Susceptibility of ESBL Escherichia coli and Klebsiella pneumoniae to fosfomycin in the Netherlands and comparison of several testing methods including Etest, MIC test strip, Vitek2, Phoenix and disc diffusion

Wouter van den Bijllaardt1,2*, Maarten J. Schijffelen3, Ron W. Bosboom4, James Cohen Stuart5, Bram Diederen6, Greetje Kampinga7, Thuy-Nga Le8, Ilse Overdevest9, Frans Stals10, Paul Voorn11, Karola Waar12, Johan W. Mouton2 and Anouk E. Muller2,13

1Microvida Laboratory for Microbiology, Amphia Hospital, Breda, The Netherlands; 2Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands; 3Laboratory for Medical Microbiology and Public Health, Hengelo, The Netherlands; 4Laboratory for Medical Microbiology and Immunology, Rijnstate Hospital, Arnhem, The Netherlands; 5Department of Medical Microbiology, Medisch Centrum Alkmaar, Alkmaar, The Netherlands; 6Microvida Laboratory for Microbiology, Bravis Hospital, Roosendaal, The Netherlands; 7Department of Medical Microbiology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands; 8Department of Microbiology, Geldersche Vallei Hospital, Ede, The Netherlands; 9Laboratory for Medical Microbiology, Stichting PAMM, Veldhoven, The Netherlands; 10Department of Medical Microbiology, Zuyderland Medical Centre, Heerlen, The Netherlands; 11Department of Medical Microbiology and Immunology, St Antonius Hospital, Nieuwegein, The Netherlands; 12Centre for Infectious Diseases Friesland, Izore, Leeuwarden, The Netherlands; 13Department of Medical Microbiology, Haaglanden Medical Centre, The Hague, The Netherlands

*Corresponding author. Tel: 0031-76-595-3015; Fax: 0031-76-595-3820; E-mail: wvandenbijl@gmail.com orcid.org/0000-0003-0676-2594

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Objectives: Fosfomycin susceptibility testing is complicated and prone to error. Before using fosfomycin widely in patients with serious infections, acquisition of WT distribution data and reliable susceptibility testing methods are crucial. In this study, the performance of five methods for fosfomycin testing in the routine laboratory against the reference method was evaluated.

Methods: Ten laboratories collected up to 100 ESBL-producing isolates each (80 Escherichia coli and 20 Klebsiella pneumoniae). Isolates were tested using Etest, MIC test strip (MTS), Vitek2, Phoenix and disc diffusion. Agar dilution was performed as the reference method in a central laboratory. Epidemiological cut-off values (ECOFFs) were determined for each species and susceptibility and error rates were calculated.

Results: In total, 775 E. coli and 201 K. pneumoniae isolates were tested by agar dilution. The ECOFF was 2 mg/L for E. coli and 64 mg/L for K. pneumoniae. Susceptibility rates based on the EUCAST breakpoint of ≤32 mg/L were 95.9% for E. coli and 87.6% for K. pneumoniae. Despite high categorical agreement rates for all methods, notably in E. coli, none of the alternative antimicrobial susceptibility testing methods performed satisfactorily. Due to poor detection of resistant isolates, very high error rates of 23.3% (Etest), 18.5% (MTS), 18.8% (Vitek2), 12.5% (Phoenix) and 12.9% (disc diffusion) for E. coli and 22.7% (Etest and MTS), 16.0% (Vitek2) and 12% (Phoenix) for K. pneumoniae were found. None of the methods adequately differentiated between WT and non-WT populations.

Conclusions: Overall, it was concluded that none of the test methods is suitable as an alternative to agar dilution in the routine laboratory.

Introduction

Infections caused by highly resistant Enterobacteriaceae continue to place a major burden on healthcare systems. Carbapenem antibiotics are increasingly used for infections caused by these microorganisms. As the use of carbapenems acts as driver for carbapenem resistance by selective pressure, alternative treatment options are necessary.1,2 Fosfomycin is a relatively old, broad-spectrum antibiotic that was first marketed in the early 1970s. Recently, interest in the use of intravenous fosfomycin has
increased as this drug appears to remain active against a broad range of otherwise highly resistant microorganisms.\(^3\)\(^-\)\(^7\)

Fosfomycin susceptibility rates are reported to be high; even in ESBL-producing *Escherichia coli*, most reported MIC\(_{50}\) values are 0.5–1 mg/L. *Klebsiella pneumoniae* is less susceptible to fosfomycin, with reported MIC\(_{50}\) values ranging between 16 and 32 mg/L.\(^6\)\(^-\)\(^10\) Based on the EUCAST susceptibility breakpoint of 32 mg/L, many *K. pneumoniae* isolates will be borderline susceptible or resistant.\(^11\)

Fosfomycin susceptibility testing is complicated and prone to error, and data on WT distributions of Enterobacteriaceae are scarce. Due to the rapid development of resistant mutants in vitro,\(^11\) resulting in isolated colonies within the disc diffusion or gradient strip inhibition zones, the way susceptibility tests should be interpreted remains uncertain.\(^13\) With fosfomycin now being used to treat life-threatening infections rather than uncomplicated urinary tract infections, reliable susceptibility testing is becoming crucial. As no large-scale evaluations of automated methods are available, a study was conducted to compare commonly used antimicrobial susceptibility testing methods for fosfomycin susceptibility testing including Vitek2, Phoenix, Etest, MIC test strip (MTS) and disc diffusion, using the agar dilution method as the reference method.\(^14\) We also determined the MIC distributions of fosfomycin in clinical ESBL-producing *E. coli* and *K. pneumoniae* as determined by the agar dilution method.

**Materials and methods**

**Isolate collection**

Ten participating microbiology laboratories equally distributed throughout the Netherlands retrospectively and randomly collected up to 80 ESBL-positive *E. coli* and 20 *K. pneumoniae* isolates. The previously characterized and stored selection contained a maximum of one isolate per species per patient, and isolates were derived from urine, blood or respiratory tract during the year 2016 or 2017.

**Susceptibility testing**

In each laboratory, fosfomycin susceptibility was retested from a single bacterial solution by Etest (bioMérieux, France), MTS (Liofilchem, Italy), disc diffusion (Oxoid Ltd/Thermo Fisher Scientific, UK) and Vitek2 (bioMérieux, France) and/or the Phoenix platform (BD Diagnostics, MD, USA), depending on the laboratory routine platform. Because only one laboratory routinely utilized the Phoenix platform, additional susceptibility testing was performed at a later stage by a single laboratory on a random selection of isolates to eventually obtain Phoenix antimicrobial susceptibility testing results of 200 *E. coli* and 200 *K. pneumoniae* isolates. All isolates were retested by the combination disc diffusion test (CDT) with cefotaxime ± clavulanic acid and ceftazidime ± clavulanic acid to confirm the ESBL status.\(^15\) In the study reference centre, all isolates were tested by the reference agar dilution method. ISO guidelines were followed for agar dilution (ISO standard 20776-1) and the new EUCAST recommendations for *E. coli* were followed for disc diffusion.\(^11\)

For all methods, after thawing and 24 h subculture on blood agar, colonies were picked and suspended in saline to obtain a suspension with a turbidity equivalent to that of a 0.5 McFarland standard. For agar dilution, 2 μL of a 10-fold dilution of this suspension (≈10\(^7\) CFU/mL) was pipetted on Mueller–Hinton II agar plates (≈10\(^5\) CFU/spot of 5–8 mm) (BBB, BD Diagnostics, MD, USA) containing 25 mg/L glucose-6-phosphate (G6P) (Sigma, Germany) and fosfomycin (Sigma, Germany) following CLSI recommendations in a concentration range of 0.25–128 mg/L.\(^16\) Positive and negative controls were included. For disc diffusion and gradient strip methods, Mueller–Hinton II agar plates were streaked with a 0.5 McFarland bacterial suspension to reach confluent growth and subsequently Etest, MTS or discs containing 200 μg of fosfomycin + 50 μg of G6P were placed. Plates were incubated at 34–37°C in ambient air and read after 16–20 h. For gradient strip methods and disc diffusion, isolated colonies within the inhibition zone were ignored. *E. coli* ATCC 25922 was used as the reference strain and was tested in every experiment.

**Interpretation of results**

For methods yielding MICs as the result the susceptibility breakpoint as set by EUCAST for fosfomycin was used: MIC ≤32 mg/L, susceptible; and MIC >32 mg/L, resistant. For *E. coli* the zone diameters were interpreted using the 2018 EUCAST disc diffusion breakpoints: zone diameter ≥24 mm, susceptible; zone diameter <24 mm, resistant.\(^13\) For *K. pneumoniae* these interpretive criteria do not apply, and therefore a tentative breakpoint was set (see the Results section).

**Evaluation**

Susceptibility categorization by the different methods was compared with results of the agar dilution method as the gold standard. Essential agreement for gradient strips was assumed when MICs of the test method were within ±1 dilution of the reference method.\(^17\) Categorical agreement was calculated, and discrepancies were referred to as very major error (VME; test method susceptible, agar dilution resistant) or major error (ME; test method resistant, agar dilution susceptible). The VME rate and ME rate were calculated by dividing the number of errors by the total number of resistant or susceptible isolates, respectively. The epidemiological cut-off value (ECOFF) was determined using the ECOFF finder.\(^18\) The correlation between agar dilution and disc diffusion results was evaluated by calculation of the Pearson’s correlation coefficient. All isolates that exhibited a VME or ME were retested by agar dilution.

**Results**

**Isolates**

The central laboratory received a total of 976 isolates for agar dilution testing, of which 775 (79.4%) were *E. coli* and 201 (20.6%) were *K. pneumoniae*. The locally performed CDT confirmed ESBL in 947 (97.0%) isolates. Antimicrobial susceptibility testing results were available for 99.5%, 98.9%, 99.9%, 41.9% and 99.6% of the isolates for Ettest, MTS, Vitek2, Phoenix and disc diffusion, respectively.

**Epidemiological cut-off and susceptibility results**

The MIC distribution for both species is shown in Figure 1. The MICs of fosfomycin were far lower for *E. coli* than for *K. pneumoniae*. Using the ECOFF finder, the ECOFF was determined at 2 mg/L for *E. coli* and 64 mg/L for *K. pneumoniae*. This coincided reasonably well with the eyeball method, although 4 mg/L for *E. coli* would also have been an option. The mode MIC for *E. coli* was 1 mg/L (MIC\(_{50/90}\) 1/2 mg/L) compared with 16 mg/L (MIC\(_{50/90}\) ≥128 mg/L) for *K. pneumoniae*. Susceptibility to fosfomycin was high when current breakpoints were applied: 95.9% of *E. coli* and 87.6% of *K. pneumoniae* had an MIC ≤32 mg/L.

**Comparison of ‘MIC’ methods**

For *E. coli*, essential agreement between Etest and the reference method and MTS and the reference method was 91% and 77%,
respectively. For *K. pneumoniae* this was 86% and 80%. Resistant isolates were poorly detected by all test methods, resulting in VME rates ranging between 12.0% (*K. pneumoniae* tested with Phoenix) and 23.3% (*E. coli* tested with Etest). Nevertheless, categorical agreement rates for *E. coli* were high, because the majority of isolates were susceptible and most susceptible isolates were correctly categorized. For *K. pneumoniae* the categorical agreement rates were lower due to more false-resistant results. Agreement and error rates for all MIC methods are presented in Table 1. Retesting of all 66 isolates exhibiting any VMEs or MEs did not alter the categorical error results except for two VME isolates. These showed a minor MIC shift of one dilution from 64 to 32 mg/L, rendering the

![Figure 1. MIC distribution of tested *E. coli* (a) and *K. pneumoniae* (b) isolates. Isolates below the ECOFF (within the WT distribution) are shaded grey. ECOFF for *E. coli* = 2 mg/L and ECOFF for *K. pneumoniae* = 64 mg/L.](https://academic.oup.com/jac/article-abstract/73/9/2380/5047839)
isolates susceptible according to the reference method. Because the MICs for both these isolates were within 1 dilution step of each other, the first measurement was not refuted.

For four isolates all test methods resulted in a VME. For four and three isolates with MEs and VMEs, respectively, three test methods gave discordant results. For the remaining 55 categorical errors, only one or two test methods showed an ME or a VME.

**Ability of ‘MIC’ methods to differentiate WT from non-WT populations**

None of the tested methods can adequately differentiate between WT and non-WT isolates as defined by the ECOFF above (Table 2). For *E. coli*, no data are given for Vitek2 and Phoenix, because the fosfomycin test range in both methods lies far above the ECOFF of 2 mg/L.

**Disc diffusion results**

Colonies within the zone were seen in ~84% of all disc diffusion tests. According to the EUCAST reading guide for disc diffusion for *E. coli* versus fosfomycin (www.eucast.org), isolated colonies within the zone were ignored. There was no major difference between the percentage of *E. coli* (83%) and *K. pneumoniae* (87%) with colonies within the inhibition zone.

Agar dilution MICs exhibited a strong linear relationship with disc diffusion inhibition zones (Figure 2). The Pearson’s correlation coefficient between the methods was −0.72 for *E. coli* and −0.70 for *K. pneumoniae* (P < 0.0001). With the provided EUCAST disc diffusion breakpoints for *E. coli*, the categorical agreement, the ME rate and the VME rate were 98.4%, 1.1% and 12.9%, respectively. For *K. pneumoniae* no error rates can be calculated because no zone diameter breakpoints are set. Based on the distribution of MIC inhibition zones (Figure 3) a tentative disc diffusion breakpoint of susceptible >16 mm/resistant <16 mm for *K. pneumoniae* is suggested. This breakpoint would result in a categorical agreement rate, ME rate and VME rate of 96.0%, 1.7% and 22.7%, respectively.

However, clinical data supporting the current clinical breakpoints are absent. We therefore also determined the performance of disc diffusion to separate WT from non-WT based on our data. The disc diffusion breakpoints that separate the WT from the non-WT population are >23 mm for *E. coli* and >15 mm for *K. pneumoniae* (Figure 3). Based on these breakpoints, disc diffusion is an unreliable method for differentiation of these populations with VME rates of 61.3% for *E. coli* and 15.0% for *K. pneumoniae* (Table 2).

**Discussion**

This study was performed to determine fosfomycin activity against a large collection of two clinically important Enterobacteriaceae and to evaluate the performance of several alternative methods for susceptibility testing in the routine laboratory. MICs were
Figure 2. Plot of agar dilution MICs (mg/L) against disc diffusion zone diameters (mm). EUCAST susceptibility breakpoints for MICs and disc diffusion are presented as continuous lines and the ECOFF is presented as a broken vertical line. The tentative disc diffusion breakpoint for *K. pneumoniae* of susceptible $>16$ mm/resistant $<16$ mm is presented as a broken horizontal line. A total of 774 *E. coli* and 198 *K. pneumoniae* isolates were tested by both methods.

Figure 3. MIC–zone diameter correlations for *E. coli* (a) and *K. pneumoniae* (b) versus 200 μg of fosfomycin + 50 μg of G6P. MICs are represented as coloured bars and zone diameters are presented on the horizontal axis.
Fosfomycin susceptibility testing by various test methods

determined by agar dilution as the reference method. Fosfomycin presented activity against 95.9% of *E. coli* and 87.6% of *K. pneumoniae* isolates. Despite high categorical agreement rates for all methods, notably in *E. coli*, none of the alternative antimicrobial susceptibility testing methods performed satisfactorily due to poor detection of resistant isolates.

For *E. coli*, the lowest VME rate was 12.5% for Phoenix, whereas Etest showed the highest VME rate (23.3%). MICs or MIC-derived values as measured by Etest, MTS, Vitek2 and Phoenix in VME isolates ranged from the low side of the test range to just below the susceptibility breakpoint, which implies that even low MICs found by these methods cannot reliably predict susceptibility. Because most susceptible isolates were identified correctly by all methods and the number of susceptible isolates for out-numbers that of resistant isolates, the categorical agreement rate was high for all methods and obscures the low sensitivity of the test to identify resistant isolates. The categorical agreement rates for *K. pneumoniae* were lower due to more false-resistant isolates. To exclude the possibility of erroneous results, all VMEs and MEs were retested by agar dilution. Two isolates that gave a VME were retested as susceptible based on an MIC of 32 mg/L compared with 64 mg/L in the first measurement. This was considered to be biological variation, and therefore the reported VME and ME rates are based on the first measurements. All other categorical errors remained after retesting, indicating that results are reproducible.

Up to now there are no EUCAST zone diameter breakpoints for Enterobacteriaceae other than *E. coli*. In 2011, Lu et al. proposed the following disc diffusion breakpoints for *K. pneumoniae*: susceptible, ≥16 mm; intermediate, 14–15 mm; and resistant, <14 mm. The present study supports the use of a susceptibility breakpoint of ≥16 mm for *K. pneumoniae* although the addition of an intermediate category did not improve the performance of the disc diffusion method (data not shown). It should be noted though that the susceptibility breakpoint does identify the two separate populations, it does not indicate whether *K. pneumoniae* infections can be treated as this required pharmacokinetic and pharmacodynamic data as well as clinical evaluations. In a study by Hirsch et al. evaluating disc diffusion and Etest with agar dilution for various species, including 150 *E. coli* and 44 *K. pneumoniae*, the overall categorical agreement was higher for disc diffusion than for Etest. In contrast to the present study, the conclusion of the authors is that Etest performs well for *E. coli*, but confirms it is less effective for *K. pneumoniae*. No VMEs were found for both species, although it should be noted that no resistant isolates were included (using CLSI breakpoints). Three other studies comparing Etest and/or disc diffusion with agar dilution also reported poor performance of Etest and disc diffusion for susceptibility testing in *K. pneumoniae*. These three studies all suffer from the limitation of only having tested small numbers of resistant isolates. In the present study, ESBL-positive isolates were selected as these would be of primary interest for treatment with fosfomycin in order to spare the use of carbapenems. (Complicated) ESBL urinary tract infections are candidates for treatment with fosfomycin. For example, intravenous fosfomycin is currently being clinically evaluated against meropenem as an alternative for bacteriamic urinary tract infections caused by ESBL *E. coli*. Further well-designed studies are still needed to confirm the efficacy of fosfomycin in these settings. The few isolates that were not confirmed to be ESBL positive were not excluded from the analysis because the performance of the fosfomycin susceptibility tests was not expected to be dependent on ESBL status. Our finding of unacceptably high VME rates is in line with that from a study by Kaase et al. evaluating the performance of Etest and disc diffusion in carbapenem-resistant Enterobacteriaceae. Scattered colonies within the inhibition zone of disc diffusion or gradient strips due to mutations causing resistance complicates the reading of these tests. The clinical significance of resistant colonies in vitro is unclear because this resistance appears to be associated with significant biological costs. As 84% of disc diffusion tests showed colonies within the inhibition zone, the decision whether to acknowledge or ignore these colonies has a major impact on the results of susceptibility testing. Because the current EUCAST guidelines recommend ignoring distinct colonies, no data were recorded on inhibition zones when colonies would have not been ignored. CLSI has no recommendation on how to deal with isolated colonies.

Both automated methods performed better than the two gradient strip methods; for *K. pneumoniae* in particular an improvement in performance was observed. No other studies have been published comparing susceptibility testing results from Vitek2 and Phoenix with agar dilution. A major disadvantage of both automated methods is that the currently available MIC ranges do not include the ECOFF for *E. coli*.

Contrary to EUCAST breakpoints that apply to both intravenous and oral therapy, CLSI has published MIC and zone diameter breakpoints that only apply to *E. coli* urinary tract isolates. Applying those breakpoints for *E. coli* instead of EUCAST breakpoints would result in an increased VME rate for Phoenix of 37.5%, whilst the error rates for the other MIC-related test methods did not change significantly. For disc diffusion, CLSI recommends reading at complete inhibition. As EUCAST instructions were followed when reading the zone diameters, we could not evaluate the CLSI method of reading.

Based on the results of this study the activity of fosfomycin against ESBL-producing *E. coli* is high. Both the MIC90 of 2 mg/L and the ECOFF of 2 mg/L lie far below the susceptibility breakpoints as set by EUCAST and CLSI for oral treatment of uncomplicated urinary tract infections. As a clinical breakpoint should never intersect the WT population, and fosfomycin efficacy has never been convincingly demonstrated for the non-WT population, it is proposed that the susceptibility breakpoint should be lowered. Setting the breakpoint at 2 mg/L, identical to the ECOFF of *E. coli*, would further increase the VME rate.

Similar to the findings for *E. coli*, the ability of all tested methods to differentiate WT *K. pneumoniae* from the non-WT population is poor, limiting the potential use of gradient strips, automated methods and disc diffusion for this purpose. Most *K. pneumoniae* MICs are just below the current susceptibility breakpoint as set by EUCAST (susceptible ≤32 mg/L). As suggested by Ito et al., a reduced susceptibility to fosfomycin can be caused by chromosomally encoded fosA genes that are present in 99.7% of the evaluated genomes of *K. pneumoniae*. fosA is rarely encountered in *E. coli*. Resistance to fosfomycin can also arise through mutations in *glpT* or *uhpT* genes, which lower the uptake of fosfomycin through the loss of active transport mechanisms. Furthermore, mutations in the *murA* gene can result in reduced
susceptibility, either due to a lower affinity of fosfomycin for its binding target enzyme MurA, or to an overexpression of MurA. These may all have caused the poor performance of the test methods.

For both *E. coli* and *K. pneumoniae* the percentage of susceptible isolates is in line with previous reports of fosfomycin susceptibility in ESBL-producing isolates. A systematic review by Vardakas et al. showed 95.1% susceptibility of ESBL *E. coli* to fosfomycin and 83.8% susceptibility of ESBL *K. pneumoniae*. Although the susceptibility rate is high, the role of fosfomycin as a component of a combination therapy for the treatment of systemic infections with *K. pneumoniae* is still being evaluated.

This study has some limitations, including a limited fosfomycin concentration range in the agar dilution method. Because the highest concentration was 128 mg/L, it was not possible to differentiate isolates with very high MICs from isolates with MICs just above the breakpoint. Another limitation is that no carbapenem-producing isolates were included, although it is unlikely that the performance of the various methods depends on the type of β-lactamase present because fosfomycin resistance is not related to β-lactam resistance. It could be hypothesized that the specifications of the agar that is used for dilution methods such as Etest, MTS and disc diffusion influences the diffusion of antibiotics through the agar and may have had an impact on the error rates. However, this is true for all tests based on diffusion. In addition, fosfomycin is a relatively small molecule and major differences are not likely to be observed. Also, the VMEs were mostly due to significant differences in test results rather than small ones. The major strength of this study is the high number of isolates tested including fosfomycin-resistant isolates. In addition, to our knowledge, this is the first study that has evaluated automated methods for fosfomycin susceptibility testing for a large number of strains.

In summary, we found that susceptibility to fosfomycin is high in ESBL-producing *E. coli* and somewhat lower for *K. pneumoniae* isolates. Susceptibility testing methods other than the reference method are unreliable because of poor detection of clinically resistant isolates and a poor ability to differentiate between WT and non-WT populations.

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