

1 The Role of *fosA* in Challenges with Fosfomycin Susceptibility Testing of Multispecies

2 *Klebsiella pneumoniae* Carbapenemase-Producing Clinical Isolates

3 Zachary S. Elliott<sup>1,2</sup>, Katie E. Barry<sup>2</sup>, Heather L. Cox<sup>1,2</sup>, Nicole Stoesser<sup>3,4</sup>, Joanne Carroll<sup>5</sup>, Kasi

4 Vegesana<sup>6</sup>, Shireen Kotay<sup>2</sup>, Anna E. Sheppard<sup>3,4</sup>, Alex Wailan<sup>2</sup>, Derrick W. Crook<sup>3,4</sup>, Hardik

5 Parikh<sup>7</sup>, Amy J. Mathers<sup>2,5#</sup>

6 <sup>1</sup>Department of Pharmacy Services, University of Virginia Health System, Charlottesville,

7 Virginia, USA.

8 <sup>2</sup>Division of Infectious Diseases and International Health, Department of Medicine, University of

9 Virginia Health System, Charlottesville, Virginia, USA.

10 <sup>3</sup>Modernizing Medical Microbiology Consortium, Nuffield Department of Clinical Medicine,

11 John Radcliffe Hospital, Oxford University, Oxford, United Kingdom.

12 <sup>4</sup>NIHR Health Protection Research Unit in Healthcare Associated Infection and Antimicrobial

13 Resistance at University of Oxford in partnership with Public Health England, Oxford, UK.

14 <sup>5</sup>Clinical Microbiology, Department of Pathology, University of Virginia Health System,

15 Charlottesville, Virginia, USA.

16 <sup>6</sup>Health Information & Technology, University of Virginia Health System, Charlottesville,

17 Virginia, USA

18 <sup>7</sup>School of Medicine Research Computing, University of Virginia, Charlottesville, USA

19 **#Corresponding Author:** Amy J. Mathers, MD, D(ABMM), P.O. Box 800255, Charlottesville, VA,

20 USA 22908-1361, Phone: (434) 982-4814, Fax: (434)-924-0075, Email : [ajm5b@virginia.edu](mailto:ajm5b@virginia.edu)

21 Running title: *FosA* Impact on Fosfomycin Susceptibility

## 22 **Abstract**

23 With multidrug resistant (MDR) Enterobacteriales on the rise, a non-toxic agent with a unique  
24 mechanism of action such as fosfomycin seems attractive. However, establishing accurate  
25 fosfomycin susceptibility testing for non-*E. coli* in a clinical microbiology laboratory remains  
26 problematic. We evaluated fosfomycin susceptibility by multiple methods with multiple strains  
27 and species of KPC-producing clinical isolates collected at a single center between 2008 and  
28 2016. In addition, we assessed the presence of fosfomycin resistance genes from whole genome  
29 sequencing (WGS) data using NCBI's AMRFinder and custom HMM search. Susceptibility  
30 testing was performed using glucose-6-phosphate supplemented fosfomycin E-Test and Kirby-  
31 Bauer disk diffusion (DD) assays, and compared to agar dilution. Clinical Laboratory and  
32 Standards Institute (CLSI) breakpoints for *E. coli* were applied for interpretation. Overall, 63%  
33 (60/96) of isolates were susceptible by E-Test, 70% (67/96) by DD, and 88% (84/96) by agar  
34 dilution. *FosA* was detected in 80% (70/88) of previously sequenced isolates, with species-  
35 specific associations and alleles, and *fosA*-positive isolates were associated with higher MIC  
36 distributions. Disk potentiation testing was performed using sodium phosphonoformate to  
37 inhibit *fosA* and showed significant increases in the zone diameter of DD testing for isolates that  
38 were *fosA*-positive compared to *fosA*-negative. The addition of sodium phosphonoformate (PPF)  
39 corrected 10/14 (71%) major errors in categorical agreement with agar dilution. Our results  
40 indicate that *fosA* influences the inaccuracy of susceptibility testing by methods readily available  
41 in a clinical laboratory when compared to agar dilution. Further research is needed to determine  
42 the impact of *fosA* on clinical outcomes.

## 43 **Introduction**

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

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

66 to fosfomycin, rendering the drug ineffective (11). Transmissible *fosA* is of most concern in  
67 Enterobacteriales, and plasmid-mediated *fosA3* has been increasingly identified in *E. coli* in  
68 Europe (12). A recent evaluation of Fos enzymes in non-*E. coli* Enterobacteriales demonstrated  
69 different *fosA* variants were shown to be chromosomally located in a species-specific manner  
70 (11) Lastly, MurA target site alternation can also confer fosfomycin resistance. Amino acid  
71 substitutions in MurA, most notably Asp369Asn and Leu370Ile, have been responsible for  
72 fosfomycin resistance (13).

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

## 87 **Materials and Methods**

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

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

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

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

118 Disk potentiation testing with sodium phosphonofomate (PPF) was performed to specifically  
119 evaluate the activity of *fosA* enzymes and the impact on susceptibility testing with the disk  
120 diffusion method. Cultures of each isolate were plated on Mueller-Hinton agar with 1mL of a  
121 50mg/mL sodium phosphonoformate (PPF; Sigma-Aldrich) solution to a 200 µg fosfomycin disk  
122 supplemented with 50µg G6P. The plates were incubated overnight at 37<sup>0</sup> C and the inhibition  
123 zone was recorded. These inhibition zones were then compared the DD inhibition zones of  
124 fosfomycin without the supplementation of PPF. For each isolate tested, a blank disk with PPF  
125 was also placed on the agar plate to serve as a negative control.

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

## 133 **Results**

### 134 **Fosfomycin Susceptibility across Species**

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

### 141 **Diffusion Method Performance**

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

### 150 **FosA presence**

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

155 without *fosA* ( $n=18$ ) had a MIC range of  $\leq 0.5$  to 1024  $\mu\text{g/mL}$ , MIC<sub>50</sub> of  $\leq 0.5$   $\mu\text{g/mL}$ , and MIC<sub>90</sub>  
156 of 2  $\mu\text{g/mL}$ . One of the 18 isolates (5.56%) was non-susceptible to fosfomycin. Isolates  
157 harboring *fosA* ( $n=70$ ) had a MIC range of  $\leq 0.5$  to  $>1024$   $\mu\text{g/mL}$ , MIC<sub>50</sub> of 16  $\mu\text{g/mL}$ , and MIC<sub>90</sub>  
158 of 128  $\mu\text{g/mL}$ . Eleven of the 70 isolates (16%) were non-susceptible to fosfomycin. Isolates  
159 carrying the *fosA* gene were associated with a higher MIC distribution as compared to those  
160 without the gene ( $P<0.00001$ ), but did not differ in rates of non-susceptibility ( $P=0.26$ ). These  
161 results are shown in **Table 3**.

### 162 **FosA inhibition effect on Susceptibility Testing by Diffusion Method**

163 Disk potentiation testing with PPF was performed on all 96 isolates. Categorical agreement with  
164 agar dilution was found in 72/96 (75%) of isolates prior to the additional of PPF and in 81/96  
165 (84%) isolates after the addition of PPF ( $p=0.11$ ). In the 88 isolates with WGS available, rates of  
166 categorical agreement were compared with and without PPF. In isolates that were negative for  
167 the *fosA* gene, categorical agreement was found in 18/18 (100%) and 17/18 (94%) of isolates  
168 before and after the additional of PPF, respectively ( $p=1$ ). In isolates that carried the *fosA* gene,  
169 categorical agreement was found in 49/70 (70%) and 59/70 (84%) before and after the addition  
170 of PPF, respectively ( $p=0.04$ ). When specifically isolating all non-susceptible isolates by DD,  
171 categorical agreement was found in 9/31 (29%) isolates and 20/31 (65%) before and after the  
172 addition of PPF, respectively ( $p=0.005$ ). Results are shown in **Table 4 and Table 5**. The  
173 presence of PPF not only increased the zone size but also greatly decreased the presence of  
174 colonies within the zone in DD testing for the *fosA*-positive isolates (**Figure 2**).

175

176



## 177 Discussion

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

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

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

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

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

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

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

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

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

244 Lastly, our data suggests that the addition of PPF may have a synergistic effect with fosfomycin  
245 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*<sub>KPC</sub>-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.

### 265 **Acknowledgements**

266 We thank UVaMC Clinical Microbiology staff for collection of study isolates.

## Appendixes

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

267

268

269

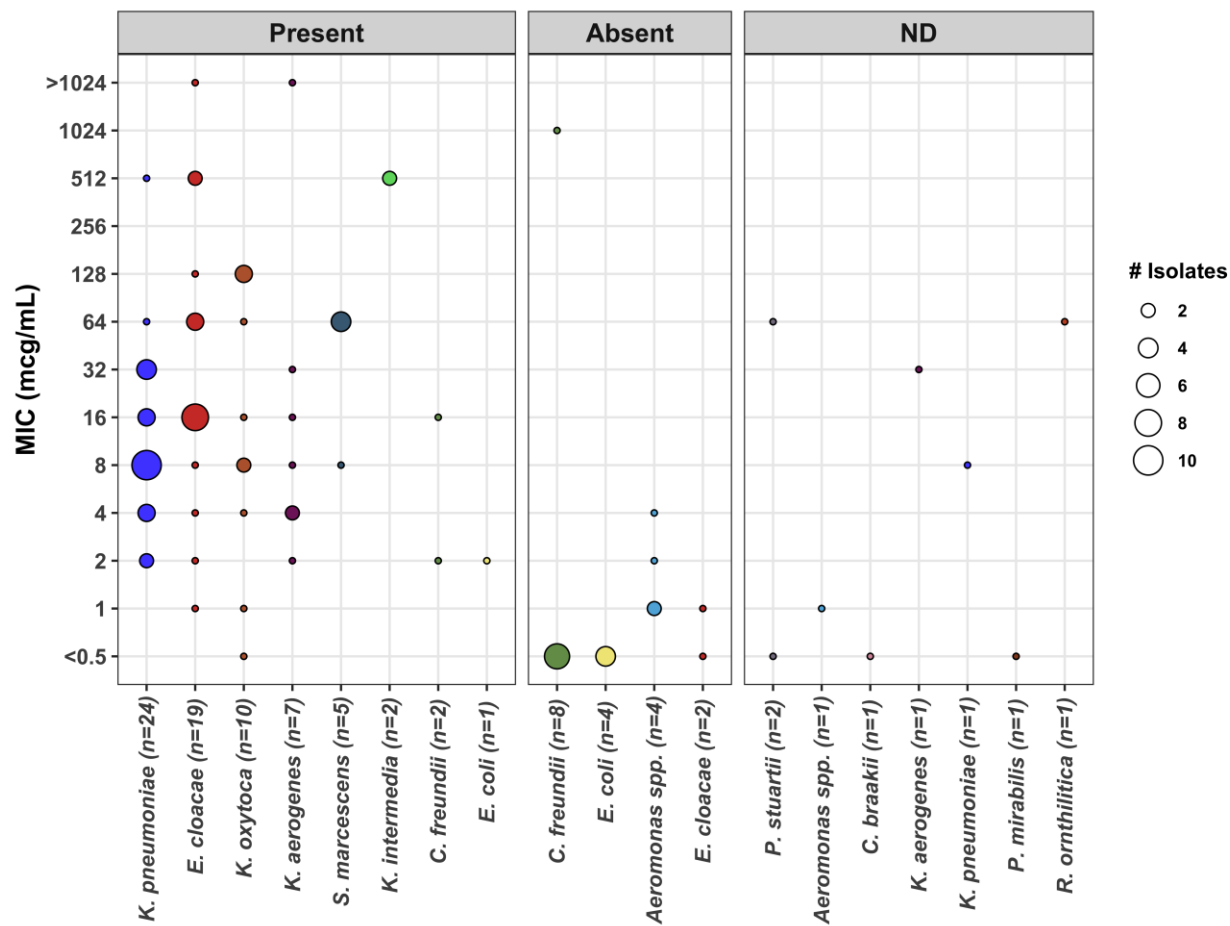
270

271

272

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

275



276

277

278 \*ND: No sequencing data

279

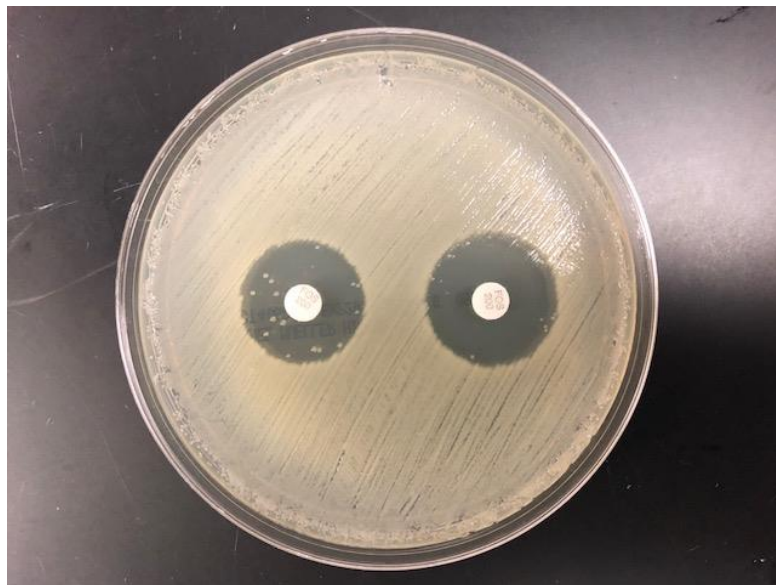
280

281

282

283  
 284 **Figure 2. Example of elimination of fosfomycin nonsusceptible sub-colonies within zone of**  
 285 **inhibition in *Klebsiella pneumoniae* CAV 1217**

286 Left: Fosfomycin alone Right: Fosfomycin + PPF



287

288

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

289 \* Isolates with WGS data available

290

<b>Table 3. Comparison of Isolates</b>						
Isolates	Range	MIC 50	MIC 90	<i>P</i> -value	No. of nonsusceptible isolates (%)	<i>P</i> -value
Not harboring <i>fosA</i> (n=18)	≤0.5 to 1024	≤0.5	2	<b><i>P</i> &lt; 0.00001</b>	1 (5.56%)	<i>P</i> =0.2627
Harboring <i>fosA</i> (n=70)	≤0.5 to >1024	16	128		11 (15.71%)	

291

<b>Table 4. Disk Potentiation testing on all isolates (n=96)</b>					
DD Fosfomycin Only			DD Fosfomycin + PPF		
Susceptibility	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%
		<b>Total: 72/96=75%</b>			<b>Total: 81/96=84%</b>
<i>P</i> =0.10644					

292

293

294

295

296

297

298

299



300

<b>Table 5. Disk Potentiation testing on WGS Isolates (n=88)</b>					
<u>FosA Negative (n=18)</u>					
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
		<b>Total: 18/18=100%</b>			<b>Total: 17/18=94.44%</b>
<b><i>P=1</i></b>					
<u>FosA Positive (n=70)</u>					
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%
		<b>Total: 49/70=70.0%</b>			<b>Total: 59/70=84.3%</b>
<b><i>P=0.04415</i></b>					

301

302

303

304

305

306

## 307 **References**

- 308  
309 1. Centers for Disease Control and Prevention (CDC). 2013. *Antibiotic resistance threats in the*  
310 *United States*. (CDC) CfDcCaP, Atlanta, GA USA.
- 311 2. Morrill HJ, Pogue JM, Kaye KS, LaPlante KL. 2015. Treatment Options for Carbapenem-  
312 Resistant Enterobacteriaceae Infections. *Open Forum Infect Dis* 2:ofv050.
- 313 3. Endimiani A, Patel G, Hujer KM, Swaminathan M, Perez F, Rice LB, Jacobs MR, Bonomo RA.  
314 2010. In vitro activity of fosfomycin against blaKPC-containing *Klebsiella pneumoniae* isolates,  
315 including those nonsusceptible to tigecycline and/or colistin. *Antimicrob Agents Chemother*  
316 54:526-9.
- 317 4. Grabein B, Graninger W, Rodriguez Bano J, Dinh A, Liesenfeld DB. 2017. Intravenous  
318 fosfomycin-back to the future. Systematic review and meta-analysis of the clinical literature. *Clin*  
319 *Microbiol Infect* 23:363-372.
- 320 5. Kaye KS, Rice LB, Dane A, Stus V, Sagan O, Fedosiuk E, das A, Skarinsky D, Eckburg P, Ellis-  
321 Grosse EJ. 2017. Intravenous Fosfomycin (ZTI-01) for the treatment of Complicated Urinary  
322 Tract Infections (cUTI) Including Acute Pyelonephritis (AP): Results from a Multi-Center,  
323 Randomized, Double-Blind Phase 2/3 Study in Hospitalized Adults (ZEUS). *Open Forum*  
324 *Infectious Diseases* 4:S528.
- 325 6. Falagas ME, Vouloumanou EK, Samonis G, Vardakas KZ. 2016. Fosfomycin. *Clin Microbiol*  
326 *Rev* 29:321-47.
- 327 7. Michalopoulos AS, Livaditis IG, Gougoutas V. 2011. The revival of fosfomycin. *Int J Infect Dis*  
328 15:e732-9.
- 329 8. Lucas AE, Ito R, Mustapha MM, McElheny CL, Mettus RT, Bowler SL, Kantz SF, Pacey MP,  
330 Pasculle AW, Cooper VS, Doi Y. 2018. Frequency and Mechanisms of Spontaneous Fosfomycin  
331 Nonsusceptibility Observed upon Disk Diffusion Testing of *Escherichia coli*. *J Clin Microbiol* 56.

- 332 9. Marchese A, Gualco L, Debbia EA, Schito GC, Schito AM. 2003. In vitro activity of fosfomycin  
333 against gram-negative urinary pathogens and the biological cost of fosfomycin resistance. *Int J*  
334 *Antimicrob Agents* 22 Suppl 2:53-9.
- 335 10. Silver LL. 2017. Fosfomycin: Mechanism and Resistance. *Cold Spring Harb Perspect Med* 7.
- 336 11. Ito R, Mustapha MM, Tomich AD, Callaghan JD, McElheny CL, Mettus RT, Shanks RMQ,  
337 Sluis-Cremer N, Doi Y. 2017. Widespread Fosfomycin Resistance in Gram-Negative Bacteria  
338 Attributable to the Chromosomal. *MBio* 8.
- 339 12. Mendes A, Rodrigues C, Pires J, Amorim J, Ramos Mh, Novais A, Peixe L. 2016. Importation of  
340 Fosfomycin Resistance *fosA3* Gene to Europe., vol 22, p 346-348. *Emerging Infectious*  
341 *Diseases*
- 342 13. Takahata S, Ida T, Hiraishi T, Sakakibara S, Maebashi K, Terada S, Muratani T, Matsumoto T,  
343 Nakahama C, Tomono K. 2010. Molecular mechanisms of fosfomycin resistance in clinical  
344 isolates of *Escherichia coli*. *Int J Antimicrob Agents* 35:333-7.
- 345 14. Hirsch EB, Raux BR, Zucchi PC, Kim Y, McCoy C, Kirby JE, Wright SB, Eliopoulos GM. 2015.  
346 Activity of fosfomycin and comparison of several susceptibility testing methods against  
347 contemporary urine isolates. *Int J Antimicrob Agents* 46:642-7.
- 348 15. Kaase M, Szabados F, Anders A, Gatermann SG. 2014. Fosfomycin susceptibility in  
349 carbapenem-resistant Enterobacteriaceae from Germany. *J Clin Microbiol* 52:1893-7.
- 350 16. Perdigao-Neto LV, Oliveira MS, Rizek CF, Carrilho CM, Costa SF, Levin AS. 2014.  
351 Susceptibility of multiresistant gram-negative bacteria to fosfomycin and performance of  
352 different susceptibility testing methods. *Antimicrob Agents Chemother* 58:1763-7.
- 353 17. Clinical Laboratory and Standards Institute. 2019. m100-S29 Performance Standards for  
354 Antimicrobial Susceptibility Testing, 29th ed. CLSI, Wayne, PA.
- 355 18. van den Bijllaardt W, Schijffelen MJ, Bosboom RW, Cohen Stuart J, Diederens B, Kampinga G,  
356 Le TN, Overdeest I, Stals F, Voorn P, Waar K, Mouton JW, Muller AE. 2018. Susceptibility of  
357 ESBL *Escherichia coli* and *Klebsiella pneumoniae* to fosfomycin in the Netherlands and

- 358 comparison of several testing methods including Etest, MIC test strip, Vitek2, Phoenix and disc  
359 diffusion. *J Antimicrob Chemother* 73:2380-2387.
- 360 19. Camarlinghi G, Parisio EM, Antonelli A, Nardone M, Coppi M, Giani T, Mattei R, Rossolini  
361 GM. 2019. Discrepancies in fosfomycin susceptibility testing of KPC-producing *Klebsiella*  
362 *pneumoniae* with various commercial methods. *Diagn Microbiol Infect Dis* 93:74-76.
- 363 20. Sheppard AE, Stoesser N, Wilson DJ, Sebra R, Kasarskis A, Anson LW, Giess A, Pankhurst LJ,  
364 Vaughan A, Grim CJ, Cox HL, Yeh AJ, Sifri CD, Walker AS, Peto TE, Crook DW, Mathers AJ,  
365 Group MMMMI. 2016. Nested Russian Doll-like Genetic Mobility Drives Rapid Dissemination  
366 of the Carbapenem Resistance Gene *blaKPC*. *Antimicrob Agents Chemother*.
- 367 21. Clinical Laboratory and Standards Institute. 2018. Performance Standards for Antimicrobial Disk  
368 Susceptibility Tests, vol M02. CLSI, 950 West Valley Rd Wayne, PA.
- 369 22. Clinical Laboratory and Standards Institute. 2018. Methods for Dilution Antimicrobial  
370 Susceptibility Tests for Bacteria That Grow Aerobically, vol M-07, p 91. Clinical Laboratory and  
371 Standard Institute, Wayne, PA.
- 372 23. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko  
373 SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA,  
374 Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell  
375 sequencing. *J Comput Biol* 19:455-77.
- 376 24. Feldgarden M, Brover V. 2019. Using the NCBI AMRFinder tool to determine Antimicrobial  
377 Resistance Genotype-Phenotype correlations within a collection of NARMS Isolates., bioRxiv  
378 doi: <https://doi.org/10.1101/550707>.
- 379 25. Eddy SR. 2011. Accelerated Profile HMM Searches. *PLoS Comput Biol* 7:e1002195.
- 380 26. Duez J-M, Mousson C, Siebor E, Pechinot A, Freysz M, Sixt N, Bador J, Neuwirth C. 2011.  
381 Fosfomycin and Its Application in the Treatment of Multidrug-Resistant *Enterobacteriaceae*  
382 Infections, vol 3, p 123-142. *Libertas Academica, Clinical Medicine Reviews in Therapeutics*.

- 383 27. Zayyad H, Eliakim-Raz N, Leibovici L, Paul M. 2017. Revival of old antibiotics: needs, the state  
384 of evidence and expectations. *Int J Antimicrob Agents* 49:536-541.
- 385 28. Testing TECoAS. 2019. Breakpoint tables for interpretation of MICs and zone diameters, version  
386 9.0, 2019. , [http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/).
- 387 29. Ito R, Tomich AD, McElheny CL, Mettus RT, Sluis-Cremer N, Doi Y. 2017. Inhibition of  
388 Fosfomycin Resistance Protein FosA by Phosphonoformate (Foscarnet) in Multidrug-Resistant  
389 Gram-Negative Pathogens. *Antimicrob Agents Chemother* 61.
- 390 30. Guo Q, Tomich AD, McElheny CL, Cooper VS, Stoesser N, Wang M, Sluis-Cremer N, Doi Y.  
391 2016. Glutathione-S-transferase FosA6 of *Klebsiella pneumoniae* origin conferring fosfomycin  
392 resistance in ESBL-producing *Escherichia coli*. *J Antimicrob Chemother* 71:2460-5.
- 393 31. Rehman MA, Yin X, Persaud-Lachhman MG, Diarra MS. 2017. First Detection of a Fosfomycin  
394 Resistance Gene,. *Antimicrob Agents Chemother* 61.
- 395