Fosfomycin Susceptibility in Carbapenem-Resistant Enterobacteriaceae from Germany

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Due to the increase in multidrug-resistant Enterobacteriaceae, the interest in older antimicrobial agents, like fosfomycin, has increased. In this study, we used agar dilution for testing susceptibilities to fosfomycin in a collection of 107 carbapenem-non-susceptible Enterobacteriaceae isolates, of which 80 produced various types of carbapenemases, including KPC, VIM, NDM, and OXA-48. Overall, 78% of the strains had fosfomycin MICs of ≤32 mg/liter and were thus considered to be susceptible according to the current EUCAST breakpoint. The MIC50 and MIC90 were 8 mg/liter and 512 mg/liter, respectively. Escherichia coli strains had significantly lower fosfomycin MICs than the Klebsiella pneumoniae and Enterobacter cloacae strains. Furthermore, comparisons of the susceptibility testing methods, like Etest and disk diffusion, were performed against agar dilution as the reference method. Essential agreement between Etest and agar dilution was 78.9%, and categorical agreement between the two 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 that reduced very major and major errors to an acceptable level was found. Etest and disk diffusion showed poor agreement with fosfomycin agar dilution.

Multidrug resistance in Enterobacteriaceae is an ever-increasing problem worldwide. 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, and class D carbapenemases, like OXA-48 (2).

Only a few antimicrobial agents with clinically significant activities 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 a single-dose oral form (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 form, it is usually combined with other antimicrobials (4). Fosfomycin inhibits the N-acetylgalactosamine-3-O-enolpyruvyl transferase, which catalyzes the conversion of UDP-N-acetlyglucosamine 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 to exist, the t-o-glycerophosphate system and the hexose monophosphate route. The hexose monophosphate route 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, plasmid-mediated mechanisms of fosfomycin resistance have also been described, which involve the expression of enzymes capable of modifying fosfomycin by adding 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.

MATERIALS AND METHODS

Strains. Previously characterized nonropy strains (n = 107) referred to our reference laboratory because of resistance to ertapenem, imipenem, or meropenem were used for the study and included strains of 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 freundii (n = 1). The strains were isolated between August 2009 and February 2011 in 48 laboratories from locations all over Germany. The main sources of isolation were urine (n = 24), lower airway specimens (n = 23), and wound swabs (n = 14). Species identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). The strains were found to be resistant to ertapenem, imipenem, or meropenem by use of disk diffusion according to EUCAST guidelines. For carbapenemase detection, a modified Hodge test (7), as well as combined disk tests with EDTA (8) and boronic acid (9), were carried out. In addition, PCRs for KPC (10), VIM (8, 11), IMP (8), NDM (12), and OXA-48 (13) were performed routinely. If the phenotypic tests suggested a carbapenemase that was not detected by PCR, the isolates were 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 was excluded.
and elevated MICs for carbapenems were most likely caused by porin deficiency combined with the expression of an extended-spectrum β-lactamase or AmpC-β-lactamase. The remaining 80 carbapenemase producers were OXA-48 (n = 24), VIM-1 (n = 15), KPC-2 (n = 12), 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 coproduced VIM-1 and KPC-2. Carbapenemases were found in the species C. farneri (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: VIM-1 (n = 2).

Susceptibility testing. All susceptibility tests were carried out from the same inoculum preparation. Agar dilution was performed according to CLSI guidelines (16). Mueller-Hinton agar plates (Oxoid) containing 25 mg/liter glucose-6-phosphate and fosfomycin in concentrations from 0.25 mg/liter to 1.024 mg/liter were prepared, and an inoculum of 10⁴ CFU was placed onto the agar plate and allowed to dry. The plates were incubated for 16 to 20 h in ambient air at 35°C.

Disk diffusion was performed according to EUCAST guidelines on Mueller-Hinton agar (Oxoid). Disks containing 50 µg fosfomycin (Oxoid) and 200 µg fosfomycin (Oxoid) were used. To determine the 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. The CLSI disk diffusion breakpoints were available for E. coli urinary tract isolates only. The 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 (susceptible, ≥14 mm; resistant, <14 mm) and the breakpoints proposed by Pasteran et al. (18) for the 200-µg fosfomycin disk (susceptible, ≥17 mm; resistant, <17 mm) and for the 50-µg fosfomycin disk (susceptible, ≥15 mm; resistant, <15 mm). We also tried to find an appropriate breakpoint by error minimization analysis.

For the fosfomycin Etest (bioMérieux), the Etest strip was placed on Mueller-Hinton agar (Oxoid) according to the manufacturer’s instructions. To determine the crossing point of the ellipse with the strip, scattered colonies were taken into account if they were either in the margin of the ellipse or had a density of >5 colonies per cm². For quality control, E. coli strain ATCC 25922, Staphylococcus aureus strain ATCC 29213, and Pseudomonas aeruginosa strain ATCC 27853 were used. The results always fell within the ranges given by EUCAST and CLSI.

Definitions and data analyses. In our study, agar dilution was used as the reference method. The MICs determined by Etest were rounded up to MIC values (mg/liter) according the Pearson-Klopper method. Agreement between agar dilution and Etest was evaluated by a Bland-Altman plot (20). The mean MIC values obtained with the two methods were plotted against the difference between the log₂-transformed MIC values. Values for the kappa coefficient, which gives a measure of the percentage of agreement between the categorical results of susceptibility testing methods, were interpreted according to classifications by Landis and Koch (21).

### RESULTS

Using agar dilution as the reference method, an MIC range between ≤0.25 mg/liter and >1.024 mg/liter, an MIC₉₀ of 8 mg/liter, and an MIC₉₀ of 512 mg/liter were found. Applying the EUCAST criteria (susceptible, ≤32 mg/liter; resistant, >32 mg/liter), 30 out of 107 strains (28%; 95% confidence interval [CI], 19.8% to 37.5%) were classified as resistant. Using the CLSI criteria for fosfomycin-tromethamine oral treatment of urinary tract infections caused by E. coli (susceptible, ≤64 mg/liter; resistant, >32 mg/liter), 21 out of 107 strains (19.6%; 95% CI, 12.6% to 28.4%) were classified as resistant. Considering the three most frequent species in our study, E. coli was resistant in four cases (16.7%) (Table 1) and had lower MICs than K. pneumoniae (Wilcoxon test, P < 0.001) and E. cloacae (Wilcoxon test, P = 0.007).

**Table 1** Fosfomycin susceptibility according to species

<table>
<thead>
<tr>
<th>Species (no. of isolates)</th>
<th>MIC data (mg/liter)</th>
<th>Resistance (per EUCAST criteria)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>MIC₉₀</td>
</tr>
<tr>
<td>All species (107)</td>
<td>≤0.25 to &gt;1.024</td>
<td>8</td>
</tr>
<tr>
<td>K. pneumoniae (50)</td>
<td>0.5 to &gt;1.024</td>
<td>16</td>
</tr>
<tr>
<td>E. coli (24)</td>
<td>≤0.25 to 256</td>
<td>1</td>
</tr>
<tr>
<td>E. cloacae (17)</td>
<td>0.5 to &gt;1.024</td>
<td>16</td>
</tr>
</tbody>
</table>

³ CI, confidence interval.

### DISCUSSION

The occurrence of sporadic colonies did not statistically differ between species. The Bland-Altman analysis (Fig. 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 the agar dilution and Etest methods 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%). EA between the two 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. Against agar dilution as the 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.

Using receiver operating characteristic (ROC) analysis of false-susceptible and true-susceptible results based on various cutoff values for inhibition zone diameters, area under the concentration-time curve (AUC) values of 0.981 and 0.976 were calculated for the 50-µg fosfomycin disk and 200-µg fosfomycin disk, respectively.

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 MEs were found (Fig. 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 19 mm for the 50-μg fosfomycin disk and ≥20 mm for the 200-μg fosfomycin disk, but there were still 26.0% (95% CI, 16.6% to 37.2%) and 27.3% (95% CI, 17.7% to 38.6%) MEs, respectively (Fig. 2 and 3).

**DISCUSSION**

In a selection of multidrug-resistant *Enterobacteriaceae* producing nine different carbapenemases, we found fosfomycin to be susceptible in 72% of the strains, according to agar dilution, the widely accepted reference method for fosfomycin susceptibility testing (7, 17, 23). These data are in a range similar to those reported by others; in Falagas et al. (23), 84.8% of the isolates were found to have an MIC for fosfomycin of ≤32 mg/liter using Etest in a strain collection without characterized carbapenemase types, but which can be assumed to be predominantly VIM and KPC, as the strains were collected between 2007 and 2009 in Greece. In Livermore et al. (24) fosfomycin MICs of ≤32 mg/liter were detected in 66.7% of the *Enterobacteriaceae* isolates producing several different carbapenemases, using agar dilution. In the study by Endimiani et al. (22), 75% of the KPC-producing *K. pneumoniae* isolates showed an MIC for fosfomycin of ≤32 mg/liter. In a strain collection comprising mainly KPC-producing *Enterobacteriaceae*, MICs of ≤32 mg/liter for fosfomycin were found in 86.7% of the isolates (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 the treatment options for 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 mechanisms carried on plasmids, which have been mainly reported so far in Asian countries (28).

Scattered colonies within the inhibition zones in disk diffusion or the ellipsoid inhibition zone in Etest were frequently found (41.1% of strains), which complicated the 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 nonreproducible results for fosfomycin susceptibility testing. In order to overcome these difficulties, we used a different approach for reading MICs and inhibition zone diameters. Our approach was supported by the fact that even for the quality-control strain \textit{E. coli} ATCC 25922, single colonies were observed, and the Etest MICs using our reading approach fell perfectly within the limits of quality control. In addition, we observed several strains with scattered colonies in the Etest ellipse, despite their susceptibility as judged by agar dilution as the 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 in combination therapy for infections caused by multidrug-resistant \textit{Enterobacteriaceae} (3), and combination therapy has been shown to prevent the development of fosfomycin resistance (30). It is an open question as to which fosfomycin susceptibility testing method best predicts treatment outcome. 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 the agar dilution method 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 the reference method and Etest is inadequate, as demonstrated by large limits of agreement in the Bland-Altman analysis covering more than 2 dilution steps. An 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 20.0% VME rate is not acceptable. Out of the six isolates with VMEs, no scattered colonies were visible in the Etest ellipse in three strains, and these strains showed Etest MICs of 24 mg/liter, 32 mg/liter, and 24 mg/liter. Even if we had used the strictest criteria for reading 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 from \textit{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. In view of this heterogeneity, we have no evidence that the high VME rate might have been influenced by the overrepresentation of certain clones.

EUCAST breakpoints for disk diffusion are in preparation and not available at the moment. The CLSI breakpoints only apply for oral therapy of urinary tract infections in \textit{E. coli}. Therefore, Lu et al. (17) recently proposed new disk diffusion breakpoints for the 200-µg fosfomycin disk. Using this threshold of $\geq 14$ 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 a proportion of VMEs in relation to all investigated strains. Using the widely accepted definition of the VME rate, with the number of resistant strains as the denominator, as was also done in this study, the VME rate would have been 25.6%. Interestingly, in those strains with VMEs, using the threshold of $\geq 14$ 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 the cause of the high VME rate.

The breakpoints of $\geq 17$ mm for the 200-µg fosfomycin disk and $\geq 15$ 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 reduced errors to an acceptable level of $< 1.5\%$ for MEs and $< 1\%$ for VMEs. The lowest cutoffs resulting in the absence of VMEs in our study were $\geq 19$ mm for the 50-µg fosfomycin disk and $\geq 20$ mm for the 200-µg fosfomycin disk. However, this would lead to unacceptably high MEs of $> 26\%$. 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, $\geq 20$ mm).

A limitation of our study is that it is debatable whether 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 a reference method and facilitates comparisons with the findings of other studies. The resistant strains should be tested in a future study for the presence of plasmid-mediated mechanisms for fosfomycin resistance.

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

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REFERENCES


