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Prevalence of fosfomycin resistance among ESBL-producing *Escherichia coli* isolates in the community, Switzerland

Linda Mueller[1,2] · Cansu Cimen[1,3] · Laurent Poirel[1,2,4] · Marie-Christine Descombes[5] · Patrice Nordmann[1,2,4,6]

Abstract

Our aim was to evaluate the prevalence of fosfomycin-resistant strains among ESBL-producing *Escherichia coli* isolates recovered from community patients in Switzerland. A total of 1225 ESBL-producing *E. coli* isolates were collected between 2012 and 2013 from a private and community laboratory. Fosfomycin resistance was assessed by using the novel rapid fosfomycin/*E. coli* NP test and agar dilution method. Resistant isolates were further investigated for acquired resistance genes *fosA1–7* by PCR and sequencing. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were performed to evaluate the clonal relationship among *fosA3*-carrying isolates. Out of the 1225 ESBL-producing *E. coli* isolates analyzed in this study, 1208 were fosfomycin susceptible while 17 were fosfomycin resistant. No discrepancy was observed between the rapid fosfomycin/*E. coli* NP test and the agar dilution method taken as the gold standard. Five out of the 17 resistant isolates carried a *fosA*-like gene. No clonal relationship was observed among those isolates. Here, the prevalence of fosfomycin resistance among ESBL-producing *E. coli* isolates in the community is reported for the first time in Switzerland, being ca. 1.4%. Among the five isolates carrying a *fosA* gene, four encoded the FosA3 enzyme, being the most prevalent fosfomycin-resistant determinant. An excellent correlation was observed between minimum inhibitory concentration–based susceptibility categorization and results of the rapid fosfomycin/*E. coli* NP test, further indicating the excellent sensitivity and specificity of this recently developed rapid test whose results are obtained in less than 2 h.

Keywords Rapid test · Enterobacterales

Introduction

On a global scale, it is estimated that urinary tract infections (UTIs) affect 150 million people per year. *Escherichia coli* is the most frequent bacterial species responsible for UTIs. It is estimated that *E. coli* causes 75% of uncomplicated and 65% of complicated UTIs [1]. Due to an increased prevalence rate of extended-spectrum β-lactamases (ESBLs) conferring resistance to broad-spectrum cephalosporins, and due to the occurrence of associated multiple resistance traits in those ESBL-producing isolates, multidrug-resistant isolates are often identified nowadays in those urinary pathogens [2–4].

Fosfomycin is a phosphonic acid-derived antibiotic owning unique structure and mechanism of action, granting for a broad-spectrum activity and without cross-resistance with other antibiotics. For these reasons, fosfomycin is a first-line treatment as oral therapy for uncomplicated UTIs. Fosfomycin shows good activity against *E. coli* isolates, exhibiting a resistance rate estimated to be < 10% [5, 6].

Chromosomally encoded fosfomycin resistance mechanisms in *E. coli* include reduced drug uptake, due to mutations in the genes encoding GlpT and UhpT transporters, and mutations in the fosfomycin target which is the enzyme catalyzing the first step in peptidoglycan biosynthesis, namely MurA. Reduced drug uptake remains the most frequently

✉ Linda Mueller
linda.mueller@unifr.ch

1 Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Faculty of Science and Medicine, University of Fribourg, Chemin du Musée 18, 1700 Fribourg, Switzerland

2 Swiss National Reference Center for Emerging Antibiotic Resistance (NARA), University of Fribourg, Fribourg, Switzerland

3 Infectious Diseases and Clinical Microbiology Clinic, Ardahan Public Hospital, Ardahan, Turkey

4 INSERM European Unit (IAME/LEA, France), University of Fribourg, Fribourg, Switzerland

5 Laboratoire Proxilis, Meyrin, Switzerland

6 University Hospital Center and University of Lausanne, Lausanne, Switzerland

encountered fosfomycin resistance mechanism not only among clinical isolates but also among in vitro obtained mutants [7, 8].

Transferable resistance mechanisms in *E. coli* entail the FosA metallo-enzymes, responsible for fosfomycin inactivation by catalyzing the conjugation of glutathione to this antibiotic molecule [8]. Among the seven FosA variants reported so far, four (FosA3, FosA4, FosA5, and FosA6) have been identified as acquired resistance determinants among *E. coli* isolates [9–11]. The FosA3 variant is the most relevant since it has been extensively reported in humans and animals, mainly in Asia but also in Europe and the USA [12–14]. The FosA3 encoding gene is located onto conjugative plasmids co-encoding ESBLs of the CTX-M-type [15]. Hence, selective pressure with fosfomycin and/or broad-spectrum cephalosporins may lead to acquisition of those plasmid and consequently to co-resistance to those drugs.

Fosfomycin resistance rates in ESBL-producing *E. coli* in Europe, Asia, and the USA is estimated to be low, the average being ca. 3.2% [5]. However, a Spanish study showed an increase in fosfomycin resistance rates among ESBL-producing *E. coli* from 2.2% in 2004 to 21.7% in 2008, in parallel to a concomitant 50% increase in fosfomycin consumption [16]. An even higher rate of fosfomycin resistance of 31.1% in ESBL producers from UTI is now reported from Israel [17]. To estimate the prevalence of fosfomycin resistance in community-acquired ESBL-producing *E. coli* isolates in Switzerland, we have screened a collection of 1225 isolates recovered in a Swiss laboratory during 2 years. This study was performed in community settings since this is the most important location of *E. coli* spread. The genetic bases of acquired fosfomycin resistance have been deciphered, and the clonal relationship among FosA producers has been evaluated.

Materials and methods

Isolate collection

A total of 1225 ESBL-producing *E. coli* isolates were collected from a Swiss private laboratory between 2012 and 2013. Samples were of different nature, including urine (~89%), genital and anal swabs (~4%), stool (~3%), and blood cultures (~2%). The remaining 2% of the samples were classified as "other" and were of various nature such as expectoration and abscesses. Samples were recovered from women, men, and newborns. Ages ranged from 0 to 103 years.

Fosfomycin resistance determination

Two techniques were used to assess fosfomycin susceptibility. First, the agar dilution method using cation-adjusted Mueller-Hinton agar (MHA-CA, reference 64884; Bio-Rad, Marnes-la-Coquette, France), supplemented with 25 μg/ml of glucose-6-phosphate, was used to determine fosfomycin minimum inhibitory concentration (MIC) over a range of dilutions (1–512 μg/ml) as recommended by Clinical Laboratory Standard Institute (CLSI) guidelines [18]. The breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used as reference [19]. *E. coli* isolates with MICs of fosfomycin ≤ 32 μg/ml were categorized as susceptible while those with MICs > 32 μg/ml were categorized as resistant. Uncertain MICs as well as MICs close to the breakpoint were repeated three times. Second, the rapid fosfomycin/*E. coli* NP test was performed as described in Nordmann et al. [20]. Results were read after 1 h and 30 min of incubation at 35 ± 2 °C. Agar dilution method and rapid fosfomycin/*E. coli* NP results were further compared.

FosA role in fosfomycin resistance

The contribution of FosA resistance proteins in fosfomycin resistance was assessed by using the disk diffusion method as described in Nakamura et al. [9]. Briefly, ~0.5 McFarland standard of fosfomycin-resistant *E. coli* isolates were inoculated onto Mueller-Hinter agar plates supplemented with 25 μg/ml of glucose-6-phosphate. Two Kirby-Bauer disks containing 200 μg of fosfomycin, with and without 0.5 mg of the FosA-inhibitor sodium phosphonoformate (PPF) (Sigma-Aldrich), were added on the plates, which were incubated at 35 ± 2 °C O/N. Actually, PPF inhibits selectively the FosA proteins that allows differentiation between plasmid-mediated FosA and chromosome-encoded resistance mechanisms to fosfomycin. An increase in the diameter of the growth inhibition zone by ≥ 5 mm in the presence of PPF is interpreted as a FosA-related resistance.

PCR amplification and sequencing

Isolates of DNA were extracted with the QIAamp DNA Mini Kit and the QIAcube workstation (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. PCR amplification followed by sequencing (Microsynth, Balgach, Switzerland) was performed to detect the plasmid-mediated *fosA* genes (*fosA1* to *fosA6*) as previously described [20]. The presence of *fosA7* gene was investigated with the following primers: fosA-7_Fw (5′-TGTGGCGACCTTTG GGTCTG-3′), fosA-7_Rv (5′-AGTTCCAGCTTGTG GCCATC-3′). PCR conditions were the same as those used for *fosA1–6* gene amplification and described previously.

FosA3-carrying isolates were further screened for CTX-M-type ESBL presence. Primers CTX-M-A1 (5′-SCS ATG TGC AGY ACC AGT AA-3′) and CTX-M-A2 (5′-GCC GCC GAC GCT AAT ACA TC-3′) were used to amplify $bla_{\text{CTX-M-1}}$-like genes (including $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-15}}$), and primers CTX-M-9-F (5′-GGT GAC AAA GAG AGT GCA AC-3′)

and CTX-M-9-R (5′-GCT GGG TAA AAT AGG TCA CC-3′) were used to amplify the $bla_{CTX\text{-}M\text{-}9}$-like genes. To detect the $bla_{CTX\text{-}M\text{-}8}$ and $bla_{CTX\text{-}M\text{-}2}$ genes, primers CTX-M-8-F (5′-ATG AGA CAT CGC GTT AAG CG-3′) and CTX-M-8-R (5′-CGT CGT ACC ATA ATC ACC GC-3′), or primers CTX-M-2-F (5′-TGA TGA CTC AGA GCA TTC GC-3′) and CTX-M-2-R (5′-AAG TAG GTC ACC AGA ACC AG-3′), were used, respectively. PCR conditions were 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 5 min. Sequences were analyzed with CloneManager Professional (Sci-Ed Software, Denver, CO, USA).

Clonal relationship

Clonal relationship of the bacterial isolates was evaluated by pulsed-field gel electrophoresis (PFGE). Total DNAs from five *E. coli* isolates carrying a plasmid-encoded *fosA3* gene were digested using XbaI enzyme (New England BioLabs, Ipswich, MA, USA). PFGE was then assessed using the CHEF-DR III System (Bio-Rad) to separate the digestion-generated fragments. Finally, multilocus sequence typing (MLST) was assessed on all *E. coli* isolates carrying a FosA-like encoding gene and sequence types (STs) assigned according to the EnteroBase database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/).

Results

A total of 1225 ESBL-producing *E. coli* isolates were investigated for fosfomycin resistance. Seventeen isolates turned to be positive according to the results of the rapid fosfomycin/*E. coli* NP test. Precise determination of their MIC values showed that, for those strains, fosfomycin MICs ranged from 64 to > 512 μg/ml, further confirming the categorization of those isolates as being truly fosfomycin resistant (Table 1). Among them, four isolates possessed the *fosA3* gene and a single isolate possessed the *fosA4*. Two out of four isolates carrying a *fosA3* gene co-harbored a $bla_{CTX\text{-}M\text{-}3}$ gene, one isolate possessed a $bla_{CTX\text{-}M\text{-}15}$ gene, and another single isolate possessed the $bla_{CTX\text{-}M\text{-}9}$ gene (Table 1). Twelve isolates remained negative for all the *fosA1–7* genes by PCR. Four out of the five isolates possessing a *fosA* gene showed accordingly an increased diameter of the growth inhibition in the presence of the FosA-inhibitor PPF. Noticeably, two isolates showing increased diameters around the fosfomycin disk with PPF remained negative for the *fosA1–7* genes (Table 1). Other mechanisms of resistance to fosfomycin were not further investigated.

The 1208 fosfomycin-susceptible isolates exhibited MICs ranging between 1 and 32 μg/ml and accordingly remained negative for the rapid fosfomycin/*E. coli* NP test. Overall, it is interesting to highlight that results of susceptibility testing and of the rapid fosfomycin/*E. coli* NP test showed 100% correlation, for both susceptible and resistant isolates. Among them, 15 isolates exhibited MIC values close to the breakpoint value ($n = 3$, MICs of fosfomycin at 64 μg/ml; $n = 4$, MICs at 128 μg/ml; $n = 8$, MICs at 32 μg/ml), but nevertheless gave concordant results with the rapid test.

PFGE and MLST analyses showed no clonal relationship among the fosfomycin-resistant isolates producing a FosA enzyme. More precisely, the four isolates carrying the *fosA3* gene were of sequence types ST117, ST359 (ST101 complex), ST69, and ST10, while that carrying the *fosA4* gene was of ST12 (Table 1).

Discussion

This is the first study evaluating the prevalence of fosfomycin resistance among ESBL-producing *E. coli* in Switzerland. Our results showed a low prevalence in the community, being ca. 1.4%. This prevalence rate is lower than that reported previously (a total of 11 distinct studies from 2004 to 2009) from the USA, Asia, and Europe where the fosfomycin resistance rate had been estimated to be 3.2% [5]. Even though studies published after 2010 reported a slight increase in fosfomycin resistance, the estimated rate is still considered to be below 10% [6]. This lower fosfomycin resistance rate observed among community-acquired pathogens in Switzerland may mirror the overall low resistance levels observed for many pathogens in Switzerland (www.anresis.ch). Additionally, differences in fosfomycin use might also reflect differences in fosfomycin resistance. A former Swiss study evaluating antimicrobial resistance among *E. coli* isolates recovered from *E. coli* in a tertiary care hospital between 2012 and 2015 reports a fosfomycin resistance rate of 0.9% [21]. However, the prevalence fosfomycin rate among ESBL vs non-ESBL *E. coli* producers had not been evaluated.

Considering the general low prevalence of fosfomycin resistance observed in this study and in other studies performed from 2004 to 2015 [5, 6], we may speculate that there is an overall low risk of rapid expansion of fosfomycin resistance among ESBL-producing *E. coli*. Nevertheless, the analysis reported in the current study was performed on samples collected between 2012 and 2013, and might not reflect the contemporary fosfomycin resistance rate. Further studies are therefore needed to confirm a low fosfomycin resistance among ESBL-producing *E. coli* in Switzerland in 2019.

Our data highlights that 29% of the fosfomycin-resistant ESBL-producing *E. coli* isolates possess a *fosA* gene. These isolates were clonally unrelated, exhibiting different clonal profiles and sequence types, suggesting that acquired plasmid-encoded FosA-related resistance occurred through independent genetic events.

Table 1 Features of the fosfomycin-resistant ESBL-producing *E. coli*

Fosfomycin phenotype	MIC (μg/l)	Rapid fosfomycin/ *E. coli* NP test	PPF potentiation test	FosA determinant	ESBL determinant	Number of isolates	Sequence type	Year of isolation
R	> 512	+	–	*fosA3*	CTX-M-3	*n* = 1	ST10	2012
R	> 512	+	+	*fosA3*	CTX-M-3	*n* = 1	ST117	2013
R	> 512	+	+	*fosA3*	CTX-M-9	*n* = 1	ST359 (ST101 complex)	2013
R	> 512	+	+	*fosA3*	CTX-M-15	*n* = 1	ST69	2013
R	> 512	+	+	*fosA4*	Na	*n* = 1	ST12	2013
R	> 512	+	*n* = 1 + *n* = 1−	–	Na	*n* = 2	NA	2013
R	256	+	–	–	Na	*n* = 3	NA	2013
R	128	+	*n* = 1 + *n* = 3−	–	Na	*n* = 4	NA	2013
R	64	+	–	–	Na	*n* = 3	NA	2012, 2013

MLST was assessed in FosA-producing *E. coli* isolates only

NA not applicable, *Na* not assessed

The FosA3 enzyme was first identified in Japan, from an *E. coli* isolate collected between 2002 and 2007 [12]. In 2010, this enzyme was observed in an *E. coli* isolate in the USA [13], then in France [15] and in Switzerland (this study) in 2012, highlighting its worldwide dissemination. Intriguingly, similar to one isolate from 2013 carrying the *fosA3* gene detected in our study, another FosA3-producing ST69 *E. coli* had been recovered in France in 2014, suggesting a clonal relationship [15].

As expected, the majority (80%) of FosA-producing *E. coli* analyzed in our study carried a *fosA3* gene, confirming that this is the most prevalent plasmid-mediated fosfomycin resistance gene variant among *E. coli*, including among ESBL producers. Isolates carrying a FosA3-encoding gene also carried produced CTX-M-type ESBLs, being of either CTX-M-3-, CTX-M-15-, or CTX-M-9-types, further highlighting that associations between *fosA3* and $bla_{\text{CTX-M}}$ genes are frequent, as widely reported [12, 14, 22].

Noteworthily, two *E. coli* ESBL-producing isolates exhibited an increased inhibition zone diameter on the agar plate in the presence of PPF (positive result), but remained negative for *fosA1–7* amplification, suggesting the possible production of still unknown FosA-related or FosA-unrelated resistance mechanism(s).

Surprisingly, for a single *E. coli* isolate carrying a *fosA3* gene, the PPF test remained negative, suggesting that the phenotypic resistance observed might be resulting from both a plasmid-encoded and a chromosomally encoded resistance mechanism(s). The other possibility is that this PPF-mediated inhibition might lack the sensitivity for detecting FosA-related resistance to fosfomycin.

It has been suggested that mutations in several chromosomal genes involved in fosfomycin resistance in *E. coli* clinical isolates might have an elevated biological cost, therefore explaining the overall low prevalence of fosfomycin resistance in *E. coli* [14]. By contrast to chromosomal mutations, production of a FosA-like enzyme has been estimated to generate a lower biological cost and therefore may be prone to be easily disseminated [23].

Finally, this study represents the first extended survey evaluating the performance of the rapid fosfomycin/*E. coli* NP test for rapid screening of fosfomycin resistance in a large collection of clinical isolates. Thanks to its excellent performance, this test whose results are given in less than 2 h and is low cost might be widely implemented to accurately monitor the evolution of fosfomycin resistance rates worldwide and is adapted to rapid determination of fosfomycin susceptibility for an adapted antibiotic stewardship of septicemia.

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