The Role of *fosA* in Challenges with Fosfomycin Susceptibility Testing of Multispecies *Klebsiella pneumoniae* Carbapenemase-Producing Clinical Isolates

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Running title: *FosA* Impact on Fosfomycin Susceptibility

Abstract

With multidrug resistant (MDR) Enterobacteriales on the rise, a non-toxic agent with a unique mechanism of action such as fosfomycin seems attractive. However, establishing accurate fosfomycin susceptibility testing for non-*E. coli* in a clinical microbiology laboratory remains problematic. We evaluated fosfomycin susceptibility by multiple methods with multiple strains and species of KPC-producing clinical isolates collected at a single center between 2008 and 2016. In addition, we assessed the presence of fosfomycin resistance genes from whole genome sequencing (WGS) data using NCBI's AMRFinder and custom HMM search. Susceptibility testing was performed using glucose-6-phosphate supplemented fosfomycin E-Test and Kirby-Bauer disk diffusion (DD) assays, and compared to agar dilution. Clinical Laboratory and Standards Institute (CLSI) breakpoints for *E. coli* were applied for interpretation. Overall, 63% (60/96) of isolates were susceptible by E-Test, 70% (67/96) by DD, and 88% (84/96) by agar dilution. *FosA* was detected in 80% (70/88) of previously sequenced isolates, with species-specific associations and alleles, and *fosA*-positive isolates were associated with higher MIC distributions. Disk potentiation testing was performed using sodium phosphonoformate to inhibit *fosA* and showed significant increases in the zone diameter of DD testing for isolates that were *fosA*-positive compared to *fosA*-negative. The addition of sodium phosphonoformate (PPF) corrected 10/14 (71%) major errors in categorical agreement with agar dilution. Our results indicate that *fosA* influences the inaccuracy of susceptibility testing by methods readily available in a clinical laboratory when compared to agar dilution. Further research is needed to determine the impact of *fosA* on clinical outcomes.

Introduction

Antimicrobial resistance among gram-negative organisms continues to increase and presents a serious threat to modern medicine with carbapenemase-producing *Enterobacteriaceae* (CPE) considered one of the most pressing issues (1). The concern with CPE is largely due to a lack of remaining therapeutic options, especially oral agents (2). This has led to the re-evaluation of older antimicrobials to combat infections due to multidrug-resistant (MDR) Gram-negative pathogens. Fosfomycin, which was originally discovered in 1969, has been shown to have *in vitro* activity against CPE (3). In the United States, the oral formulation is available for the treatment of uncomplicated urinary tract infections due to susceptible strains of *Escherichia coli* and *Enterococcus feacalis.* Outside of the United States, the intravenous (IV) formulation is approved and available for the management of systemic infections (4). Zavante Pharmaceuticals received Fast Track designation from the FDA in 2015 for IV fosfomycin and have completed Phase III clinical trials for the United States market (5).

Fosfomycin is a bactericidal antibiotic that binds to the cysteine residue of UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and inhibits peptidoglycan biosynthesis (6). Fosfomycin has activity against a range of bacterial pathogens, including highly drug resistant *Enterobacteriaceae* (6). Fosfomycin resistance in Enterobacteriales has been primarily driven by mutations in the *glpT* and *uhpT* genes, preventing active transport of fosfomycin into the cell (7). These mutations are however thought to be associated with a fitness cost in *E. coli* and are thus unstable (8, 9). The other major mechanism of resistance is hydrolysis of the drug via diverse Fos enzymes: FosA (FosA2, FosA3, FosA4, FosA5, FosA6, FosA7), FosB, and FosX are metalloenzymes, whereas FosC is a serine enzyme (10). FosA was originally discovered on a transposon, Tn2921, in a *Serratia marcescens* plasmid, and catalyzes the addition of glutathione

to fosfomycin, rendering the drug ineffective (11). Transmissible *fosA* is of most concern in Enterobacteriales, and plasmid-mediated *fosA3* has been increasingly identified in *E. coli* in Europe (12). A recent evaluation of Fos enzymes in non-*E. coli* Enterobacteriales demonstrated different *fosA* variants were shown to be chromosomally located in a species-specific manner (11) Lastly, MurA target site alternation can also confer fosfomycin resistance. Amino acid substitutions in MurA, most notably Asp369Asn and Leu370lle, have been responsible for fosfomycin resistance (13).

Susceptibility testing of fosfomycin for non-*E. coli* Enterobacteriales is difficult for clinical microbiology labs (14-16). Both CLSI and EUCAST specifically recommend against the use of broth microdilution methods which likely impacts the inaccuracies with most automated susceptibility testing platforms for *E. coli* or *Klebsiella pneumoniae* (17, 18). Agar dilution is considered the reference method, and endorsed by EUCAST, however this is difficult to execute routinely in a clinical microbiology laboratory. Kirby-Bauer Disk Diffusion (DD) and E-Tests are more attractive options, as they can be performed easily in a clinical laboratory, but colonies often grow within the zones of inhibition making interpretation difficult (19). Attempting to change the zone cutoff to better align with agar dilution has not proved successful in non-*E. coli* Enterobacteriales (15). With agar dilution as the only accurate method for non-*E. coli* Enterobacteriales, we aimed to characterize some of the molecular mechanisms (by whole genome sequencing for a subset of isolates) that may be contributing to the inaccuracies with these diffusion methods, using a set of clinical, non-*E. coli* carbapenemase producing strains and agar dilution-based reference phenotyping.

Materials and Methods

Retrospective samples of *Klebsiella pneumoniae* carbapenemase KPC-producing *Gammaproteobacteria* isolates were selected from those collected at University of Virginia Health System since August 2008. Isolates were chosen to represent diverse species and strains for which Illumina sequence data were available. Species identification had been performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF, VITEK-MS, VITEK-2, bioMêrieux) and all isolates were bla_{KPC} PCR positive as previously described (20).

E-Test was performed using glucose-6-phosphate (G6P) supplemented fosfomycin E-Test strips (BioMêrieux Durham, NC) according to the manufacturer instructions. DD was performed with a 200 µg fosfomycin disk with 50µg G6P (Becton and Dickinson, Franklin Lakes, NJ) on Mueller-Hinton agar as per CLSI guidelines(21). Agar dilution was performed using G6P supplemented (25 µg/mL) Mueller-Hinton agar with fosfomycin concentrations ranging from 0.5 to 1,024 µg/mL per CLSI methods(22). A 0.5 McFarland inoculum for each isolate was placed in triplicate on the agar, placed in an incubator at 35^0 C for 16 to 20 hours, and then interpreted. Plates were prepared without isolates at each concentration to serve as the control.

Per the package insert for interpretation of E-Test, the crossing point of the ellipse was used to identify the minimum inhibitory concentration (MIC) where colonies within the zone of the ellipse were accounted for if 5 colonies were present within 3mm of the strip within the zone (BioMêrieux Durham, NC). DD diameters were measured as the shortest distance between 2 separate colonies (17, 21). For agar dilution, the median interpreted MIC was recorded as the result. All fosfomycin susceptibilities were interpreted according to CLSI breakpoints for *E. coli* urinary isolates as there are no breakpoints available for non-*E. coli* Enterobacteriales (17).

With agar dilution as the reference method, essential agreement for E-test and DD were defined as MIC variation within on dilution. Categorical agreement was defined as matching susceptible/intermediate/resistant interpretation criteria for the two respective tests, as per CLSI guidelines for *E. coli* urinary isolates. Falsely susceptible results were deemed to be very major errors and falsely resistant results to be major errors. All other disagreements were deemed minor errors. Chi-Square and Fisher's Exact Tests were used to compare rates of non-susceptibility and categorical agreement. The Mann-Whitney test was utilized for statistical analysis of MIC distributions and DD zone diameter changes.

Disk potentiation testing with sodium phosphonofomate (PPF) was performed to specifically evaluate the activity of *fosA* enzymes and the impact on susceptibility testing with the disk diffusion method. Cultures of each isolate were plated on Mueller-Hinton agar with 1mL of a 50mg/mL sodium phosphonoformate (PPF; Sigma-Aldrich) solution to a 200 µg fosfomycin disk supplemented with 50µg G6P. The plates were incubated overnight at 37^0 C and the inhibition zone was recorded. These inhibition zones were then compared the DD inhibition zones of fosfomycin without the supplementation of PPF. For each isolate tested, a blank disk with PPF was also placed on the agar plate to serve as a negative control.

Molecular mechanisms of resistance to fosfomycin were investigated in a subset of isolates previously whole genome sequenced by Ilumina Sequencing (HiSeq, 2000) as previously described (20). The quality filtered short reads were *de novo* assembled using SPAdes v3.11 (23), and the contigs were screened for *fos* resistance genes using NCBI's AMRFiner (id $\geq$ 0.9; cov $\geq$ 0.5)(24). For isolates where AMRFinder failed to detect *fos* genes, we screened the contigs using a custom HMM model built from distinct *fosA* protein sequences published by Ito *et. al.*, with an e-value threshold of 1e-20 (11, 25).

Results

Fosfomycin Susceptibility across Species

Ninety-six bla_{KPC}-positive isolates across twelve species were included in the study (**Table 1** for species breakdown). Eighty-eight of the 96 isolates had undergone whole genome sequencing (WGS). The MIC_{50} across all isolates was 8 µg/mL and the MIC_{90} was 128 µg/mL by agar dilution. Using the 2019 CLSI breakpoints (≤64 µg/mL=S), 84 of 96 isolates (88%) were susceptible, 11 of 96 (11%) were resistant, and 1 of 96 (1%) was classified as intermediate. The MIC distributions by agar dilution are shown in **Figure 1.**

Diffusion Method Performance

Sixty of 96 isolates (63%) were susceptible by E-Test and 65/96 (68%) were susceptible by DD. Categorical agreement of E-Test to agar dilution occurred in 69/96 isolates (72%) with 1 very major error, 16 major errors, and 10 minor errors. Essential agreement occurred in 55 of 96 isolates (57%) overall and in 4 of 5 (80%) *E. coli* isolates. Categorical agreement of DD to agar dilution occurred in 72/96 isolates (75%) with 2 very major errors, 14 major errors, and 8 minor errors. Of note, when testing the non-*E. coli* species, colonies within the zone were frequently present thus making interpretation challenging but adhered to package insert and CLSI guidance for E-test and DD respectively(21).

FosA presence

Of the isolates with WGS data, no isolate harbored *fosC*, while 70/88 isolates (80%) harbored an allele of *fosA*. All *K. pneumoniae* isolates (*n*=24) carried *fosA*, with 23 of 24 isolates (96%) harboring the *fosA6* or *fosA6*-like variant. Interestingly, only *K. aerogenes* isolates (n=7; 100%) carried the same variant. Detection of *fosA* in all tested isolates is shown in **Figure 1.** Isolates

without *fosA* (*n*=18) had a MIC range of ≤0.5 to 1024 µg/mL, MIC_{50} of ≤0.5 µg/mL, and MIC_{90} of 2 µg/mL. One of the 18 isolates (5.56%) was non-suseptible to fosfomycin. Isolates harboring *fosA* (*n*=70) had a MIC range of ≤0.5 to >1024 µg/mL, MIC_{50} of 16 µg/mL, and MIC_{90} of 128 µg/mL. Eleven of the 70 isolates (16%) were non-susceptible to fosfomycin. Isolates carrying the *fosA* gene were associated with a higher MIC distribution as compared to those without the gene (*P*=<0.00001), but did not differ in rates of non-susceptibility (*P*=0.26). These results are shown in **Table 3.**

FosA inhibition effect on Susceptibility Testing by Diffusion Method

Disk potentiation testing with PPF was performed on all 96 isolates. Categorical agreement with agar dilution was found in 72/96 (75%) of isolates prior to the additional of PPF and in 81/96 (84%) isolates after the addition of PPF (p=0.11). In the 88 isolates with WGS available, rates of categorical agreement were compared with and without PPF. In isolates that were negative for the *fosA* gene, categorical agreement was found in 18/18 (100%) and 17/18 (94%) of isolates before and after the additional of PPF, respectively (p=1). In isolates that carried the *fosA* gene, categorical agreement was found in 49/70 (70%) and 59/70 (84%) before and after the addition of PPF, respectively (p=0.04). When specifically isolating all non-susceptible isolates by DD, categorical agreement was found in 9/31 (29%) isolates and 20/31 (65%) before and after the addition of PPF, respectively (p=0.005). Results are shown in **Table 4 and Table 5.** The presence of PPF not only increased the zone size but also greatly decreased the presence of colonies within the zone in DD testing for the *fosA*-positive isolates (**Figure 2)**.

Discussion

We demonstrate that fosfomycin susceptibility testing by routinely used laboratory diffusion-based methods (E-test and DD) largely overcalls resistance, when compared to agar dilution as a gold standard (see **Table 1**). Fosfomycin susceptibility appears to be influenced by the presence of *fosA* among these KPC-producing Enterobacteriales isolates. This is highly relevant to the clinical microbiologist who is frequently fielding requests for fosfomycin susceptibility testing for non-*E. coli* Enterobacteriales. A prior study by Kaase *et al.* tested 107 carbapenem-non-susceptible *Enterobacteriaceae* isolates, of which 80 produced various carbapenemases (KPC, VIM, NDM, OXA-48) and similarly found 81% of isolates to be susceptible to fosfomycin with a MIC of ≤64 mcg/mL by agar dilution. This study also found similar issues of discordance with diffusion testing methods (15), as was also seen in Hirsch *et al*. (14).

Fosfomycin has been promoted as a useful, safe medication for the treatment of urinary tract infections against multidrug resistant non-*E. coli Enterobacteriaceae* (2, 3, 7, 26, 27) but susceptibility testing by agar dilution is practically difficult for a clinical microbiology lab. The CLSI cutoffs for *E. coli* were applied for E-test and DD interpretation for all species tested in this experiment, which requires accounting for scattered colonies within the ellipse per the package insert or zone per CLSI (17). Fosfomycin susceptibility testing for *E. coli* was recently reviewed by CLSI in 2018. At that time the recommendation that susceptibility cut-offs only apply to *E. coli* was strengthened and, since scattered colonies are rare within the zone for this species the practice of measuring the zone from the innermost colonies was upheld (17). This differs from the new European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for fosfomycin and *E. coli*, which suggests ignoring scattered colonies within diffusion-based inhibition zones, and utilizing agar dilution approaches for non-*E.coli* Enterobacteriales (28).

This decision for the former was based on the findings that sub-colonies of *E. coli* within inhibition zones are rare (<1% of isolates) and largely less fit with channel or transporter mutations (8, 9).

We postulate based on our findings that the colonies within the inhibition zone seen more frequently with some non-*E. coli* species may be driven by the presence of a chromosomal *fosA* rather than channel or transporter mutations. Thus the advice to ignore the colonies in the zone in *E. coli* may not apply to non-*E. coli* Enterobacteriales and clinical microbiologists should proceed with caution. The discordance among commercially available DD and E-test with agar dilution we observed was largely due to bacterial colonies that grow within the zone of inhibition. Based on the change in zone size with the addition of a FosA inhibitor as well as the work of others demonstrating the activity of chromosomal expressed FosA, it may unwise to ignore sub-colonies as the clinical implications of this finding remain unknown (29).

In our subset of *E. coli* isolates (n=5), categorical agreement was found in 5 of 5 (100%) isolates. All *E. coli* isolates were susceptible to fosfomycin by both DD and agar dilution, and no colonies were observed within the zones of inhibition. Although our numbers are small, this is consistent with other reports (8).

FosA (alleles 1-7) was identified in the majority of the clinical isolates in this study. *FosA* was present in all *Klebsiella spp.* isolates, with a large portion harboring the *fosA6* allele. *FosA6* was first reported in 2016, from an ESBL-producing, fosfomycin-resistant *E. coli* strain in Pennsylvania, USA. It shared 96% identity with *fosA5* and 79% identity with *fosA3* but was located on a plasmid, unlike the chromosomally-encoded *fosA* in *K. pneumoniae* (30). It has been suggested that *fosA6* was mobilized from the chromosome of *K. pneumoniae* to an *E. coli* plasmid (30). However, in our study, no *E. coli* harbored *fosA6*, but rather one isolate harbored

FosA7, which has been described on the chromosome of *Salmonella enterica* (31). We postulate that this gene may have been acquired via plasmid transfer with *Salmonella enterica* serving as the reservoir for this allele. No *fosC* was detected, as expected, as it is a gene found most commonly within *Pseudomonas* spp. which were not included in this study.

In this subset of isolates, the presence of *fosA* resulted in a trend towards higher MIC values compared to isolates not harboring the gene. Despite the higher distribution of MIC values, there was no statistical difference in fosfomycin susceptibility when performed by the agar dilution method within the range of CLSI range of susceptible for *E. coli*.

Disk potentiation testing with PPF was performed to specifically evaluate the activity of *fosA* enzymes and the impact on susceptibility testing with DD. The addition of PPF significantly increased zone diameter size and, subsequently, improved categorical agreement of disk diffusion with agar dilution, particularly in *fosA*-positive isolates in which most major errors were eliminated. These improvements were largely due to the elimination of sub-colonies within the zone of inhibition, as illustrated for isolate CAV 1217 (**Figure 2**). As expected, there was no statistical change in categorical agreement in isolates that did not harbor a *fosA* allele. The results of the disk potentiation testing indicate that *fosA* impacts fosfomycin activity and limits rapid, diffusion-based susceptibility testing. Some isolates had substantial zone diameter increases (6-8mm) after the addition of PPF, yet did not alter the susceptibility interpretation. This is likely due to alternative mechanisms of fosfomycin resistance, such as transporter mutations or MurA mutations. An alternative explanation is that certain alleles of *fosA* may have the ability to overcome the inhibition of PPF that was added to the disk.

Lastly, our data suggests that the addition of PPF may have a synergistic effect with fosfomycin against *fosA*-positive organisms. This is corroborated by a recently published study that found

246 significant MIC reductions and restored fosfomycin susceptibility in *fosA*-positive Gram-
247 negative organisms (29). PPF is available as the antiviral foscarnet but would likely be
248 unattractive as an adjuvant therapy due to toxicity.

249 Our study is limited by a small sample size of bla_{KPC}-positive multidrug-resistant isolates
250 collected from a single center. However, the clinical utility of fosfomycin is primarily against
251 MDR isolates and our study highlights the difficulty in accurately providing fosfomycin AST for
252 these organisms. A further limitation is the lack of exploration of other molecular mechanisms
253 of resistance which were not evaluated in all isolates. Our study also lacks outcome data, and
254 therefore we can make no conclusions on the clinical implications of fosfomycin susceptibility
255 testing results.

256 In conclusion, fosfomycin appears to have reliable *in vitro* activity against KPC-producing
257 Gram-negative organisms by agar dilution. However, methods readily available in a clinical
258 microbiology laboratory, E-test and DD, generate frequent major errors for the same isolates,
259 with the presence of *fosA* impacting the interpretation of these diffusion-based methods. Caution
260 is advised when interpreting and releasing AST results derived from diffusion-based methods for
261 non-*E. coli Enterobacteriales*. Regardless, further research is needed to establish correlations
262 between antimicrobial susceptibility testing, *fosA* presence and clinical outcomes.

263 **Disclosures**

264 Fosfomycin was provided and a portion of the study was funded by Zavante Therapeutics.

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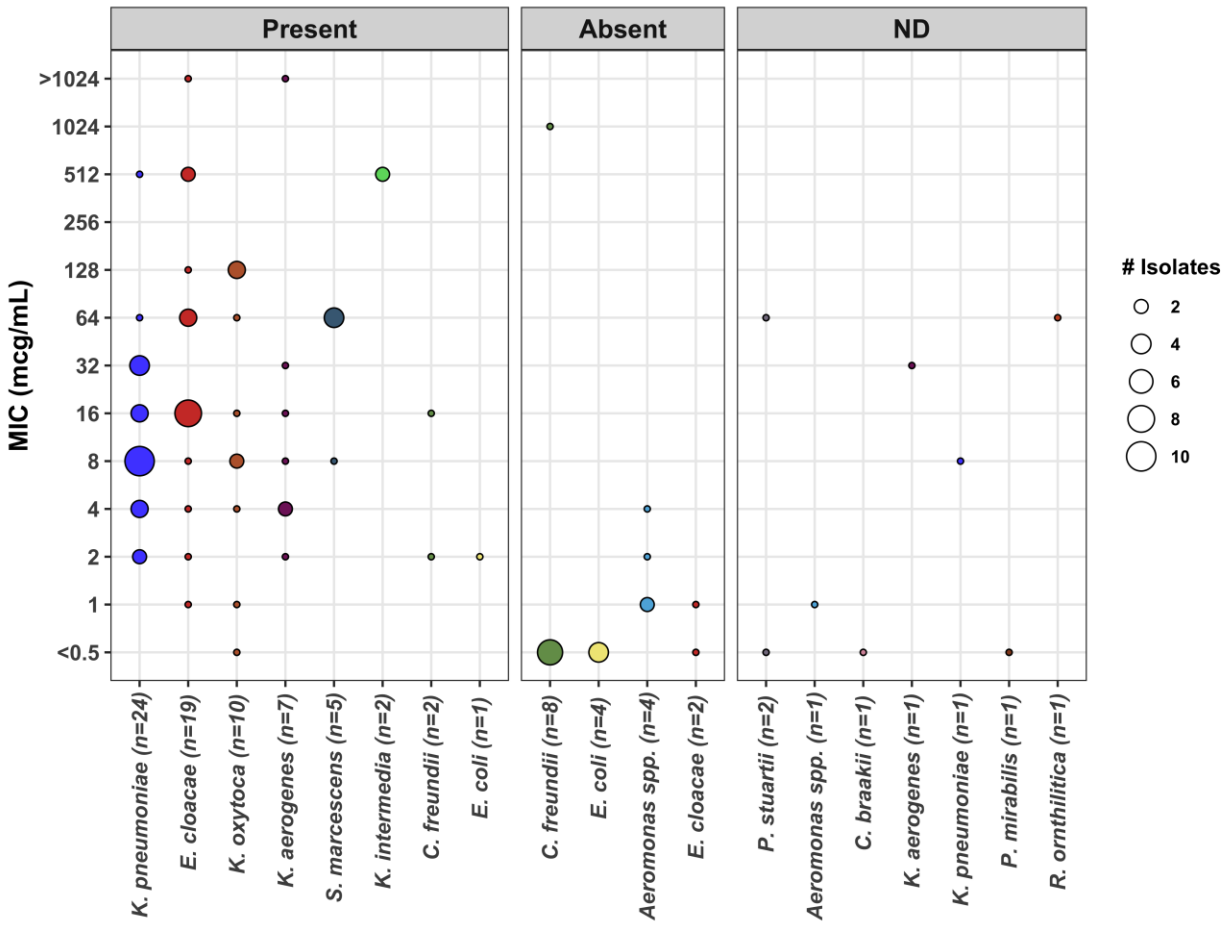
Appendixes

Table 1: Fosfomycin Susceptibility (n=96)

Organism	**Susceptibility**		
	E-Test (%)	DD (%)	Agar Dilution (%)
Klebsiella. pneumoniae (n=25)	16 (64%)	16 (64%)	24 (96%)
Enterobacter cloacae (n=21)	10 (48%)	15 (71%)	17 (81%)
Citrobacter spp. (n=11)	10 (91%)	9 (82%)	10 (91%)
Klebsiella oxytoca (n=10)	7 (70%)	8 (80%)	7 (70%)
Klebsiella. aerogenes (n=8)	3 (38%)	3 (37.5%)	7 (88%)
Escherichia coli (n=5)	5 (100%)	5 (100%)	5 (100%)
Serratia marcescens (n=5)	2 (40%)	2 (40%)	5 (100%)
Aeromonas spp. (n=5)	5 (100%)	5 (100%)	5 (100%)
Klebsiella intermedia (n=2)	0 (0%)	0 (0%)	0 (0%)
Raoultella ornithinolytica (n=1)	0 (0%)	1 (100%)	1 (100%)
Providencia stuartii (n=2)	1 (50%)	1 (50%)	2 (100%)
Proteus mirabilis (n=1)	1 (100%)	0 (0%)	1 (100%)
Totals:	**60 (63%)**	**65 (68%)**	**84 (88%)**

Figure 1. MIC distribution of KPC-producing isolates, grouped by *fosA* resistance gene presence screened from whole-genome sequencing data.



*ND: No sequencing data

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284 **Figure 2. Example of elimination of fosfomycin nonsusceptible sub-colonies within zone of**

285 **inhibition in *Klebsiella pneumonia* CAV 1217**

286 Left: Fosfomycin alone Right: Fosfomycin + PPF

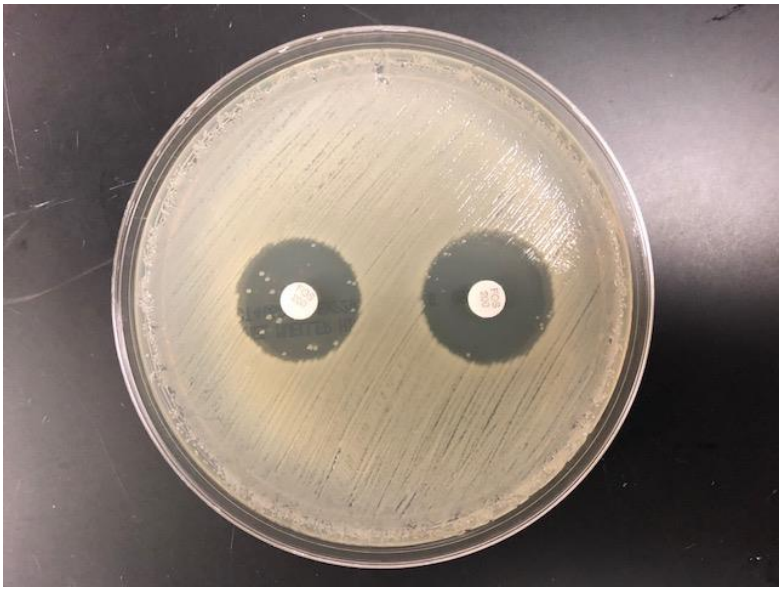

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Table 2. MIC Distributions Among Most Frequent Isolates

		MIC data (mcg/mL)			Nonsusceptibility	
Species (n)	FosA Positive*	Range (mcg/mL)	MIC_{50}	MIC_{90}	No. of Isolates	%
All species (96)	70/88 (79.55%)	≤ 0.5 to >1024	16	128	12	12.5%
K. pneumoniae (25)	24/24 (100.00%)	2 to 512	8	32	1	4.0%
E. cloacae (21)	19/21 (90.48%)	≤ 0.5 to >1024	16	512	4	19.05%
Citrobacter freundii (10)	2/10 (20.00%)	≤ 0.5 to >1024	0.5	16	1	10.0%

289 * Isolates with WGS data available

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Table 3. Comparison of Isolates

Isolates	Range	MIC 50	MIC 90	*P*-value	No. of nonsuceptible isolates (%)	*P*-value
Not harboring *fosA* (n=18)	≤0.5 to 1024	≤0.5	2	***P*=<0.00001**	1 (5.56%)	*P*=0.2627
Harboring *fosA* (n=70)	≤0.5 to >1024	16	128		11 (15.71%)	

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Table 4. Disk Potentiation testing on all isolates (n=96)

DD Fosfomycin Only			DD Fosfomycin + PPF		
Susceptiblity	n=	Categorical agreement	Susceptibility	n=	Categorical agreement
Susceptible	65	63/65=96.9%	Susceptible	80	76/80=95.0%
Intermediate	7	0/7=0%	Intermediate	6	0/6=0%
Resistant	24	9/24=37.5%	Resistant	10	5/10=50%
		Total: 72/96=75%			**Total: 81/96=84%**
					P=0.10644

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Table 5. Disk Potentiation testing on WGS Isolates (n=88)

FosA Negative (n=18)

DD Fosfomycin Only			DD Fosfomycin + PPF		
Susceptibility	n=	Categorical agreement	Susceptibility	n=	Categorical agreement
Susceptible	17	17/17=100%	Susceptible	17	17/17=100%
Intermediate	0	n/a	Intermediate	1	0/1=100%
Resistant	1	1/1=100%	Resistant	0	n/a
		Total: 18/18=100%			**Total: 17/18=94.44%**
					***P*=1**

FosA Positive (n=70)

DD Fosfo Only			DD Fosfo + PPF		
Susceptibility	n=	Categorical agreement	Susceptibility	n=	Categorical agreement
Susceptible	43	41/43=95.3%	Susceptible	58	54/58=93.1%
Intermediate	6	0/6=0%	Intermediate	4	0/4=0%
Resistant	21	8/21=38.1%	Resistant	8	5/8=62.5%
		Total: 49/70=70.0%			**Total: 59/70=84.3%**
					***P*=0.04415**

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