lactam antibiotics. We found that most isolates were susceptible to c/t (58.3%), while 38.5% showed intermediate resistance, and 3.2% were resistant. We also found that K. pneumoniae isolates were more resistant to c/t than E. coli isolates, and that c/t may be a good alternative to carbapenems, in that that some carbapenem resistant isolates were susceptible to c/t. Genotypic analysis showed blaSHV and blaCTX-M are independently associated with elevated c/t resistance. Although c/t demonstrated strong activity against Enterobacteriaceae, the high percentage of isolates with intermediate susceptibility emphasizes the need for close monitoring and continued surveillance for c/t resistance among ESBLs.

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Introduction

Ceftolozane/tazobactam (c/t) is an antibiotic/inhibitor combination that received FDA approval in 2014 for treatment of complicated intra-abdominal infections (cIAI) and complicated urinary tract infections (cUTI) [1-4]. This combination therapy is useful for treating extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae and multidrug-resistant (MDR) Pseudomonas aeruginosa, but is not recommended for carbapenem resistant Enterobacteriaceae (CREs) [5,6]. Ceftolozane is a 5th generation cephalosporin β-lactam that targets penicillin binding proteins, while tazobactam is an inhibitor that prevent hydrolysis of ceftolozane by blocking the hydrolytic activity of β-lactamase enzymes [2-4]. The activity of ceftolozane against Gram-negative bacteria is maintained or enhanced upon by the addition of the inhibitor tazobactam [7,8]. Studies show that the addition of tazobactam to ceftolozane reduced the minimum inhibitory concentrations (MICs) against Enterobacteriaceae harboring the ESBLs blaCTX-M, blaSHV, blaTEM, and blaPER-1 [9].

Resistance to c/t has been observed in multiple surveillance studies although most bacteria are susceptible. Surveillance of 500 Enterobacteriaceae and 500 P. aeruginosa from patients in Spain, showed 94.4% of P. aeruginosa and 99.6% of Enterobacteriaceae were susceptible to c/t [10]. Additionally surveillance of 30,582 Enterobacteriaceae isolates across Europe showed 94.5% and 79.4% susceptibility in Western and Eastern Europe, respectively [11]. In the US, surveillance of 1,428 Enterobacteriaceae isolates collected across 70 medical centers found 91.9% of K. pneumoniae isolates and 96.4% of E. coli isolates were susceptible to c/t [2]. However, a study in China of 1,774 Enterobacteriaceae isolates from 30 medical centers, found similar frequencies of c/t susceptibility in E. coli but a much lower frequency of susceptibility in K. pneumoniae: 91.4% E. coli isolates and 56.7% K. pneumoniae isolates [12]. These frequency differences observed in c/t susceptibility highlight the geographic disparities in the possible effectiveness of this treatment option.

Pseudomonas aeruginosa has evolved resistance to c/t through well-characterized mechanisms. Point mutations in the outer membrane protein OprD, AmpR, and AmpC decrease susceptibility [13]. Likewise, horizontal transfer of the genes for GES, BEL, PER, and OXA-2 have each been linked to elevated resistance to c/t [14,15]. In Enterobacteriaceae, resistance is less well characterized. It has been shown that E. coli harboring the carbapenemases blaKPC-2 and blaVIM-2 are resistant to c/t [16]. Moreover, recombinant E.

coli strains producing GES-1, GES-6, PER-1, BEL-1, and BEL-2 remained resistant to c/t with increased MICs [15]. For GES-5 and PER-1 producers, it is possible that c/t resistance is driven by a high hydrolysis rate of ceftolozane and lower inhibition by tazobactam [15]. However, c/t resistance can also occur in strains that are not CREs. Early experiments looking at ceftolozane's activity against E. coli strains harboring narrow spectrum β-lactamase and ESBLs such as blaTEM-1-9, blaSHV-1-4, blaOXA-1, -2, and blaCTX-M-3, -18, showed reduced activity for c/t when blaTEM-3-9, blaSHV-2-44, blaOXA-2, or blaCTX-M-3, -18 were present, while the activity of imipenem, a carbapenem, was not affected by the presence of any ESBLs [4]. To further investigate the occurrence of c/t resistance in ESBLs, we conducted a surveillance study of c/t resistance using a repository of fresh ESBL positive isolates collected from Dignity Health Mercy Medical Center (DHMMC) in Merced.

Methods

Isolates

We assessed resistance to c/t from a repository of 1,250 Enterobacteriaceae ESBL positive isolates from patients at DHMMC in Merced, California (USA), collected from 2013-2020. These isolates are about 90% E. coli, 5% K. pneumoniae, and 5% other Enterobacteriaceae. Most isolates (~90%) are collected from urinary tract infections. At DHMMC, isolates were tested for antibiotic susceptibility on the Vitek2 for nine common β-lactam antibiotics: ampicillin, ampicillin-sulbactam, piperacillin-tazobactam, cefazolin, ceftazidime, ceftriaxone, and cefepime, and ertapenem and imipenem and added to our repository based on a positive identification as ESBL by Vitek2 [17]. We determined the empirical and conditional frequencies for resistance to these antibiotics and summarized the data in Table 1.

Susceptibility Testing

We performed Kirby-Bauer disk diffusion susceptibility tests of c/t in triplicate on 993 ESBL positive Enterobacteriaceae isolates (899 E. coli and 94 K. pneumoniae) from our collection [18]. Isolated colonies were grown in Mueller Hinton broth for 16-18 hours, then plated as a lawn on Mueller Hinton II agar with c/t disk for 16-18 hours. The zone of inhibition (ZI) diameter was measured in millimeters, rounding to the nearest millimeter. We followed the Clinical & Laboratory Standards Institute (CLSI) for c/t ZI breakpoints as follows: resistant ZI ≤ 17mm, intermediate ZI = 18-20mm, and susceptible ZI ≥ 21mm (Table 1) [18].

Statistical methods

From another study, we were already in possession of genotype data for the four common β-lactamase genes, blaTEM, blaOXA, blaSHV, and blaCTX-M for 855 isolates of the 993 tested for c/t resistance. We analyzed the c/t ZI measurements for these isolates as a function of these genes. We performed an ANOVA to assess statistical deviation from equal mean ZI measurements by resistance gene combinations, pairwise mean ZI measurement comparisons by gene resistance combinations using Welch's t-test, and considered elevated resistance as a function of ZI. We used a Bonferroni-type multiple testing procedure to control for false discoveries in performing multiple statistical tests (776 total tests). Only the results that remained significant after application of the False Discovery Rate (FDR) controlling procedure with false discovery control level q = 0.05 are reported as significant. Statistical analyses were performed using MATLAB R2020a [19-23].

Results

We found that 3.2% (n = 32) of the isolates are resistant (ZI ≤ 17mm), 38.5% (n = 382) are intermediate (ZI = 18-20mm), and 58.2% (n = 578) are susceptible (ZI ≥ 21mm) to c/t according to CLSI breakpoints [18]. The distribution of Enterobacteriaceae c/t resistance in our collection are summarized in Table 1 (Column 2). We compared the resistance phenotypes of the nine β-lactam antibiotics from MICs provided by DHMMC (Table 1, Column 2) to our c/t resistance profile [17]. We observed a low frequency of resistance to piperacillin/tazobactam (5.5%) in our collection, which was only slightly higher than c/t (3.2%). We also observed a low frequency of resistance to the carbapenems, ertapenem (0.5%) and imipenem (0.1%). We used these resistance profiles to compute conditional frequencies of c/t resistance when other resistance phenotypes are observed (Table 1, Column 3). We found that isolates resistant to each of the antibiotics had mostly low frequencies of resistance to c/t. For carbapenems however, we found that isolates resistant to ertapenem and imipenem had higher frequencies of c/t resistance (50% and 60%, respectively). Lastly, we looked at the resistance profile of the 32 c/t resistant isolates with respect to the nine other antibiotics by computing the conditional frequency of resistance to other antibiotic treatments when resistance to c/t is observed (Table1, Column 4). We found that c/t resistant isolates had higher frequencies of resistance to all other antibiotics, except the carbapenems ertapenem and imipenem, which had relatively lower frequencies of resistance (12.5% and 9.4%, respectively). These results mean that while c/t is less likely to treat carbapenem resistant strains, carbapenems may still be a good choice to treat c/t resistant strains.

Table 1: Empirical Frequency of Resistance. Column 2: Empirical frequency of resistance to 10 β-lactam antibiotics. Column 3: conditional empirical frequency of c/t resistance given resistance to each of the other 9 antibiotics. Column 4: conditional empirical frequency of resistance to each of the other 9 antibiotics given resistance to c/t.

Antibiotic	**Resistance frequency**		**Frequency of c/t resistance given resistance to each antibiotic**		**Frequency of resistance to each antibiotic given c/t resistance**	
Enterobacteriaceae	(%)	n	(%)	n	(%)	n
ceftolozane-tazobactam	3.2	993	100.0	32	100.0	32
ampicillin	99.8	985	3.1	983	93.8	32
ampicillin-sulbactam	61.2	978	4.0	598	75.0	32
piperacillin-tazobactam	5.5	967	17.0	53	28.1	32
cefazolin	98.2	985	3.1	967	93.8	32
ceftazidime	96.0	984	3.1	945	90.6	32
ceftriaxone	97.2	985	3.1	957	93.8	32
cefepime	95.9	983	3.1	943	90.6	32
ertapenem	0.8	984	50.0	8	12.5	32
imipenem	0.5	982	60.0	5	9.4	32
K. pneumoniae	(%)	n	(%)	n	(%)	n
ceftolozane-tazobactam	10.5	95	100.0	10	100.0	
ampicillin	100.0	95	10.5	95	100.0	10
ampicillin-sulbactam	70.2	94	12.1	66	80.0	10
piperacillin-tazobactam	16.3	92	40.0	15	60.0	10
cefazolin	100.0	95	10.5	95	100.0	10
ceftazidime	97.9	95	10.8	93	100.0	10
ceftriaxone	96.8	95	10.9	92	100.0	10
cefepime	96.8	94	11.0	91	100.0	10
ertapenem	4.2	95	75.0	4	30.0	10
imipenem	4.3	94	75.0	4	30.0	10
E. Coli	(%)	n	(%)	n	(%)	n
ceftolozane-tazobactam	2.3	887	100.0	20	100.0	20
ampicillin	99.8	883	2.2	881	95.0	20
ampicillin-sulbactam	60.0	877	2.9	526	75.0	20
piperacillin-tazobactam	4.0	869	5.7	35	10.0	20
cefazolin	98.0	883	2.2	865	95.0	20
ceftazidime	95.9	882	2.1	846	90.0	20
ceftriaxone	97.4	883	2.2	860	95.0	20
cefepime	95.9	882	2.1	846	90.0	20
ertapenem	0.5	883	25.0	4	5.0	20
imipenem	0.1	882	0.0	1	0.0	20

We investigated the frequency of c/t resistance over time since its FDA approval in 2014 to determine whether it was increasing in our collection. The yearly isolate resistance breakdown is presented in Table 2.

Table 2: Yearly c/t resistant isolate frequency and number of isolates collected per year

Year	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Resistance Frequency (%)	8.7	2.0	2.4	2.7	0.9	0.0	11.5
Total isolates collected (n)	104	204	249	187	109	79	61

We considered the frequency of resistant isolates prior to FDA approval of c/t in 2014 as a baseline and did not observe any significant increase in the frequency of c/t resistant isolates from pre-approval years suggesting that the c/t resistance we observe in this study is not likely to be the result of c/t selection, but rather pre-existing resistance to c/t that is likely to exist in other ESBL populations. Compared to our baseline in 2013, isolates from 2014 to 2018 show a significant decrease in c/t resistance, while isolates from 2019 show no significant difference. Continued surveillance will show if the frequency of resistance in 2019 is an anomaly similar to 2013,

or a steady trend of increasing frequency of resistance to c/t.

The occurrence of c/t resistance in ESBL isolates that did not exhibit carbapenem resistance prompted us to further investigate the relationship of ESBL resistance genes to c/t. For this aspect of the study we directly used c/t ZI measurements rather than phenotypic categorizations of resistance because there is greater resolution and additional statistics are made available. We analyzed the association between the ZI measures and the presence and absence combinations of the four β-lactamase genes: blaSHV, blaTEM, blaCTX-M, and blaOXA. From our collection of 1,250 clinical isolates, 855 were screened for these genes [20]. The distributions of these resistance genes over the 855 isolates are presented in Figure 1.

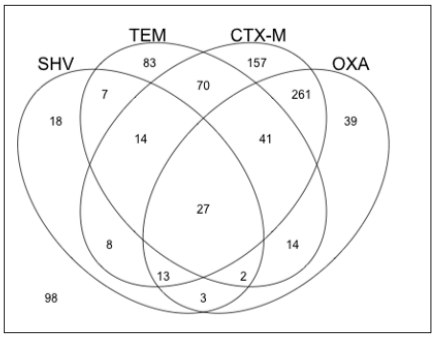

Figure 1: Combinations of Resistance Genes in 855 Clinical Isolates from DHMMC determined by PCR screening. There are 98 isolates that did not contain any of the four resistance genes we screened for. (see Methods for more details)

First, we broadly analyzed the differences in ZI measure between isolates that harbor ([+]) or lack ([−]) each of the four β-lactamase genes: bla_{TEM}, bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$ (Figure 2). We found a statistically significant association between bla_{SHV}, $bla_{CTX\text{-}M}$ and a decrease in average ZI measurement (p-value < 0.05 and q = 0.05) when looking at all Enterobacteriaceae (independent of species). However, when we perform this analysis by species (E. coli and K. pneumoniae), only the association of $bla_{CTX\text{-}M}$ and a decrease in ZI measure remained significant only for E. coli (Figure 2G, p-value < 0.05, and q = 0.05). The difference in mean ZI measurement between E. coli isolates with $bla_{CTX\text{-}M}$ and those lacking it is small ~0.60mm with a 95% confidence interval of (0.30mm, 0.91mm) (Table S1) and is almost visually undetectable in Figure 2G.

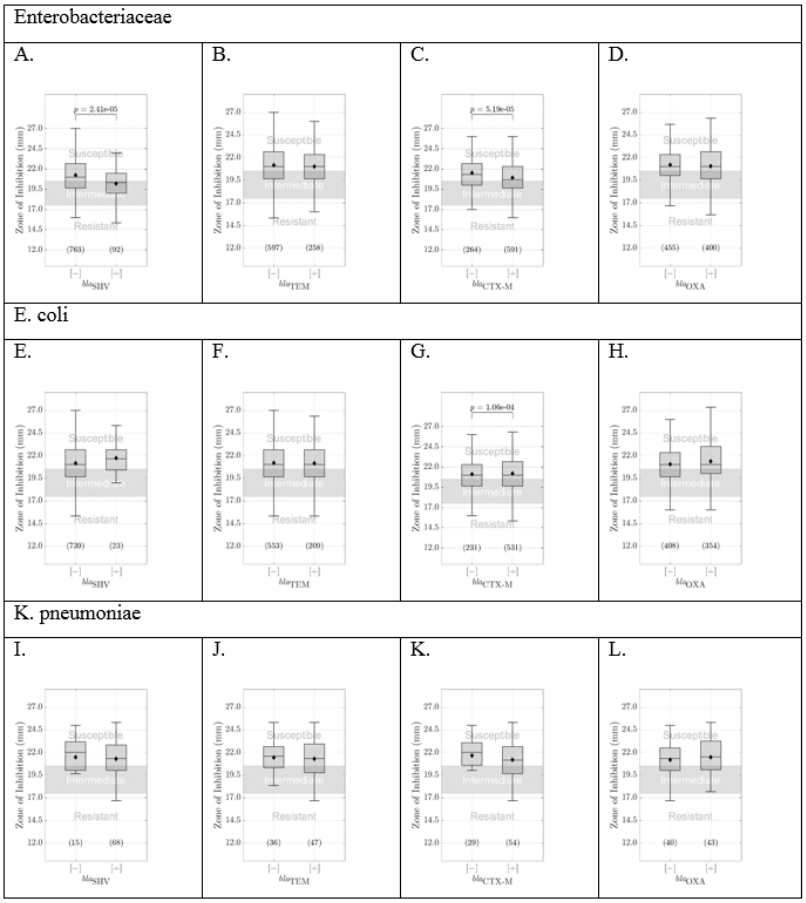

Figure 2: Distribution of Zone of Inhibition (ZI) Measurements (mm) by Resistance Gene Presence/Absence. Resistance gene presence is indicated by [+] and resistance gene absence is indicated by [−]. The diamond (◆) indicates the average zone of inhibition measurement for each condition and the number below each boxplot indicates the number of samples under that condition. The

p-value for a Welch's t-test is presented for statistically significant mean differences (p-value < 0.05 and false discovery control level q = 0.05). The CLSI regions for c/t resistance classification are labeled: susceptible, intermediate, and resistant.

Because resistance genes may interact, considering the relationship between ZI and each resistance gene in isolation is insufficient. To detect more complex associations, we must consider all the possible combinations of presence and absence of all four resistance genes at the same time (16 possible genetic combinations, Figure 3). To be thorough in our analysis, our supplemental section includes the analyses where we consider: the combinations of two genes and three genes. We note that significance was found only when either blaSHV and/or blaCTX-M were present (Figures S1-S5, p-value < 0.05, and q = 0.05).

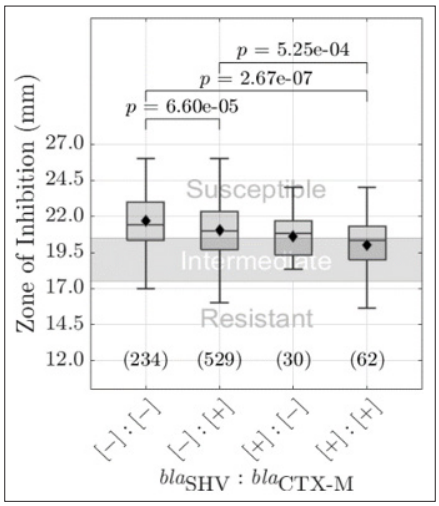

Table S1: Distribution of Zone of Inhibition (mm) Measurements by Resistance Gene Combination (Enterobacteriaceae). Resistance gene presence is indicated by [+] and resistance gene absence is indicated by [−]. The diamond (◆) indicates the average zone of inhibition measurement for each condition and the number below each boxplot indicates the number of samples under that condition. The p-value for a Welch's t-test is presented for statistically significant mean differences (p-value<0.05 and false discovery control level q=0.05). The CLSI regions for c/t resistance classification are labeled: susceptible, intermediate, and resistant.

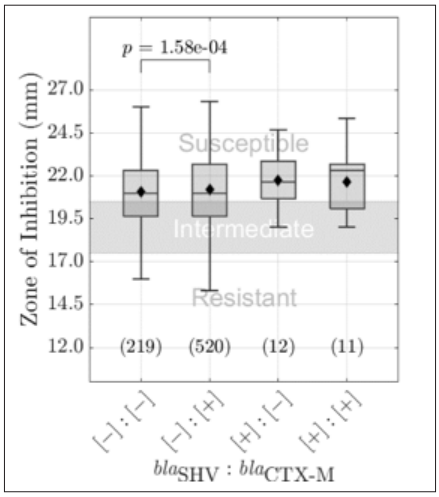

Table S2: Distribution of Zone of Inhibition (mm) Measurements by Resistance Gene Combination (E. coli). Resistance gene presence is indicated by [+] and resistance gene absence is indicated by [−]. The diamond (◆) indicates the average zone of inhibition measurement for each condition and the number below each boxplot indicates the number of samples under that condition. The *p*-value for a Welch's t-test is presented for statistically significant mean differences (*p*-value<0.05 and false discovery control level *q*=0.05). The CLSI regions for c/t resistance classification are labeled: susceptible, intermediate, and resistant.

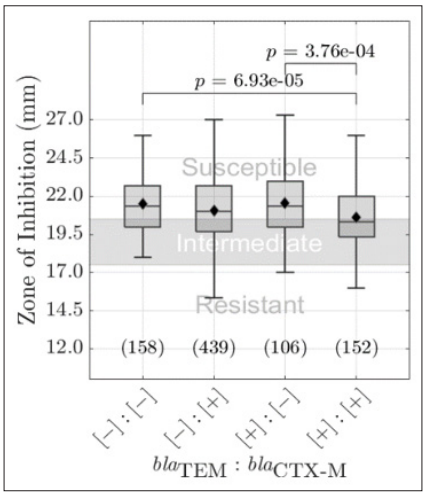

Table S3: Distribution of Zone of Inhibition (mm) Measurements by Resistance Gene Combination (Enterobacteriaceae). Resistance gene presence is indicated by [+] and resistance gene absence is indicated by [−]. The diamond (◆) indicates the average zone of inhibition measurement for each condition and the number below each boxplot indicates the number of samples under that condition. The p-value for a Welch's t-test is presented for statistically significant mean differences (p-value<0.05 and false discovery control level q=0.05). The CLSI regions for c/t resistance classification are labeled: susceptible, intermediate, and resistant.

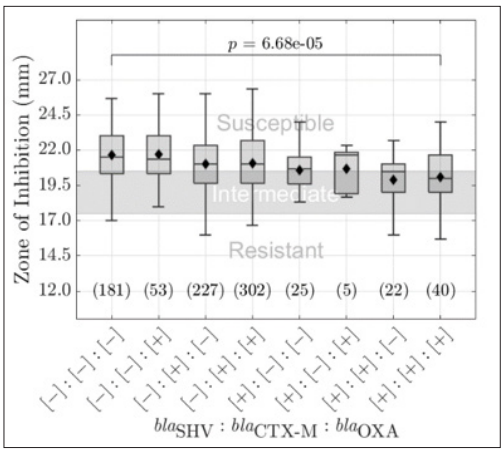

Table S4: Distribution of Zone of Inhibition (mm) Measurements by Resistance Gene Combination (Enterobacteriaceae). Resistance gene presence is indicated by [+] and resistance gene absence is indicated by [−]. The diamond (◆) indicates the average zone of inhibition measurement for each condition and the number below each boxplot indicates the number of samples under that condition. The p-value for a Welch's t-test is presented for statistically significant mean differences (p-value<0.05 and false discovery control level q=0.05). The CLSI regions for c/t resistance classification are labeled: susceptible, intermediate, and resistant.

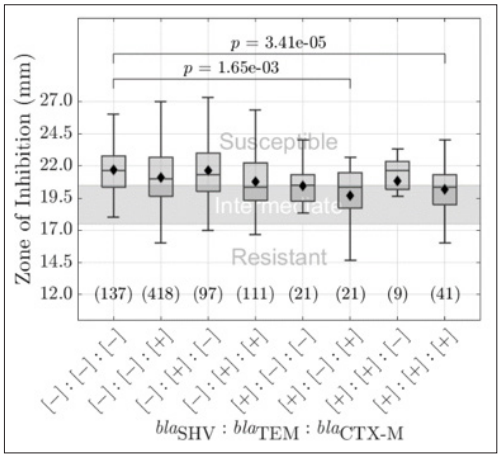

Table S5: Distribution of Zone of Inhibition (mm) Measurements by Resistance Gene Combination (Enterobacteriaceae). Resistance gene presence is indicated by [+] and resistance gene absence is indicated by [−]. The diamond (◆) indicates the average zone of inhibition measurement for each condition and the number below each boxplot indicates the number of samples under that condition. The p-value for a Welch's t-test is presented for statistically significant mean differences (p-value<0.05 and false discovery control level q=0.05). The CLSI regions for c/t resistance classification are labeled: susceptible, intermediate, and resistant.

Table S1: Comparisons of four resistance genes in all Enterobacteriaceae [ALL], E. coli [EC], and K. pneumoniae [KP]. Bold denotes significant assoications

Genes	Comparisons	Sample Sizes	Difference	p-value	95% CI
SHV[ALL]	$\mu([-])-\mu([+])$	[763][92]	1.03	2.41e-05	(0.57,1.50)
TEM[ALL]	$\mu([-])-\mu([+])$	[597][258]	0.17	2.76e-01	(-0.14,0.49)
CTX-M[ALL]	$\mu([-])-\mu([+])$	[264][591]	0.61	5.19e-05	(0.32,0.90)
OXA[ALL]	$\mu([-])-\mu([+])$	[455][400]	0.13	3.51e-01	(-0.15,0.42)
SHV[EC]	$\mu([-])-\mu([+])$	[739][23]	0.14	7.58e-01	(-0.78,1.05)
TEM[EC]	$\mu([-])-\mu([+])$	[553][209]	0.12	5.02e-01	(-0.22,0.46)
CTX-M[EC]	$\mu([-])-\mu([+])$	[231][531]	0.60	1.06e-04	(0.30,0.91)
OXA[EC]	$\mu([-])-\mu([+])$	[408][354]	0.15	3.00e-01	(-0.14,0.45)
SHV[KP]	$\mu([-])-\mu([+])$	[15][68]	1.60	1.75e-02	(0.31,2.89)
TEM[KP]	$\mu([-])-\mu([+])$	[36][47]	-0.40	4.16e-01	(-1.36,0.57)
CTX-M[KP]	$\mu([-])-\mu([+])$	[29][54]	0.87	9.60e-02	(-0.16,1.90)
OXA[KP]	$\mu([-])-\mu([+])$	[40][43]	0.25	6.00e-01	(-0.70,1.21)

To rigorously assess the possibility of combined genetic associations with c/t resistance using the ZI measure and the genetic information from all four β-lactamase genes, we separate the isolate data (n = 855) into disjoint resistance gene combinations (Figure 3). ANOVA of these disjoint conditions indicate a statistically significant difference in means (p-value = 1.874e-04, q = 0.05). Following the FDR controlling procedure, only a few associations remained significant: the absence of all four β-lactamase genes vs. the presence of all four β-lactamase genes (conditions 1 vs. 16), the absence of all genes vs. the absence of only bla_{SHV} (conditions 1 vs. 8), the single presence of bla_{OXA} vs. the presence of all four β-lactamase genes (conditions 2 vs. 16), the single presence of bla_{TEM} vs. the absence of only bla_{SHV} (conditions 5 vs. 8), and the presence of bla_{TEM} vs. the presence of all genes (conditions 5 vs. 16) (Welch's t-test, all p-values < 0.05, q = 0.05).

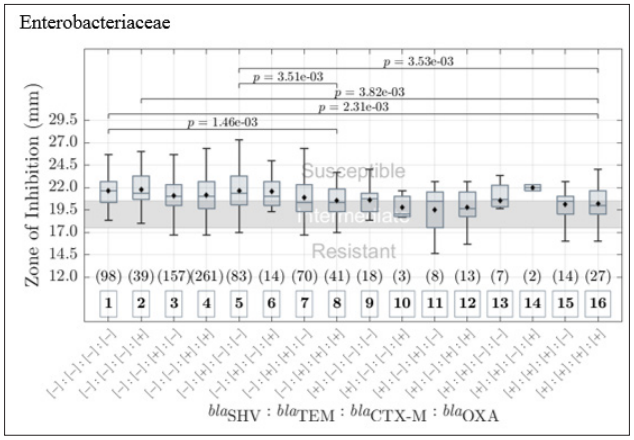

Figure 3: Distributions of Zone of Inhibition (mm) Measurements by Resistance Gene Combination (Enterobacteriaceae). Resistance gene presence is indicated by [+] and resistance gene absence is indicated by [−]. The diamond (◆) indicates the average zone of inhibition measurement for each condition and the number below each boxplot in parentheses indicates the number of samples under that condition and boxed numbers refer to that condition for easier reference. The p-value for a Welch's t-test is presented for statistically significant mean differences (p-value < 0.05 and false discovery control level q = 0.05). The CLSI regions for c/t resistance classification are labeled: susceptible, intermediate, and resistant.

Conclusions

Our surveillance of the frequency of c/t resistance is consistent with the low frequencies of c/t resistance observed in similar studies across the United States and Europe [2, 10]. We found that resistance in these clinical isolates has been consistently low over the past 6 years, giving us reason to believe that this treatment option will continue to be effective in the immediate future. The cumulative frequency of resistance across all Enterobacteriaceae clinical isolates is likely lower than 3.2% because our collection emphasizes ESBL resistance genes which can contribute to c/t resistance. The emphasis on ESBLs likely explains the relatively high percentage of isolates with intermediate resistance to c/t (38.2%).

The conditional frequencies of resistance for c/t and nine other antibiotics showed that we generally have low frequencies of resistance to carbapenems and to combination therapies that include the inhibitor tazobactam. Even when the frequency of resistance to cephalosporins is high, the frequency of resistance to c/t remains low. As expected, isolates that are carbapenem resistant showed increased c/t resistance frequency, indicating that co-resistance between carbapenems and c/t is common. In the case of the 32 c/t resistant isolates with carbapenems, 12.5% were resistant to ertapenem, and 9.4% were resistant to imipenem, suggesting that if c/t resistance is observed, carbapenems may be the best treatment option, though the opposite relationship is not true.

Through the analysis of PCR test results for four resistance genes (bla_{TEM}, bla_{OXA}, bla_{SHV}, and $bla_{CTX\text{-}M}$) and ZI measurements in the presence of c/t, we found that isolates containing blaSHV or $bla_{CTX\text{-}M}$ or their co-presence were associated with a smaller c/t ZI measurement, indicating a possible relationship between these genes and c/t resistance; a relationship which has been previously suggested and one which indicates caution in prescribing c/t in settings where the frequencies of these genes are known to be high [24, 25].

While $bla_{CTX\text{-}M}$ and bla_{SHV} variants contribute to c/t resistance, their contributions alone do not appear to be sufficient for clinical resistance as shown through the many $bla_{CTX\text{-}M}$ positive isolates in our collection that remain susceptible to c/t (Figure 1). Additionally, when we looked at the disjoint resistance gene combination, we did not find significance when $bla_{CTX\text{-}M}$ was present vs. its absence (Figure 1, Conditions 1 & 3, and 1 & 10). Similarly, for bla_{SHV}, we did not find significance between its sole presence and absence with c/t resistance (Figure 1, Conditions 1 & 9, and 1 & 10). This suggests that the presence of these two β-lactamase genes may promote c/t resistance but is not alone sufficient. It is possible that other generalized resistance determinants, such as porins and efflux pumps, could play a role in the co-occurrence of resistance to c/t and carbapenems [26].

In conclusion, while there is a low frequency of c/t resistance in our collection, it is likely that if the usage of c/t increases, there will be a corresponding increase and selection for isolates with $bla_{CTX\text{-}M}$ or bla_{SHV} genes and their co-occurrence in ESBL clinical populations. We recommend continuous surveillance for c/t resistance in ESBL clinical populations and recommend further investigations into candidate genes that drive c/t resistance [27-38].

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