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BRIEF REPORT

Antibiotic susceptibility and fosfomycin resistance characterization in a cohort of children older than 6 years of age with urinary tract infection

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Abstract Fosfomycin tromethamol (FT) was reintroduced as an option for the treatment of low urinary tract infection (UTI) in children. In this study, we described the antibiotic sensitivity and mechanisms of resistance to fosfomycin in isolates from children older than 6 years with UTI. Urine culture and antibiotic susceptibility study were performed. In fosfomycin resistant strains, PCR for *fos*, $bla_{CTX\text{-}M}$ was performed followed by classification by phylogenetic group and sequencetyping. *Escherichia coli* was the most frequent etiological agent (89.2%). The susceptibility percentages were: fosfomycin 97.9%; amoxicillin-clavulanate 92.7%; cefuroxime and ceftriaxone 99%; nitrofurantoin 94.4%. An *E. coli* strain (ST69, phylogenetic group D) was resistant to fosfomycin (MIC 256 mg/l) and carried the $bla_{CTX\text{-}M\text{-}14}$ and *fosA3* genes in a 45 kb IncN-type plasmid. This is the first report of *E. coli* ST69 with $bla_{CTX\text{-}M\text{-}14}$/*fosA3* of human origin.

PALABRAS CLAVE
Escherichia coli;
Resistencia a fosfomicina;
Infección urinaria;
fosA3;
Niños

Sensibilidad antibiótica y caracterización de la resistencia a fosfomicina en una cohorte de niños mayores de 6 años con infección urinaria

Resumen La fosfomicina-trometamol (FT) se reintrodujo como una opción para el tratamiento de la infección del tracto urinario (ITU) baja en niños. En este estudio describimos la sensibilidad antibiótica y los mecanismos de resistencia a FT en aislamientos de niños mayores de 6 años con ITU. Se realizaron urocultivos y estudios de sensibilidad antibiótica. En las cepas resistentes

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a fosfomicina se realizó la técnica de PCR para *fos*, bla_{CTX-M}, y su identificación según su grupo filogenético y secuenciotipo. *Escherichia coli* fue el agente etiológico más frecuente (89,2%). Los porcentajes de sensibilidad fueron: fosfomicina 97,9%; amoxicilina-clavulánico 92,7%; cefuroxima y ceftriaxona 99%; nitrofurantoína 94,9%. Una cepa de *E. coli* (ST69, grupo filogenético D) fue resistente a fosfomicina (CIM 256 mg/l) y portaba los genes $bla_{CTX-M-14}$ y *fosA3* en un plásmido de 45 kb del tipo IncN. Este es el primer reporte de *E. coli* ST69 con $bla_{CTX-M-14}$/*fosA3* de origen humano.

Fosfomycin is an old antibiotic that has reemerged as a new strategy to overcome antibiotic resistance. Fosfomycin spectrum of action includes gram-positive (*Staphylococcus aureus*, *Enterococcus* spp.) and gram-negative bacteria (*Enterobacterales*, *Pseudomonas* spp.), including multi-resistant microorganisms such as extended-spectrum beta-lactamase (ESBL) and carbapenemase producers[6].

Its mechanism of action is cytosolic inactivation of N-acetylglucosamine enolpyruvyl transferase (MurA), which prevents the formation of N-acetylmuramic acid from N-acetylglucosamine and phosphoenolpyruvate, being the initial step of peptidoglycan synthesis. Fosfomycin crosses the bacterial wall using the transport system glycerol-3-phosphate (GlpT) and the transport system for hexose phosphates (UhpT)[6].

There are two presentations of this antibiotic, fosfomycin disodium (for parenteral use) and fosfomycin trometamol (FT) with good oral bioavailability and only indicated for lower UTI[8].

The increase in the clinical use of fosfomycin has led to the development of different resistance mechanisms, particularly, when used as monotherapy[8].

Fosfomycin resistance can result from reduced permeability (mutations in the chromosomal *glpT* and *uhpT* genes), amino acid mutations in the active site of the MurA target, and the production of fosfomycin-inactivating enzymes (*fos* genes). Different fosfomycin-modifying enzymes have been described. FosA (glutathione *S*-transferase), the first to be described, is a metalloenzyme transferred through plasmids in *Enterobacterales*. The FosA3 enzyme is the most commonly identified FosA-like determinant found as an acquired mechanism in *Escherichia coli*[6]. The *fosA3* gene is usually located in conjugative plasmids also carrying CTX-M-type ESBL-encoding genes[8], and related to different plasmid incompatibility groups (IncN, IncF, IncFII, IncI1, among others)[2]. Other *fos* genes, such as *fosA4*, *fosA5*, and *fosA6* have also been identified in *E. coli*, although less frequently[8]. In some *Enterobacterales* (i.e, *Serratia marcescens*, *Klebsiella* spp., *Enterobacter* spp., *Kluyvera georgiana*) there are homologous chromosomal *fosA* genes and these isolates can present reduced fosfomycin susceptibility[6]. Other fosfomycin-modifying enzymes are FosB (in gram-positive bacteria), FosX (chromosomal enzyme in *Listeria monocytogenes*) and FosC (in *Pseudomonas syringae*). Kinases that cause fosfomycin degradation through phosphorylation (FomA and FomB) have been identified in *Streptomyces* spp[8].

In relation to the clinical use of fosfomycin, although it is an old antibiotic, the experience in children is scarce. Recently, a review of fosfomycin use in pediatrics recommends it for UTI treatment, osteomyelitis (such as in cases caused by methicillin-resistant *S. aureus*), and in combination for multidrug-resistant gram-negative bacilli in bacteremias (especially enterobacteria harboring carbapenemases)[1]. In the last years, FT was introduced as an option for the treatment of lower UTI in children older than 6 years of age and adults[12].

Despite the fact that fosfomycin use is increasing, susceptibility to FT is not routinely tested in all laboratories and there is sparse data available about susceptibility profiles in *E. coli* isolates from UTI in children. Hereby we propose to describe the antibiotic susceptibility of isolates obtained from urinary tract infections in children older than 6 years old, and to characterize FT resistance mechanisms involved in resistant strains.

Children aged 6–14 years old with UTI, assisted at a hospital in Uruguay during 5/15/2018–8/15/2019, were included. UTI was defined as the presence of suggestive UTI clinical symptoms (dysuria, increased frequency of urination, fever) and confirmed by a significant bacterial count (1×10^5 CFU/ml) and monomicrobial growth in urine culture. Urine culture, bacterial identification and antibiotic susceptibility were assessed by automated systems either Vitek®2 (bioMerieux, Marcyl'Etoile, Francia, ASTN250) or Phoenix® (Becton Dickinson Diagnostics, Sparks, MD, EEUU, panel BGN 448879) and results were interpreted according to CLSI.[4]

Susceptibility to fosfomycin was tested by disk diffusion (200ug disc OXOID®, Hampshire United Kingdom), in accordance with CLSI guidelines[4]. Unfortunately, CLSI breakpoints to fosfomycin only apply to *E. coli* urinary tract isolates, which makes the study difficult for other *Enterobacterales*. In this work, we employed the susceptible *E. coli* breakpoint $\geq$16 mm for fosfomycin to all *Enterobacterales*. For resistant isolates, minimum inhibitory concentration was determined (MIC) by agar dilution, using Muller Hinton agar (OXOID®, Hampshire United Kingdom) supplemented with glucose-6-phosphate (25 mg/l)[4].

Polymerase chain reaction (PCR) for *fosA* and *fosA3* was performed in Fosfomycin-resistant isolates[10]. Isolates

Table 1 Antibiotic susceptibility results for *Enterobacterales* (data exposed as susceptible to each antibiotic). Percentages are expressed for *E. coli* isolates.

Antibiotic	*E. coli* (n = 255)	*Proteus mirabilis* (n = 14)	*Klebsiella pneumoniae* (n = 7)
Ampicillin	203 (79.6%)	13	0
Amoxicillin-clavulanate	234 (91.8%)	14	7
Trimethoprim-sulfamethoxazole	187 (73.3%)	13	7
Cefazolin	247 (96.9%)	14	7
Cefuroxime	253 (99.2%)	14	7
Ceftriaxone	253 (99.2%)	14	7
Ciprofloxacin	236 (92.5%)	13	7
Gentamicin	247 (96.8%)	14	7
Nitrofurantoin	254 (99.6%)	0	6
Fosfomycin	254 (99.6%)	14	7

harboring a fosfomycin resistant gene were further characterized by PCR for extended spectrum beta-lactamases of the CTX-M family: $bla_{\text{CTX-M-group-1}}$, $bla_{\text{CTX-M-group-2}}$, $bla_{\text{CTX-M-group-3}}$, $bla_{\text{CTX-M-group-4}}$, $bla_{\text{CTX-M-group-25}}$ and transferable quinolone resistance genes: *qnrA*, *B*, *C*, *D*, *S*, *VC* and *aac(6′)Ib-cr*[10].

Positive results were confirmed by Sanger sequencing, and sequences were analyzed using the BLASTn database (https://blast.ncbi.nlm.nih.gov).

Their phylogenetic group was characterized according to Clermont et al.[3], and sequence type (ST) classification was done following the scheme to identify major *E. coli* ST causing urinary tract and bloodstream infections (ST69, 73, 95, and 131) proposed by Doumith et al[7].

Mobility of resistance genes was analyzed by conjugation assays using rifampicin-resistant *E. coli* J53-2 as recipient[9]. Transconjugants were selected on Luria Bertani agar plates supplemented with fosfomycin (1 mg/l) and glucose-6-phosphate (25 mg/l) plus rifampicin (150 mg/l).

Incompatibility groups and toxin–antitoxin mechanisms (i.e.: addiction systems) were detected by PCR using donor and transconjugant genomic DNA as template as reported previously[9].

Plasmid size was estimated in donor strain and transconjugant by treatment with S1 nuclease followed by pulsed-field gel electrophoresis (PFGE)[9].

Clinical data of patients with UTI caused by fosfomycin-resistant bacteria were collected by revision of medical records. Variables collected were age, sex, symptoms and signs, urine exam results, whether it was the first episode of UTI or recurrence, presence of morphological or functional abnormalities of the urinary tract and antibiotic consumption in the last 6 months. The study was approved by the ethics committee and board of directors.

From May 2018 to August 2019, 286 urine cultures with significant growth (1×10^5 CFU/ml) were obtained. *E. coli* was the most frequent etiological agent in 255 urine cultures (89.2%), followed by *P. mirabilis* in 14 (4.9%), *K. pneumoniae* in 7 (2.5%), *Staphylococcus saprophyticus* in 5 (1.7%), and others in 5 (1.7%).

Antibiotic susceptibility for the total of isolates was (susceptible results): 280 to fosfomycin (97.9%), 158 to ampicillin (55.2%), 265 to amoxicillin-clavulanate (92.7%), 279 to cefazolin (97.6%), 283 to cefuroxime and ceftriaxone (99%), 266 to ciprofloxacin (93%), 270 to nitrofurantoin (94.4%), 215 to trimethoprim-sulfamethoxazole (75.2%) and 279 to gentamicin (97.6%). Six isolates were resistant to fosfomycin (5 *S. saprophyticus* with fosfomycin natural resistance and 1 *E. coli*). Antibiotic susceptibility results for main enterobacteria species are shown in Table 1. *E. coli* showed high susceptibility levels (above 90%) to amoxicillin-clavulanate, cephalosporins, fluoroquinolones, aminoglycosides, nitrofurantoin and fosfomycin.

ESBLs were phenotypically detected in 2 *E. coli* isolates, but only one isolate (Ec870) was resistant to (Table 1 and 2). Ec870 was obtained from the urine of a 6-year old girl with dysuria and abdominal pain. This was her fifth UTI episode in the last 2 years, all caused by *E. coli* isolates with the same resistance profile (only resistant to ampicillin and trimethoprim-sulfamethoxazole). In the last 6 months she had received multiple antibiotic treatments with amoxicillin-clavulanate and cefuroxime. No morphological or functional urinary tract abnormalities were detected.

Ec870 harbored the ESBL gene $bla_{\text{CTX-M-14}}$, which explains the resistant phenotype to ampicillin, cefazolin, cefuroxime and ceftriaxone, and the fosfomycin resistance gene *fosA3* responsible for fosfomycin resistance (Table 2). The isolate belonged to phylogenetic group D and ST69. Two plasmids of approximately 45 kb and 150 kb in size, incompatibility groups F, FII, FIA, FIB and N, and addiction systems *vagCD*, *hok/sok*, *ccdAB*, were detected in Ec870. Conjugation assays were positive, and $bla_{\text{CTX-M-14}}$/*fosA3* were co-transferred and detected in transconjugants (TcEc870). TcE870 presented only a 45 kb plasmid, IncN type. None of the toxin-antitoxin genes studied were detected in transconjugants. MIC to fosfomycin was 256 mg/l for donor and transconjugant strains.

Community-acquired UTI caused by antibiotic-resistant bacteria is increasing worldwide, leading to the need for alternative antibiotic plans. Even though fosfomycin is an old antibiotic, FT was introduced in the last years for the treatment of lower UTI in children older than 6 years old. Fosfomycin susceptibility is not always tested in clinical microbiology laboratories, and scarce data are available in children.

In this study, *E. coli* was the predominant etiological agent (89.2%) of UTI in children aged 6–14 years, followed

Table 2 Phenotypic and genotypic characteristics of fosfomycin-resistant Ec870.

	Ec870	TcEc870	*E. coli* J53-2
Source	Urine culture	–	–
Isolation date	15/5/2018	–	–
Sequence type	69	–	–
Phylogenetic group	D	–	–
ESBL synergy test	(+)	(+)	–
MIC (mg/l) by Phoenix			
Ampicillin	>16	>16	4
Amoxicillin-clavulanate	8/4	8/4	8/4
Cefazolin	>16	>16	≤4
Cefoxitin	≤4	≤4	≤4
Cefuroxime	>16	>16	4
Ceftriaxone	≥4	≥4	≤1
Meropenem	≤0.5	≤0.5	≤0.5
Imipenem	≤0.25	≤0.25	≤0.25
Amikacin	≤8	≤8	≤8
Gentamicin	≤2	≤2	≤2
Ciprofloxacin	≤0.125	≤0.125	≤0.125
Trimethoprim-sulfamethoxazole	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5
Nitrofurantoin	≤16	≤16	≤16
Fosfomycin agar dilution (mg/l)	256	256	0.125
Fosfomycin disk (mm)	12	6	28
Resistance genes	$bla_{\text{CTX-M-14}}$ *fosA3*	$bla_{\text{CTX-M-14}}$ *fosA3*	–
Plasmids			
Incompatibility groups	IncN IncF-FII-FIA-FIB	IncN	–
Addiction systems	*vagCD-hoksok-ccdAB*	(–)	–
Size	45 kb 150 kb	45 kb	–

Susceptibility test results, resistance genes, and plasmid characteristics.

by *P. mirabilis* (4.9%) and *K. pneumoniae* (2.5%). The distribution of the etiological agents in the same age group in Spain is similar to that reported here[14].

With regard to antibiotic susceptibility, 97.9% were susceptible to fosfomycin (resistance was detected in five *S. saprophyticus* with natural resistance and one *E. coli* with acquired resistance to fosfomycin). In Spain, susceptibility to fosfomycin from UTI isolates in children aged 5–15 years was 88%, most of the resistant isolates corresponded to *K. pneumoniae* and only 2% of *E. coli* isolates were resistant to fosfomycin[14].

In this work, we identified one conjugative plasmid from the IncN family harboring *fosA3* and $bla_{\text{CTX-M-14}}$ genes. IncN plasmids harboring $bla_{\text{CTX-M-14}}$ were reported in *Salmonella* spp. strains in our country[5]. However, this is not only the first report of *fosA3* but also the association of $bla_{\text{CTX-M-14}}$/*fosA3* in an *E. coli* isolate in Uruguay.

Although *E. coli* ST69, harboring $bla_{\text{CTX-M-9}}$ (another member of the $bla_{\text{CTX-M-9-group}}$ along with $bla_{\text{CTX-M-14}}$) has been previously detected in adults in Uruguay[15], its isolation in children is novel. Contrary to these results, commonly, ST69 isolates are considered susceptible to almost all the antibiotic families[7]. As far as we know, this is the first detection of *fosA3*/$bla_{\text{CTX-M-14}}$ in a ST69 *E. coli* isolate of human origin worldwide.

In this sense, the occurrence of these resistance determinants in a conjugative plasmid brings the possibility to transfer to other sequence types with resistance to more antibiotic families such as ST131[7].

On the other hand, this finding is worrisome due to the possible dissemination of these resistance mechanisms to susceptible strains. Considering this, the UTI treatment with second or third-generation cephalosporins or fosfomycin tromethamine could exert selective pressure for the occurrence of isolates resistant to both antibiotic families, as such was the case with this patient who had received cefuroxime for the treatment of previous UTI episodes.

Most of the strains studied in this work showed high susceptibility to different antibiotics, except ampicillin and trimethoprim-sulfamethoxazole in which resistance exceeded 20%. In a cohort of children in Spain, resistance rates to ampicillin, amoxicillin-clavulanate and trimethoprim-sulfamethoxazole were above 20%[14]. Our local results showed high susceptibility rates to amoxicillin-clavulanate, endorsing its use for oral UTI empirical treatment. In the cohort of children included in our study,

ESBL enterobacteria were detected in only 2 urine cultures (0.7%), a frequency that was lower than that reported in France (0.8-10%)[11] and Spain (1.8%)[14].

The high susceptibility to many oral antimicrobial agents in our setting broadens empirical treatment options. For lower UTI, amoxicillin-clavulanate and nitrofurantoin could be suitable options. Fosfomycin shows several advantages for its use, such as once-daily dosing, low side effects, suitable clinical and microbiology results, and little effect on intestinal microbiota[13]. However, considering that fosfomycin is active against ESBL and carbapenemase enterobacteria producers, its use is not recommended when there are other treatment options of a narrower spectrum. As its use increases, active monitoring of resistance levels will be necessary.

Conflict of interest

The authors declare that they have no conflicts of interest.

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