Title: Fosfomycin Susceptibility in Carbapenem-Resistant Enterobacteriaceae from Germany

Running title: Fosfomycin Susceptibility in Enterobacteriaceae

Keywords (3-5): carbapenemase, fosfomycin, Etest, disk diffusion

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Abstract
Due to the rise in multidrug-resistant Enterobacteriaceae the interest into older antimicrobial agents like fosfomycin has increased. In this study we used agar dilution for testing susceptibility to fosfomycin in a collection of 107 carbapenem non-susceptible Enterobacteriaceae, of which 80 produced various types of carbapenemases including KPC, VIM, NDM and OXA-48. Overall 78% of the strains had fosfomycin MICs ≤ 32 mg/L and were thus considered susceptible according to the current EUCAST breakpoint. The MIC\textsubscript{50} and MIC\textsubscript{90} were 8 mg/L and 512 mg/L, respectively. Escherichia coli strains had significantly lower fosfomycin MICs than Klebsiella pneumoniae and Enterobacter cloacae strains. Furthermore, a comparison of susceptibility testing methods like Etest and disk diffusion against agar dilution as reference method was performed. Essential agreement between Etest and agar dilution was 78.9%, categorical agreement between both methods was 92.5% with 20% very major errors and 2.6% major errors. Disk diffusion was studied with 50 µg and 200 µg fosfomycin disks, but no inhibition zone breakpoint could be found, that could reduce very major and major errors to an acceptable level. Etest as well as disk diffusion showed only poor agreement to fosfomycin agar dilution.
Multidrug-resistance in *Enterobacteriaceae* is an ever-increasing problem worldwide. Of particular concern is the spread of carbapenemases because these β-lactamases mediate resistance to all or almost all β-lactam antibiotics. In addition, strains carrying carbapenemases very often harbor resistance mechanisms against several unrelated antibiotics (1). Three groups of carbapenemases have been described in *Enterobacteriaceae* to date, namely Ambler class A carbapenemases like KPC, metallo-β-lactamases like VIM, GIM or NDM or class D carbapenemases like OXA-48 (2). Only few antimicrobial agents with clinically significant activity against resistant strains of Gram-negative bacteria are currently at or beyond phase III of development. This might lead to serious therapeutic limitations in treatment of many severe hospital-acquired infections. Fosfomycin is used for the treatment of uncomplicated urinary tract infections as single-dose oral treatment (3). Of note, in its intravenous form the drug is also considered for the treatment of severe infections like bacteremia and pneumonia due to multidrug-resistant Gram-negative bacteria. In this case it is usually combined with other antibiotics (4). Fosfomycin inhibits the N-acetylglucosamine-3-O-enolpyruvyl transferase, which catalyzes the conversion of UDP-N-acetylmuramic acid to UDP-N-acetylmuramic acid (5). This enolpyruvyl transferase is essential for any bacterium possessing muramic acid in its cell wall structure. Fosfomycin can enter the bacterial cell only by active transport. Two transport systems are known: the L-α-glycerophosphate system and the hexose monophosphate route. The latter system is more important and has to be induced, especially by glucose-6-phosphate (5). Fosfomycin resistance is mainly due to chromosomal mutations. Decreased drug uptake can be caused by mutations affecting the expression of the two transporter systems (6). In addition, resistance can be caused by mutations in the gene coding for MurA, the target of fosfomycin (6). Recently also plasmid-
mediated mechanisms of fosfomycin resistance have been described, which involve expression of enzymes capable of modifying fosfomycin by addition of glutathione, L-cysteine or H₂O (6).

The aim of this study was to determine the susceptibility of fosfomycin in a strain collection including various carbapenemase-producing Enterobacteriaceae and to compare susceptibility testing methods like Etest and disk diffusion with agar dilution in these strains.

Strains
Previously characterized non-copy strains (n = 107) referred to our reference laboratory because of resistance to ertapenem, imipenem or meropenem were used for the study and included Klebsiella pneumoniae (n = 50), Escherichia coli (n = 24), Enterobacter cloacae (n = 17), Klebsiella oxytoca (n = 6), Citrobacter freundii (n = 4), Serratia marcescens (n = 3) Proteus mirabilis (n = 1), Enterobacter aerogenes (n = 1) and Citrobacter farmeri (n = 1). The strains were isolated between August 2009 and February 2011 in 48 laboratories from different locations all over Germany. The main materials of isolation were urine (n = 24), lower airway specimens (n = 23) and wound swabs (n = 14). Species identification was performed by MALDI-TOF (Bruker Daltonics, Bremen, Germany). The strains were resistant to ertapenem, imipenem or meropenem by use of disk diffusion according to EUCAST. For carbapenemase detection a modified Hodge Test (7) as well as combined disk tests with EDTA (8) and boronic acid (9) had been carried out. In addition PCRs for KPC (10), VIM (8, 11), IMP (8), NDM (12) and OXA-48 (13) had been performed routinely. If the phenotypic tests suggested a carbapenemase which could not be detected by PCR the isolates had been analyzed by a microbiological bioassay (14) and additional PCRs for rarely occurring carbapenemases like GIM-1 (15). In 27 strains of the species E. coli (n = 9), E. aerogenes (n = 1), E. cloacae (n = 5), K. oxytoca (n = 3),
K. pneumoniae (n = 8) and P. mirabilis (n = 1) a carbapenemase could be excluded and elevated MICs for carbapenems were most likely caused by porin deficiency combined with expression of an extended-spectrum β-lactamase or AmpC-β-lactamase. The remaining 80 carbapenemase producers comprised OXA-48 (n = 24), VIM-1 (n = 16), KPC-2 (n = 13), KPC-3 (n = 11), VIM-4 (n = 4), NDM-1 (n = 4), OXA-162 (n = 4), GIM-1 (n = 3) and VIM-2 (n = 2). One K. pneumoniae isolate co-produced VIM-1 and KPC-2. Carbapenemases were found in the species C. farmeri (n = 1), C. freundii (n = 4), E. coli (n = 15), E. cloacae (n = 12), K. oxytoca (n = 3), K. pneumoniae (n = 42) and S. marcescens (n = 3).

Susceptibility testing

All susceptibility tests were carried out from the same inoculum preparation. Agar dilution was performed according to CLSI (16). Mueller-Hinton agar plates (Oxoid) containing 25 mg/L glucose-6-phosphate and fosfomycin in concentrations from 0.25 mg/L to 1024 mg/L were prepared, an inoculum of 10⁴ cfu was placed onto the agar plate and allowed to dry. Plates were incubated for 16-20 h in ambient air at 35°C.

Disk diffusion was performed according to EUCAST on Mueller-Hinton agar (Oxoid). Disks containing fosfomycin 50 µg (Oxoid) and fosfomycin 200 µg (Oxoid) were used. For the determination of inhibition zone diameters scattered colonies were taken into account if they were either in the margin of the inhibition zone or in a density of >5 colonies per cm². CLSI disk diffusion breakpoints are only available for E. coli urinary tract isolates. EUCAST disk diffusion breakpoints are in preparation. Therefore we applied the breakpoints proposed for disk diffusion by Lu et al. (17) for the 200 µg fosfomycin disk (S: ≥ 14 mm; R: < 14 mm) as well as the breakpoints proposed by Pasteran et al. (18) for the 200 µg fosfomycin disk (S: ≥ 17 mm; R: < 17 mm) and for the 50 µg fosfomycin disk (S: ≥ 15 mm; R: < 15 mm). We also tried to find an appropriate breakpoint by error minimization.
Fosfomycin Etest (bioMerieux) was placed on Mueller-Hinton agar (Oxoid) according to manufacturer's instructions. For the determination of the crossing point of the ellipsis with the strip scattered colonies were taken into account if they were either in the margin of the ellipse or in a density of >5 colonies per cm². For quality control E. coli ATCC 25922, S. aureus ATCC 29213 and P. aeruginosa ATCC 27853 were used. The results fell always within the ranges given by EUCAST and CLSI.

Definitions and data analyses
Agar dilution was considered the reference method in our study. MICs determined by Etest were rounded up to a standard twofold agar dilution scale. Essential agreement (EA) was defined as agreement of MIC measurements within one dilution step. Strains with MIC results which fell within the test range of the reference method and had an opportunity for a result on the Etest method that could be on-scale were considered evaluable as proposed by the U.S. Food and Drug Administration (19). Categorical agreement (CA) was defined as susceptible and resistant results that matched between the methods based on the interpretative breakpoint proposed by EUCAST. False susceptible results were considered as very major errors (VME) and false resistant results as major errors (ME), respectively. All analyses were performed using R (http://www.R-project.org). The Wilcoxon rank-sum test was used for comparison of MIC distributions and statistical significance was established at P < 0.05. 95% confidence intervals of proportions were calculated according the Pearson-Klopper method. Agreement between agar dilution and Etest was evaluated by a Bland-Altman plot (20). Briefly, the mean MIC values obtained with the two methods were plotted against the difference between the log2-transformed MIC values. Values for Kappa coefficient, which gives a measure of the percentage of agreement between categorical results of susceptible testing methods, were interpreted.
according to Landis and Koch classification (21).

Results

By agar dilution as reference method a MIC range between $\leq 0.25$ mg/L and $> 1024$ mg/L, a MIC$_{50}$ of 8 mg/L and a MIC$_{90}$ of 512 mg/L was found. Applying EUCAST criteria (S: $\leq 32$ mg/L; R: $> 32$ mg/L) 30 out of 107 strains (28%; 95% CI: 19.8% to 37.5%) could be classified as resistant. Using CLSI criteria for fosfomycin-tromethamin for oral treatment of urinary tract infections caused by *E. coli* (S: $\leq 64$ mg/L) 21 out of 107 strains (19.6%; 95% CI: 12.6% to 28.4%) would be classified as resistant. Considering the three most frequent species in our study *E. coli* was resistant in four cases (16.7%) (Table 1) and has lower MICs than *K. pneumoniae* (Wilcoxon test: $P < 0.001$) and *E. cloacae* (Wilcoxon test: $P = 0.007$).

Sporadic colonies within the Etest ellipse, which were ignored for reading of the MIC, were seen in 41.1%. The occurrence of sporadic colonies did not statistically differ between species. The Bland-Altman analysis (Figure 1) revealed large limits of agreement between -2.9 and +2.6 of the difference of the log2-transformed data of both measurements. EA between agar dilution and Etest among the 90 evaluable strains was found in 71 strains (78.9%; 95% CI: 69.0% to 86.8%). EA among all strains was found in 88 strains (82.2%; 95% CI: 73.7% to 89.0%). CA between both methods was found in 99 isolates (92.5%; 95% CI: 85.8% to 96.7%) with a Kappa value of 0.807 (95% CI: 0.68 to 0.94), which indicates a substantial to almost perfect agreement. Compared to agar dilution as reference method six VMEs (20.0%; 95% CI: 7.7% to 38.6%) and two MEs (2.6%; 95% CI: 0.3% to 9.1%) were observed.

By ROC analysis of false-susceptible and true-susceptible results based on various cut-off values for inhibition zone diameters an AUC of 0.981 and 0.976 was calculated for the
Applying the breakpoint proposed by Lu et al. (17) for the 200 µg fosfomycin disk nine VMEs (30%; 95% CI: 14.7% to 49.4%) but no ME were found (Figure 2). When using the breakpoint suggested by Pasteran et al. (18) for the 200 µg fosfomycin disk we observed five VMEs (16.7%; 95% CI: 5.6% to 34.7%) and four MEs (5.2%; 95% CI: 1.4% to 12.8%). Applying the breakpoint suggested by Pasteran et al. (18) for the 50 µg fosfomycin disk we found four VMEs (13.3%; 95% CI: 3.8% to 30.7%) and four MEs (5.2%; 95% CI: 1.4% to 12.8%). The lowest cutoff not resulting in VMEs was \( \geq 19 \) mm for the 50 µg fosfomycin disk and \( \geq 20 \) mm for the 200 µg fosfomycin disk, however with 26.0% (95% CI: 16.6% to 37.2%) and 27.3% (95% CI: 17.7% to 38.6%) MEs, respectively (Figures 2 and 3).

**Discussion**

In a selection of multidrug-resistant *Enterobacteriaceae* producing nine different carbapenemases we found fosfomycin to be susceptible in 72% of strains according to agar dilution, the widely accepted reference method for fosfomycin susceptibility testing (17, 22, 7). These data are in a similar range to those reported by others: Falagas et al. (23) found 84.8% of the isolates to have an MIC for fosfomycin \( \leq 32 \) mg/L using Etest in a strain collection without characterization of its carbapenemase types, but which can be assumed to be predominantly VIM and KPC as the strains were collected between 2007 and 2009 in Greece. Livermore et al. (24) detected fosfomycin MICs \( \leq 32 \) mg/L in 66.7% of *Enterobacteriaceae* isolates producing several different carbapenemases using agar dilution. In the study by Endimiani et al. (22) 75% of the KPC-producing *K. pneumoniae* showed an MIC for fosfomycin \( \leq 32 \) mg/L. In a strain collection comprising mainly KPC-producing *Enterobacteriaceae* 86.7% with an MIC of \( \leq 32 \) mg/L for fosfomycin were found (18).
In our study *E. coli* strains had significantly lower fosfomycin MICs than *K. pneumoniae* and *E. cloacae* strains, which is in accordance with previous reports (17, 23–25). As treatment options in case of infections due to carbapenemase producing *Enterobacteriaceae* are severely limited fosfomycin therapy has been considered for infections caused by multidrug-resistant *Enterobacteriaceae* (4, 26, 27). Monitoring fosfomycin resistance is important since resistance is not only caused by chromosomal mutations but can also be mediated by plasmid encoded mechanisms which have been mainly reported from Asian countries so far (28).

Scattered colonies within inhibition zones in disk diffusion or the ellipsoid inhibition zone in Etest were frequently found (41.1% of strains), which complicated reading of the tests. According to the EUCAST disk diffusion reading guide colonies that are not contaminations should be taken into account. However, as it can be assumed that single scattered colonies within the fosfomycin inhibition zone are due to resistance conferring mutations and the location of colonies within the inhibition zone is mainly determined by chance, this reading guide would lead to non-reproducible results for fosfomycin susceptibility testing. In order to overcome these difficulties we used a different approach for reading MICs and inhibition zone diameters, respectively. Our approach was supported by the fact that even for the quality control strain *E. coli* ATCC 25922 single colonies could be observed and Etest MICs using our reading approach perfectly fell into the limits of quality control. In addition, we observed several strains with scattered colonies in the Etest ellipse despite susceptibility as judged by agar dilution as reference method. A similar approach for reading disk diffusion has been proposed by others (18, 29). It can be argued that also from a clinical point of view it might be appropriate to ignore a certain subpopulation of fosfomycin resistant mutants when reading susceptibility tests because intravenous fosfomycin is mostly used as combination therapy for infections caused by...
multidrug-resistant Enterobacteriaceae (5) and combination therapy has been shown to prevent the development of fosfomycin resistance (30). It is an open question, which fosfomycin susceptibility testing method predicts treatment outcome best. The absolute number of bacteria exposed to the antibiotic in the inhibited area around the Etest strip is possibly larger than the number of $10^4$ cfu of bacteria placed on single spots during agar dilution. Therefore it can be assumed, that a substantial proportion of fosfomycin resistant mutants are systematically overlooked in agar dilution compared to Etest and vice versa. It is not known, however, if these resistant mutants correlate with therapeutic failure, since the fitness of fosfomycin resistant mutants observed in vitro has been questioned (31).

The correlation between MICs determined by agar dilution as reference method and Etest is inadequate as demonstrated by large limits of agreement in the Bland-Altman analysis covering more than two dilution steps. EA of only 78.9% is far lower than the value of 96.67% proposed by the FDA for this sample size. In addition the rate of VMEs of 20.0% is not acceptable. Out of the six isolates with VMEs no scattered colony was visible in the Etest ellipse in three strains and these strains showed Etest MICs of 24 mg/L, 32 mg/L and 24 mg/L, respectively. Even if we had used the strictest criteria for reading of the Etest, this would result in an unacceptably high rate of 10.0% VMEs, far exceeding the limit proposed by the FDA. Five of the six isolates with VMEs were K. pneumoniae, but they all differed regarding their carbapenemases: one strain was carbapenemase negative and its carbapenem-resistance was most likely explained by porin deficiency combined with CTX-M-15 expression. The other strains harbored genes coding for OXA-48, KPC-2 combined with VIM-1, OXA-162 and KPC-2, respectively. In view of this heterogeneity we have no evidence that the high VME rate might have been influenced by overrepresentation of certain clones.

EUCAST breakpoints for disk diffusion are in preparation, but not available at the moment.
CLSI breakpoints only apply for oral therapy of urinary tract infections in *E. coli*. Therefore, Lu et al. recently proposed new disk diffusion breakpoints for the 200 µg fosfomycin disk (17). Using this threshold of ≥ 14 mm for a susceptible result we found an unacceptably high VME rate of 30% which is in contrast to the lower value of 3.7% VME reported by Lu et al. (17). However, the reported value was calculated as proportion of VMEs in relation to all investigated strains. Using the widely accepted definition of VME rate with the number of resistant strains as denominator as also done in this study the VME rate would have been 25.6%. Interestingly, the strains with VMEs using the threshold of ≥ 14 mm for a susceptible result included all the strains with VMEs using Etest. Again, the heterogeneity of their carbapenemase content argues against a single clone as cause of the high VME rate.

The breakpoints of ≥ 17 mm for the 200 µg fosfomycin disk and ≥ 15 mm for the 50 µg fosfomycin disk as proposed by Pasteran et al. (18) would result in VME rates of 16.7% and 13.3%, respectively, in our collection. By error minimization analysis we were not able to find a fosfomycin zone breakpoint, that could reduce errors to an acceptable level of <1.5% ME and <1% VME. The lowest cutoffs resulting in the absence of VMEs in our study were ≥ 19 mm for the 50 µg fosfomycin disk and ≥ 20 mm for the 200 µg fosfomycin disk, respectively. However, this would lead to unacceptably high MEs exceeding 26%.

Therefore we conclude, that disk diffusion is not an appropriate method for fosfomycin susceptibility testing. At most disk diffusion can be used to determine, for which isolates susceptibility testing with an alternative test method does not make sense because of a high likelihood of resistance (zone diameter <10 mm) or susceptibility (zone diameter ≥ 20 mm).

A limitation of our study is, that it can be debated if agar dilution is the appropriate reference method. No fosfomycin susceptibility testing method including agar dilution has
ever been correlated with clinical outcome. However, this holds true also for many other
antimicrobial agents. Susceptibility testing methods for fosfomycin can be difficult to
interpret due to a high rate of chromosomal mutations leading to single resistant colonies. 
Due to the small area onto which the inoculum is applied when performing agar dilution
resistant subpopulations might be taken into consideration more often than in Etest or disk
diffusion. In this work agar dilution was chosen because it is widely accepted as reference
method and in order to facilitate comparability to other studies. The resistant strains should
be tested for the presence of plasmid-mediated mechanisms for fosfomycin resistance in a
future study.

In conclusion, a considerable proportion of 72% of multidrug-resistant Enterobacteriaceae
with diverse resistance mechanisms including carbapenemase production were tested
susceptible to fosfomycin using agar dilution. The occurrence of scattered colonies within
inhibition zones complicates reading of Etest and disk diffusion results, which are also in
poor agreement to agar dilution results.

Acknowledgements
The authors would like to thank Anja Kaminski, Susanne Friedrich, Doris Jaromin, Anke
Albrecht, Brigitte Hemmerle, Marion Schmidt for excellent technical assistance.

Funding
This work was supported by the Robert Koch-Institute with funds provided by the German
Ministry of Health (grant no. 1369-402) and by Infectopharm, Heppenheim, Germany.

Transparency declaration
M. K. has received speaker or consultancy fees or research grants from Amplex,
AstraZeneca, Bayer, Becton Dickinson, BioMérieux, BioRad, Bruker, Cepheid,
Infectopharm, MSD, Pfizer, Roche Diagnostics and Siemens Healthcare. S. G. G. has
received speaker fees by BioMérieux, MSD, Pfizer, Roche Diagnostics. F. S. has received speaker fees by Becton Dickinson. All other authors: none to declare.


Falagas ME, Maraki S, Karageorgopoulos DE, Kastoris AC, Mavromanolakis E,


Table 1: Fosfomycin susceptibility according to species

<table>
<thead>
<tr>
<th>species</th>
<th>MIC range (mg/L)</th>
<th>MIC₅₀ (mg/L)</th>
<th>MIC₉₀ (mg/L)</th>
<th>resistant [EUCAST] number (% and 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>all species (n = 107)</td>
<td>≤ 0.25, &gt; 1024</td>
<td>8</td>
<td>512</td>
<td>30ᵃ 28.0 (19.8 – 37.5)</td>
</tr>
<tr>
<td>K. pneumoniae (n = 50)</td>
<td>0.5, &gt; 1024</td>
<td>16</td>
<td>256</td>
<td>16 32.0 (19.5 – 46.7)</td>
</tr>
<tr>
<td>E. coli (n = 24)</td>
<td>≤ 0.25, 256</td>
<td>1</td>
<td>256</td>
<td>4 16.7 (4.7 – 37.4)</td>
</tr>
<tr>
<td>E. cloacae (n = 17)</td>
<td>0.5, &gt; 1024</td>
<td>16</td>
<td>512</td>
<td>4 23.5 (6.8 – 49.9)</td>
</tr>
</tbody>
</table>

ᵃ E. coli: carbapenemase negative (n = 1), KPC-3 (n = 1), VIM-1 (n = 1), OXA-48 (n = 1);
E. cloacae: VIM-1 (n = 3), OXA-48 (n = 1); K. oxytoca: carbapenemase negative (n = 2),
VIM-1 (n = 1); K. pneumoniae: carbapenemase negative (n = 4), KPC-2 (n = 3), KPC-2 and VIM-1 (n = 1), KPC-3 (n = 1), VIM-4 (n = 1), OXA-48 (n = 5), OXA-162 (n = 1);
P. mirabilis: carbapenemase negative (n = 1); S. marcescens: VIM-1 (n = 2)
Figure 1: Bland-Altman analysis of agreement between Etest and agar dilution

The differences of the log2-transformed MICs obtained by agar dilution and Etest are plotted against their mean MIC values. The solid line represents the mean of the differences, the dotted lines indicate the lower and upper limit of the 95% confidence interval. The dashed line represents the linear fit between mean MIC values and difference between log2-transformed MICs obtained with the two methods.
Figure 2: Scattergram of MICs versus zone diameters for the 200 µg fosfomycin disk
The horizontal dashed line represents the EUCAST breakpoint for fosfomycin. The vertical
dash-dot line indicates the breakpoint proposed by Lu et al. (17), the vertical dotted line
indicates the breakpoint proposed by Pasteran et al. (18)
Figure 3: Scattergram of MIC versus zone diameters for 50 µg fosfomycin disk
The horizontal dashed line represents the EUCAST breakpoint for fosfomycin. The vertical
dotted line indicates the breakpoint proposed by Pasteran et al. (18)
ERRATUM

Fosfomycin Susceptibility in Carbapenem-Resistant Enterobacteriaceae from Germany

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Volume 52, no. 6, p. 1893–1897, 2014. Page 1893, abstract, line 4: “78% of the strains had fosfomycin MICs of ≤32 mg/liter” should read “72% of the strains had fosfomycin MICs of ≤32 mg/liter.”