 ctx-m-15-producing urinary escherichia coli o25b-st131-phylogroup b2 has acquired resistance to fosfomycin

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objectives: to describe trends in fosfomycin resistance in urinary isolates of escherichia coli producing extended-spectrum β-lactamases (esbls) in relation to fosfomycin consumption and to characterize representative fosfomycin-resistant isolates.

methods: in 2007–08, an unexpected increase in fosfomycin resistance in esbl-producing urinary e. coli was observed. laboratory records were reviewed and a prospective surveillance study was initiated on all urinary tract infections caused by esbl-producing, fosfomycin-resistant e. coli. bla_{esbl} types, phylogroups, genetic environment and afa/dra operon were determined by pcR and sequencing. molecular epidemiology was analysed by pfge and multilocus sequence typing. to elucidate possible mechanisms of fosfomycin resistance, uhpt, glpt, uhpa, ptsl, cyaα and murα genes were analysed. fosfomycin consumption was determined as recommended by who.

results: from 2004 to 2008, fosfomycin consumption increased by 50%, while fosfomycin resistance in esbl producers increased from 2.2% to 21.7%. of 26 isolates studied, 24 produced ctx-m-15 and belonged to the o25b-st131-phylogroup b2 clonal strain. pfge revealed two clusters. cluster i included 18 isolates, 16 of them indistinguishable from strains producing ctx-m-15 previously described in madrid. the five isolates of cluster ii had the is_{26} linked to bla_{ctx-m-15} and the afa/dra operon. in cluster i isolates, no mutations in glpt, uhpt, uhpa, ptsl, cyaα and murα were detected. cluster ii isolates showed a 15 bp deletion (a_{169}−c_{183}) in uhpa.

conclusions: fosfomycin resistance in urinary e. coli has increased due to the acquisition of this resistance by a previously circulating ctx-m-15-producing e. coli o25b-st131-phylogroup b2 strain. this happened during a period when the use of fosfomycin increased by 50%.

keywords: urinary tract infections, antibiotic consumption, extended-spectrum β-lactamases

introduction

escherichia coli is the most prevalent uropathogen in community-acquired urinary tract infections (utis). increased antibiotic resistance in this pathogen may limit the therapeutic options for the treatment of utis. extended-spectrum β-lactamase (esbl) production, ciprofloxacin resistance and amoxicillin/clavulanic acid resistance have increased in the last few years in spain and in other european countries.1,2 resistance to ciprofloxacin and trimethoprim/sulfamethoxazole in spanish e. coli isolates was ~30% in 2007.1

oral antibiotics are the treatment of choice for community-acquired utis and infections caused by multiresistant, esbl-producing e. coli are difficult to treat. fosfomycin, an orally dispensed antibiotic, is a phosphonic acid, cell-wall-active antibiotic agent with excellent in vitro activity against e. coli and little cross-resistance with other antibiotics. despite many years of use in europe1–5 for the treatment of uncomplicated
UTIs, fosfomycin remains very active against *E. coli*. However, its use may have increased in recent years due to the limited availability of other therapeutic options.

MurA (UDP-N-acetylgalactosamine enolpyruvyl transferase) is an essential enzyme in the biosynthesis of the peptidoglycan layer of the bacterial cell. MurA is the fosfomycin target, which covalently attaches to Cys-115 in the active site of the enzyme. Mutations of Cys-115 to Asp exist in pathogens such as *Mycobacterium* or *Chlamydia*, rendering these organisms resistant to fosfomycin.

The expression of the *gltP* and *uhpT* genes requires the presence of cyclic AMP; mutations in *cyaA* and *ptsI* genes can decrease the cyclic AMP level in the cell, generating fosfomycin resistance. In addition, *uhpA* is a regulatory gene of the *Uhpt* system.

We report, for the first time, the acquisition of fosfomycin resistance in a clonal group of urinary isolates of *CTX-M-15*-producing *E. coli*. We examined trends in ESBL production and fosfomycin resistance in *E. coli* from UTIs in one large community health area of Madrid, Spain, and compared them with trends in fosfomycin consumption. Fosfomycin resistance appeared amid increased fosfomycin consumption at the community level. Using molecular methods, we characterized 26 ESBL-producing fosfomycin-resistant *E. coli* strains isolated from UTIs.

### Materials and methods

#### Study design and bacterial strains

In 2007 and 2008, microbiologists from the Centro de Especialidades de Argüelles (catchment population of 665422 in 2007), a community healthcare of the Autonomous Community of Madrid, Spain, observed an unexpected increase in fosfomycin resistance in UTIs caused by *ESBL*-producing *E. coli*. We examined trends in ESBL production and fosfomycin resistance in *E. coli* from UTIs in one large community health area of Madrid, Spain, and compared them with trends in fosfomycin consumption. Fosfomycin resistance appeared amid increased fosfomycin consumption at the community level. Using molecular methods, we characterized 26 ESBL-producing fosfomycin-resistant *E. coli* strains isolated from UTIs.

Fosfomycin susceptibility was determined independently by the two participating centres. The methods used were disc diffusion, microdilution, Etest (AB BIODISK, Sweden) and agar dilution; in all of them the fosfomycin susceptibility was determined by adding glucose-6-phosphate as recommended. *E. coli* ATCC 25922 and *CTX-M-15*-producing *E. coli* 222C belonging to ST131 were used as quality control strains. The CLSI criteria for fosfomycin susceptibility (S ≤ 64 mg/L; R > 128 mg/L) as well as the EUCAST criteria (S ≤ 32 mg/L; R > 64 mg/L) were considered.

A representative sample of 26 consecutive fosfomycin-resistant, ESBL-producing *E. coli* isolated in 2008 was studied further.

### Characterization of antibiotic resistance genes

Characterization of *bla<sub>ESBL</sub>, bla<sub>QAC-1</sub>, aac(3)-Ila aminoglycoside resistance gene and aac(6')-Ib-cr aminoglycoside–quinoines resistance gene were carried out by PCR amplification with specific primers and DNA sequencing.

In order to elucidate the molecular mechanisms of fosfomycin resistance, the *uhpT* and *glpT* structural genes (coding for transport systems), the *uhpA* regulatory gene, and the *ptsI* and *cyaA* cyclic AMP control-system genes were amplified and sequenced with specific primers supplied by Professor Dan I. Andersson (Swedish Institute for Infectious Disease Control).

The *murA* gene was amplified and sequenced using specific primers designed according to sequences of *E. coli* K12 (GenBank accession number NC000913), UMN026 (GenBank accession number NC011751) and UTI189 (GenBank accession number NC007946), as follows: murAF1-5'-TTCGGCGCTAAATTATCTGCTC-3' (primer location 54...76); murAR1-5'-GCCGCTAAACGTCCACACTCTC-3' (primer location 669...646); murAF2-5'-GCAGTCCGGTTGATCTACATT-3' (primer location 355...378); and murAR2-5'-TACTCGCCTTTCACAGCTCAA-3' (primer location 1259...1238).

#### Conjugation assay

Conjugation experiments were carried out using the kanamycin–azide-resistant *E. coli* BM101 as a recipient. Putative transconjugants were selected on Mueller–Hinton agar plates containing kanamycin (100 mg/L), azide (160 mg/L) and cefotaxime (4 mg/L). A CTX-M-14-producing *E. coli* isolate was used as a positive control strain for conjugation experiments.

#### Phylogenetic groups and O25b O type detection

The phylogenetic groups of ESBL-producing *E. coli* isolates were determined by a multiplex PCR assay described by Clermont et al.

The O25b O type detection was carried out by an allele-specific PCR.

#### Molecular epidemiology

The genetic relatedness among *E. coli* isolates was determined by PFGE after total chromosomal DNA digestion with XbaI.

Multilocus sequence typing (MLST) types were determined in 10 selected strains according to the Max-Planck Institute scheme for *E. coli* developed by M. Achtman and others.

#### Genetic environment characterization

The genetic environment of *bla<sub>CTX-M-15</sub>* was investigated by searching for the presence of and linkage with sequences previously reported to be associated with this gene as ISEcp1 and IS26. PCR and sequencing using previously described primers were used to investigate these surrounding regions.

#### afa/dra operon detection

The presence of the *afa/dra* operon that encodes Dr family adhesins was evaluated using PCR with specific primers.

#### Fosfomycin consumption

Outpatient consumption of fosfomycin (WHO code J01XX01) for the period 2004–08 was analysed in the area of study, including consumption in elderly nursing homes. The defined daily dose (DDD) and the number of DDD per 1000 inhabitants per day (DID) were calculated following WHO methodology.
Results and discussion

From January 2003 to July 2008, a total of 25869 UTIs were diagnosed: 17602 of them (68%) were caused by E. coli, of which 575 (3.3%) were ESBL producers. ESBL-producing E. coli increased from 1.5% in 2003 to 5.9% in 2008 (χ² for trend 111.4; P<0.0001) (Figure 1). In the same time period, a total of 446 (2.5%) E. coli isolates were fosfomycin resistant by microdilution (MIC > 64 mg/L; CLSI and EUCAST), a method that has an excellent agreement with the agar dilution method in E. coli.24 Fosfomycin resistance increased from 1.6% in 2003 to 3.8% in 2008 (χ² for trend 49.8; P<0.0001) (Figure 1). In addition, fosfomycin resistance in ESBL-producing isolates was very infrequent in 2003/2004, with only two isolates identified (2.2%); however, a great increase was experienced in 2007, with 31 isolates detected (19.7%), and through July of 2008, with 28 isolates detected (21.7%) (χ² for trend 30.9; P<0.0001).

Community consumption of fosfomycin increased from 0.122 DID in 2004 to 0.191 in 2008 (56.6% increase) (P=0.001 by Poisson regression analysis) (Figure 1); the number of fosfomycin packages prescribed also increased from 12406 in 2004 to 18966 in 2007 (52.9% increase).

A sample of 26 consecutive fosfomycin-resistant, ESBL-producing E. coli isolated in 2008 was studied further. Previous isolates were unavailable for study. Twenty-five (96.2%) isolates were from patients >65 years old and 23 (88.5%) were from females. Sixteen (61.5%) were isolated from UTIs in patients admitted to elderly nursing homes and 10 (38.5%) were community-acquired infections. By the Etest, fosfomycin susceptibility was MIC₉₀ = 1024 mg/L, MIC₅₀ > 1024 mg/L and range 128 to >1024 mg/L. All isolates had an MIC > 64 mg/L by microdilution; E. coli control strains ATCC 25922 and 222C had an MIC of 2 and 0.5 mg/L, respectively. These results were confirmed by the agar dilution method. Additional non-susceptibility percentages were trimethoprim/sulfamethoxazole, 96.2%; tobramycin, 96.2%; amoxicillin/clavulanic acid, 63.4%; gentamicin, 57.7%; amikacin, 38.5%; nitrofurantoin, 19.2%; and cefoxitin, 3.8%. All isolates were ciprofloxacin resistant but imipenem susceptible. The most common multiresistance pattern was ampicillin–ciprofloxacin–trimethoprim/sulfamethoxazole–fosfomycin–cefotaxime–ceftazidime–tobramycin (90.3%).

In 25 (96.2%) isolates, blaCTX-15 was detected and blaCTX-14 was detected in 1 (Table 1). All 25 CTX-M-15-producing isolates had the blaOXA-1 and the aac(6’)-Ib-cr aminoglycoside–quinolones resistance genes; 11 of them (44%) had the aac(3)-Ib aminoglycoside resistance gene (Table 1). Twenty-four (96%) of 25 CTX-M-15-producing isolates were phylogroup B2 and 1 was phylogroup A. The CTX-M-14-producing isolate was phylogroup D (Table 1). All the 24 phylogroup B2 isolates belonged to the O25b type.

PFGE revealed two well-defined clusters at a genetic linkage of ≥90% (Figure 2); 18 (72%) CTX-M-15-producing B2 isolates comprised Cluster I, while Cluster II contained 5 CTX-M-15-producing B2 isolates. These two clusters had a genetic relationship of 80%, corresponding to three to four bands of difference. Two additional isolates producing CTX-M-15 showed unrelated patterns (Figure 2). All 16 isolates that caused UTIs in patients admitted to elderly nursing homes belonged to Cluster I. Cluster I was identical to the predominant PFGE pattern described by our group in the first clonal outbreak of CTX-M-15-producing E. coli described in the Madrid area;13 at that time, the pathogen was fosfomycin susceptible (strain 222C).

MLST analysis showed that eight of nine CTX-M-15-producing isolates tested were ST131 (Table 1), while isolate 4862 was ST23; isolate 4761, producing CTX-M-14, was ST117 (Table 1).
Table 1. Molecular characteristics of 26 urinary ESBL-producing fosfomycin-resistant *E. coli* isolates

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PG, phylogenetic group; ND, not determined; UP, unrelated pattern.

*Linkage between IS*Ecp1* and bla*CTX-M-15.*

*a*Linkage between IS26 and bla*CTX-M-15.*

*E. coli* isolate representative of the clonal CTX-M-15-producing strain (Cluster I) described previously in the Madrid area, Spain (see Clermont et al.19).

*DNA did not cut.*
No transconjugants were obtained from representative ST131 isolates of the two different PFGE clusters. The insertion sequence IS\_Ecp1 was situated 48 bp upstream of bla\_CTX-M-15 in all 25 isolates, as previously described. Five PFGE Cluster II isolates had the IS\_Ecp1 truncated at nucleotide 24 from the end by an IS\_26 (Table 1) and those isolates had lower ceftazidime MICs (Table 1). The afadra operon was detected in six isolates (23.1%) (Table 1); five of the six had the IS26 element flanking bla\_CTX-M-15 and were isolated from community-acquired UTIs. Four CTX-M-15-producing fosfomycin-resistant isolates (two of Cluster I and two of Cluster II) were tested for molecular mechanisms of fosfomycin resistance. E. coli 222C, a previous CTX-M-15-producing fosfomycin-susceptible isolate with the same PFGE profile as the Cluster I isolates, was also tested. No mutations were detected in glpT, uhpT, ptsI and cyaA genes, as the sequences of these genes were identical in all five isolates, including 222C. However, Cluster II isolates showed a 15 bp deletion (A169–C183) in the uhpA gene in comparison with the sequence of strain 222C and E. coli K12. Fosfomycin resistance in E. coli has been previously associated with a number of different chromosomal mutations in ptsI, cyaA, glpT, uhpT and uhpA genes, although no cyaA or ptsI mutations could be found in vivo. The analysis of the murA sequences did not show changes between 222C and the four fosfomycin-resistant isolates. In addition, the amino acid sequences of the protein MurA of the five isolates tested were identical to those of the K12, UMN026 and UTB89 E. coli strains. Fosfomycin covalently attaches to the Cys-115 in the active site of E. coli MurA and irreversibly inhibits its enzymatic function, but this position was conserved in the fosfomycin-resistant isolates tested in the present study. Little is known about molecular mechanisms of fosfomycin resistance in E. coli. Although we sequenced five genes previously described as implicated in this resistance, as well as the murA gene, the mechanisms of fosfomycin resistance in Cluster I isolates were not identified.

In conclusion, we describe a recent fosfomycin resistance increase in E. coli clinical urinary isolates in a large community health area of Madrid, mainly due to the acquisition of this resistance by a previously circulating CTX-M-15-producing E. coli ST131/phylogroup B2 strain. Although the emergence of fosfomycin resistance may have occurred as a local outbreak in the area of study, this fact is of clinical and epidemiological concern as fosfomycin was one of the very few oral antibiotic treatments available for UTIs caused by multi-resistant E. coli. The same clone detected in Madrid in 2004 was fosfomycin susceptible. Since 2004, fosfomycin use increased in the area of study by 50%, a factor that may have contributed to the emergence of fosfomycin resistance in 2007 and 2008.
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**Transparency declarations**

None to declare.

**References**
