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Susceptibility profile, resistance mechanisms & efficacy ratios of fosfomycin, nitrofurantoin & colistin for carbapenem-resistant *Enterobacteriaceae* causing urinary tract infections

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***Background & objectives*: The escalation in carbapenem resistance among *Enterobacteriaceae* has resulted in a lack of effective therapeutic alternatives. Older antimicrobials, fosfomycin, nitrofurantoin and colistin for urinary tract infections (UTIs) caused by carbapenem-resistant *Enterobacteriaceae* (CRE) may be effective treatment options. The objectives of this study were to evaluate the utility of fosfomycin, nitrofurantoin and colistin in treating UTI caused by CRE and molecular characterization of the plasmid-mediated carbapenem resistance mechanisms.**

***Methods*: Consecutive, non-duplicate isolates of CR *Escherichia coli* and *Klebsiella* spp. from urine cultures were included (n=150). Minimum inhibitory concentrations (MIC) were determined by *E*-test (fosfomycin and nitrofurantoin) and broth microdilution (colistin). Efficacy ratios were derived by dividing susceptibility breakpoints by observed MIC values of the drugs for the isolates. Isolates were screened for genes coding for carbapenemases using multiplex PCR. Fosfomycin, nitrofurantoin and colistin-resistant isolates were screened for plasmid-borne resistance genes *fos*A3, *oqx*AB and *mcr*-1, respectively using PCR.**

***Results*: Among *E. coli*, 98.9, 56 and 95 per cent isolates were susceptible to fosfomycin, nitrofurantoin and colistin, respectively, while 94 and 85 per cent of *Klebsiella* spp. were susceptible to fosfomycin and colistin, respectively. The efficacy ratios indicated fosfomycin as the drug of choice for UTI caused by CR *E. coli* and *Klebsiella* spp., followed by colistin. The bla_{NDM} gene was most common, followed by $bla_{OXA48\text{-}like}$. Plasmid-borne genes encoding resistance to fosfomycin, nitrofurantoin and colistin were absent.**

***Interpretation & conclusions*: With increasing resistance against the current treatment options, older drugs may emerge as effective options. Molecular screening of resistant isolates is essential to prevent the spread of plasmid-borne resistance against these drugs.**

Key words Carbapenem-resistant *Enterobacteriaceae* - colistin - fosfomycin - nitrofurantoin - therapeutic efficacy

Urinary tract infections (UTIs) constitute a major burden of bacterial infections world over[1]. Carbapenems are the drug of choice for treating UTIs caused by multidrug-resistant *Enterobacteriaceae* such

as *Escherichia coli* and *Klebsiella* species. Increasing carbapenem resistance among *Enterobacteriaceae* has resulted in a lack of effective therapeutic alternatives for UTIs caused by carbapenem-resistant *Enterobacteriaceae* (CRE). Therefore, revival of older antimicrobial agents such as fosfomycin, nitrofurantoin and colistin for treating these UTIs seems to be a feasible option[2,3]. However, plasmid-mediated mechanisms against these older drugs have also been reported[4,5]. The spread of these resistance plasmids further complicates the situation. Molecular screening for plasmid-borne isolates has become important in controlling their spread.

The choice of an appropriate agent to treat an infection is generally based on the minimum inhibitory concentrations (MIC) values whenever available. However, deriving the efficacy ratio, which is the ratio of susceptibility breakpoint value by the MIC value obtained, is a better indicator of an agent that should be chosen against a pathogen[6]. A higher efficacy ratio is indicative of a drug being more effective than those with a lower efficacy ratio.

The aim of this study was to evaluate the role of fosfomycin, nitrofurantoin and colistin in treating UTIs caused by CR *E. coli* and *Klebsiella* species. The objectives included to collate and analyze susceptibility profile of the first- and second-line antimicrobials used for UTIs; to determine MIC of fosfomycin, nitrofurantoin and colistin for *E. coli* and *Klebsiella* spp.; to calculate the efficacy ratios of these antimicrobials; to perform molecular characterization of CR *E. coli* and *Klebsiella* spp. and to screen the fosfomycin, nitrofurantoin and colistin-resistant isolates for carriage of plasmid-borne *fos*A3, *oqx*AB genes and *mcr*-1 genes coding for fosfomycin, nitrofurantoin and colistin resistance, respectively.

Material & Methods

This prospective study was conducted in the department of Clinical Microbiology, Christian Medical College and Hospital, Vellore, India, between January 2016 and June 2017. The study was approved by the Institutional Review Board (IRB Min No: 9830).

Urine samples received from hospitalized patients for culture were processed using the routine semi-quantitative culture method[7]. The types of samples included were catheter samples, mid-stream clean catch samples, percutaneous nephrostomy, suprapubic catheter, suprapubic aspirate, ileal conduit and condom catheter. A total of 150 consecutive, non-duplicate isolates of *E. coli* and *Klebsiella* spp., satisfying the inclusion criteria (isolates found in probably significant or significant colony counts and resistant to imipenem, meropenem and ertapenem by disc diffusion method) and exclusion criteria (repeat urine culture from patients already included) were included in this study.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing by disk diffusion was performed as a part of the routine testing and interpretation was done according to the Clinical and Laboratory Standard Institute (CLSI) guidelines[8]. Isolates were tested for susceptibility to cefpodoxime (10 µg), amoxicillin-clavulanate (20/10 µg), piperacillin-tazobactam (100/10 µg), cefoperazone-sulbactam (75/30 µg), gentamicin (10 µg), amikacin (30 µg), netilmicin (30 µg), ciprofloxacin (5 µg) and trimethoprim-sulphamethoxazole (1.25/23.75 µg) by Kirby-Bauer disk diffusion method[9].

The MICs of fosfomycin (n=150) and nitrofurantoin (n=81) were determined using *E*-test strips (AB Biodisk BioMérieux, France) following manufacturer's protocol. The nitrofurantoin MIC (µg/ml) results were interpreted using CLSI guidelines (≤32 susceptible, 64 intermediate and ≥128 resistant)[8]. The CLSI guidelines do not provide fosfomycin susceptibility clinical breakpoints for *Klebsiella* spp. thus, the results were interpreted using breakpoints provided for *E. coli*. For fosfomycin, the MIC value (µg/ml) interpretation was: ≤64 susceptible, 128 intermediate and ≥256 resistant[8].

For colistin susceptibility testing, the isolates (n=150) were subjected to the broth microdilution (BMD) method, with susceptible *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *mcr*-1 positive *E. coli* NCTC13846 (Courtesy: Dr Olga Perovic, National Institute for Communicable Diseases, Johannesburg, South Africa) as the quality control strains[10]. The antibiotic pure substance, colistin sulphate powder, was obtained from Sigma-Aldrich, USA. The results were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines version 8, 2018 (*http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Tigecycline_Guidance_document_20181223.pdf*) (susceptible ≤2 mg/l and resistant >2 mg/l).

Molecular characterization: Molecular detection of resistance genes coding for carbapenemases: Multiplex PCR was performed for detection of carbapenem resistance genes coding for carbapenemases. Isolates

were screened for the production of Class A *Klebsiella pneumoniae* carbapenemase (KPC)[11], Class B carbapenemases active on imipenem (IMP), verona integron-encoded metallo-β-lactamase (VIM) and New Delhi metallo-β-lactamase (NDM)[12,13] and Class D carbapenemase OXA48-like[12]. The amplification of genes was performed using Veriti Thermal cycler (Applied Biosystems, USA) using the following cycling condition; initial denaturation at 95°C for 15 min, 30 cycles of 94°C for 30 sec, 59°C for 1.5 min, 72°C for 1.5 min and final extension at 72°C for 10 min followed by hold at 4°C. Amplicons were visualized on two per cent agarose gel using electrophoresis. Control strains were obtained from International Health Management Associates, USA.

Molecular detection of plasmid-borne *oqx*AB genes coding for nitrofurantoin resistance: The nitrofurantoin resistant *E. coli* were screened for carriage of plasmid-borne resistance genes *oqx*AB using a modified version of the PCR protocol described earlier[14]. Briefly, amplification of genes was performed using Veriti Thermal cycler with the following cycling condition; initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 30 sec, 52°C for 60 sec, 72°C for 60 sec and final extension at 72°C for 7 min followed by hold at 4°C. Amplicons were visualized on two per cent agarose gel using electrophoresis.

Molecular detection of plasmid-borne colistin resistance gene *mcr*-1: The colistin-resistant isolates were screened for plasmid-borne resistance gene *mcr*-1 using a modified version of the PCR protocol described earlier[5]. Briefly, amplification of genes was performed using Veriti Thermal cycler using the following cycling condition; initial denaturation at 95°C for 10 min, 25 cycles of 95°C for 60 sec, 58°C for 30 sec, 68°C for 60 sec and final extension at 72°C for 10 min followed by hold at 4°C. Amplicons were visualized on two per cent agarose gel using electrophoresis.

Calculation of efficacy ratio: The efficacy ratio of the drugs was derived by dividing the susceptible breakpoint MIC as per CLSI guidelines[8] by the MIC result obtained by *E*-test (fosfomycin and nitrofurantoin) or broth microdilution (BMD, colistin)[6]. The efficacy ratios were compared to predict the most effective agent for UTI caused by *E. coli* and *Klebsiella* species. Since nitrofurantoin MIC by *E*-test was determined only for *E. coli*, the nitrofurantoin efficacy ratio for *Klebsiella* spp. could not be calculated.

Results

On routine antimicrobial susceptibility testing of the CR *E. coli,* 98.8 per cent were found to be resistant to cefpodoxime, amoxicillin-clavulanate, cefoperazone-sulbactam each and 96.3 per cent were resistant to piperacillin-tazobactam. The resistance rates of the aminoglycosides were as follows: netilmicin - 77.8 per cent, amikacin - 76.5 per cent and gentamicin 86.4 per cent, while 88.9 per cent were resistant to trimethoprim/sulphamethoxazole and 95.1 per cent to ciprofloxacin. Among the CR *Klebsiella* spp., all the isolates (100%) were resistant to cefpodoxime, cefoperazone-sulbactam and piperacillin-tazobactam, and 99 per cent of them were resistant to amoxicillin-clavulanate. Resistance to aminoglycosides was 87 per cent for gentamicin and amikacin, and 96 per cent for netilmicin, while 80 and 93 per cent of the isolates were resistant to trimethoprim/sulphamethoxazole and ciprofloxacin, respectively.

The MICs of fosfomycin and colistin for all 150 isolates and of nitrofurantoin for *E. coli* isolates are summarized in Table I. The susceptibility profile of the isolates is shown in Table II. The efficacy ratios calculated for fosfomycin, colistin and nitrofurantoin are shown in Figs 1 and 2. The fosfomycin efficacy ratio for *E. coli* was ≥4 for majority of the isolates (96%, n=78), while, the fosfomycin efficacy ratio for *Klebsiella* spp. was ≥4 for 74 per cent of isolates

Table I. Minimum inhibitory concentration (MIC) range, MIC_{50} and MIC_{90} of the carbapenem-resistant organisms

Organism	Fosfomycin MIC (n=150) (µg/ml)				Nitrofurantoin MIC (n=81) (µg/ml)				Colistin MIC (n=150) (µg/ml)			
	MIC range	MIC_{50}	MIC_{90}	Mean MIC	MIC range	MIC_{50}	MIC_{90}	Mean MIC	MIC range	MIC_{50}	MIC_{90}	Mean MIC
Escherichia coli (n=81)	0.25-96	0.75	8	9.8	2->128	32	>128	34	0.12-16	0.5	2	1.6
Klebsiella spp. (n=69)	0.25->256	16	48	12.5	NA	NA	NA	NA	0.25-32	1	8	2.1

NA, not available

(n=51). Fosfomycin was more active against *E. coli* than *Klebsiella* spp. Similarly, the number of isolates with high colistin efficacy ratio was more for *E. coli* than *Klebsiella* spp., making colistin a better agent for CR *E. coli* than CR *Klebsiella* species. However, for nitrofurantoin, the number of *E. coli* isolates with nitrofurantoin efficacy ratio of one or less than one was 62 per cent (n=50). Thus, among the three agents tested for CR *E. coli*, the order of preference was fosfomycin, followed by colistin and nitrofurantoin; while for CR *Klebsiella* spp., fosfomycin was preferred over colistin.

Table II. Susceptibility profile of carbapenem resistant organisms

Isolate	Non-susceptible (%)		
	Fosfomycin	Nitrofurantoin	Colistin
Escherichia coli (n=81)	1.2 (n=1)	44 (n=36)	5 (n=4)
Klebsiella spp. (n=69)	6 (n=4)	NA	15 (n=10)

NA, not available

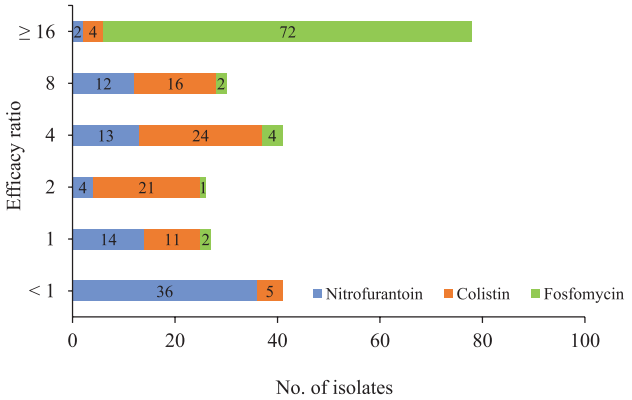

Fig. 1. Comparison of efficacy ratio of fosfomycin, nitrofurantoin and colistin for *E.coli.*

The results of the PCR for detection of genes encoding carbapenemase enzymes bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{KPC} and $bla_{OXA48\text{-}like}$ are summarized in Table III. Among *E. coli* isolates, the most common gene detected was bla_{NDM} followed by $bla_{OXA48\text{-}like}$, whereas in *Klebsiella* spp., $bla_{OXA48\text{-}like}$ was more common than bla_{NDM}. Co-carriage of more than one carbapenemase genes was also detected in 16 isolates. Plasmid-borne fosfomycin resistance gene *fos*A3 was absent in *E. coli* (n=1) isolate that showed intermediate susceptibility to fosfomycin. There was no co-carriage of plasmid-borne nitrofurantoin resistance gene *oqx*AB in the nitrofurantoin resistant *E. coli*. Co-carriage of plasmid-borne colistin resistance gene *mcr*-1 was absent in the colistin resistant *E. coli* or *Klebsiella* species.

Discussion

On routine antimicrobial susceptibility testing, amikacin resistance was found to be lower than most other drugs. Hu *et al*[15] reported 87 per cent amikacin resistance in CRE, whereas Moemen and Masallat[16] reported 52.4 per cent amikacin resistance which was much lower as compared to resistance against

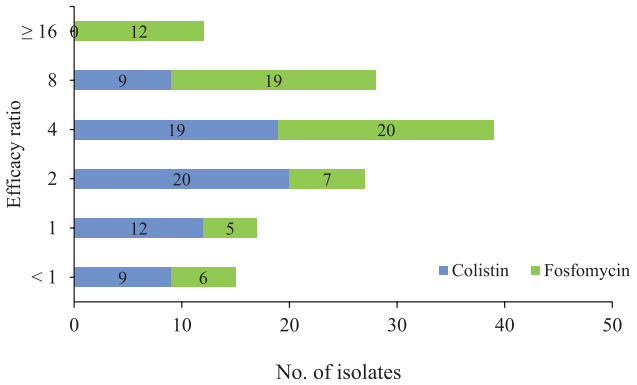

Fig. 2. Comparison of the efficacy ratio of fosfomycin and colistin for *Klebsiella* spp.

Table III. Molecular profile of study isolates

Isolate	Genes detected	bla_{IMP}	bla_{VIM}	bla_{NDM}	bla_{KPC}	$bla_{OXA48\text{-}like}$	Co-producers
Escherichia coli (n=81)	Carbapenemases	-	Positive 1.2% (n=1)	Positive 43% (n=35)	-	Positive 14% (n=11)	Positive 6% (n=5)
	Co-occurrence of *mcr*-1 gene (n=4) or *oqx*AB genes (n=36) or *fos*A3 (n=1)	-	Negative	Negative	-	Negative	Negative
Klebsiella spp. (n=69)	Carbapenemases	-	Positive 1.4% (n=1)	Positive 23% (n=16)	-	Positive 35% (n=24)	Positive 17% (n=12)
	Co-occurrence of *mcr*-1 gene (n=10)	-	Negative	Negative	-	Negative	Negative

other drugs. Piperacillin-tazobactam resistance was; 96.3 per cent for *E. coli* and 100 per cent for *Klebsiella* species. Other groups[15,16] reported 98.7 and 97.6 per cent piperacillin-tazobactam resistance for CRE, respectively. When tested against cefoperazone-sulbactam, 98.8 per cent *E. coli* and 100 per cent *Klebsiella* spp. were found to be resistant. Hu *et al*[15] reported 88.3 per cent cefoperazone-sulbactam resistance.

The susceptibility testing for fosfomycin showed good *in vitro* activity with 98.8 and 94 per cent susceptibility among *E. coli* and *Klebsiella* spp., respectively. Livermore *et al*[17] reported fosfomycin susceptibility of 100 per cent in CR *E. coli* but only 48.1 per cent in CR *Klebsiella* species. Falagas *et al*[18] reported 95 per cent susceptibility among carbapenemase-producing *Enterobacteriaceae*. In India, Sahni *et al*[19] reported susceptibility rate of 83 per cent among the *E. coli* (n=2416) isolated from urine between 2009-2010 whereas Rajenderan *et al*[20] found that fosfomycin inhibited 90 per cent of *E. coli* and *Klebsiella* spp. from various samples, including urine. The efficacy ratio of fosfomycin for CR *E. coli* and CR *Klebsiella* spp. also indicated good activity of this agent.

Nitrofurantoin MIC was determined only for the *E. coli* isolates. Only 56 per cent of the isolates were susceptible to nitrofurantoin. Shanmugam *et al*[21] reported 51 per cent susceptibility to nitrofurantoin among CR *E. coli*. Although studies specific to nitrofurantoin susceptibility in CRE are limited, various studies done on multidrug resistance (MDR) or extended-spectrum beta-lactamases producing *E. coli* from urine samples, report the considerable difference in susceptibility to nitrofurantoin[22,23]. The efficacy ratios of nitrofurantoin for *E. coli* were not as promising as those for fosfomycin or colistin. The colistin susceptibility rates for *E. coli* and *Klebsiella* spp. were 95 and 85 per cent respectively. Overall, 90.7 per cent of the isolates were susceptible to colistin. Süzük *et al*[24] from Turkey reported 96.8 per cent colistin susceptibility among CRE, while a study from Pakistan[25] found 84.1 per cent colistin susceptibility among CRE.

From the efficacy ratios of the three drugs calculated, fosfomycin appeared to be a good treatment option in the management of CR *E. coli* followed by colistin and nitrofurantoin. For CR *Klebsiella* spp., fosfomycin followed by colistin appeared to be good therapeutic options.

The most common carbapenemase gene detected was bla_{NDM} in 37 per cent followed by $bla_{OXA48\text{-}like}$ in 23 per cent isolates, with a predominance of bla_{NDM} *in E. coli* (68.5) and of $bla_{OXA48\text{-}like}$ in *Klebsiella* spp. (42.6%). A study from Bangladesh reported bla_{NDM} (50%) followed by $bla_{OXA48\text{-}like}$ (20%) on CR uropathogens[26]. Sharma *et al*[27] reported bla_{NDM} (32%) and $bla_{OXA48\text{-}like}$ (32%) in septicaemia cases. Co-carriage of more than one gene was detected in 14 per cent isolates, with 75 per cent of them co-carrying bla_{NDM} and $bla_{OXA48\text{-}like}$. Similar findings were reported by other investigators[26,28].

Nitrofurantoin resistance in *E. coli* may be chromosomal or plasmid-mediated. The mutations in chromosomal genes *nfs*A and *nfs*B (encoding oxygen-insensitive nitroreductases) and the deletion in *rib*E (encoding lumazine synthase involved in biosynthesis of flavin mononucleotide) contribute to nitrofurantoin resistance[29,30] whereas the plasmid-borne resistance genes *oqx*AB encode an efflux pump active for nitrofurantoin[4]. None of the isolates was positive for *oqx*AB genes. The resistance in these isolates could be chromosome-mediated. For fosfomycin, chromosomal mutations can affect the function of the transport systems, involved in its uptake by the bacteria. Fosfomycin inactivating enzymes (metalloenzymes and kinases) produced by bacteria also lead to resistance. In *E. coli*, *fos*A3 gene coding for fosfomycin inactivating enzyme was reported to be co-carried on the same plasmid as *oqx*AB[31,32].

Colistin resistance is mainly due to alteration in the lipopolysaccharide of bacterial outer membrane. Mutations in genes involved in lipid A modifications, such as mutations in *mgr*B gene are known to cause colistin resistance. Plasmid (*mcr*-1, *mcr*-2, *mcr*-, *mcr*-3, *mcr*-4 and *mcr*-5) mediated resistance has been reported among *E. coli* and *Klebsiella* spp.[5]. The *mcr-1* gene codes for phosphoethanolamine transferase that modifies the pEtN moiety of lipid A, conferring resistance to colistin[33]. The colistin-resistant isolates in this study were not found to carry *mcr*-1 gene. This could be due to chromosome-mediated mechanisms or presence of other variants of *mcr* gene not evaluated in this study.

Currently, in the midst of widespread plasmid-mediated resistance, the absence of plasmid-mediated fosfomycin, nitrofurantoin and colistin resistance is a relief. However, continuous screening and surveillance of isolates to prevent the rapid spread of plasmid-borne resistance are essential.

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***Conflicts of Interest*:** None.

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