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Short Communication

Faropenem resistance causes in vitro cross-resistance to carbapenems in ESBL-producing *Escherichia coli*

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ABSTRACT

Objective: Faropenem is an oral penem drug with activity against Gram-positive and Gram-negative bacteria, including CTX-M-15-type extended spectrum beta-lactamase (ESBL)-producing Enterobacteriales and anaerobic bacteria. As there are structural similarities, there is concern for the development of carbapenem cross-resistance; however, there are no studies confirming this. This study examined whether in vitro development of faropenem resistance in *Escherichia coli* isolates would result in cross-resistance to carbapenems.

Methods: Four well-characterized *E. coli* isolates from the US Centers for Disease Control and Prevention antibiotic resistance isolate bank were utilized. Three isolates (NSF1, NSF2 and NSF3) are ESBL producers (CTX-M-15) and one (NSF4) is pan-susceptible. Faropenem minimum inhibitory concentrations (MICs) were determined and resistance was induced by serial passaging in increasing concentrations of faropenem. Susceptibility to carbapenems was determined and whole-genome sequencing (WGS) was performed to identify the underlying genetic mechanism leading to carbapenem resistance.

Results: Faropenem MIC increased from 1 mg/L to 64 mg/L within 10 days for NSF2 and NSF4 isolates, and from 2 mg/L to 64 mg/L within 7 days for NSF1 and NSF3 isolates. Reduced carbapenem susceptibility (ertapenem MIC ≥8 mg/L, doripenem/meropenem ≥2 mg/L and imipenem ≥1 mg/L) developed among three CTX-M-15-producing isolates that were faropenem-resistant, but not in NSF4 isolate that lacked ESBL enzyme. WGS analysis revealed non-synonymous changes in the *ompC* gene among three CTX-M-15-producing isolates, and a single nucleotide polymorphism (SNP) in the *envZ* gene in NSF4 isolate.

Conclusion: Induced resistance to faropenem causes cross-resistance to carbapenems among *E. coli* isolates containing CTX-M-15-type ESBL enzymes.

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1. Background

Faropenem is an oral penem, a type of unsaturated beta-lactam with a similar structure to carbapenems [1]. Drugs in the penem class have a sulfur atom at position one in the thiazolidine ring instead of carbon in the pyrrolidine ring of the carbapenem [1]. Faropenem has broad antimicrobial activity, including against Gram-positive and Gram-negative bacteria, anaerobic bacteria, and Enterobacteriales containing TEM-, SHV-, and CTX-M-type extended spectrum beta-lactamases (ESBLs) [1]. Faropenem was approved in India in 2010 by the Central Drugs Standard Control Organization for the treatment of respiratory tract, urinary tract, skin and soft tissue, and gynecological infections [2]. Since its approval, faropenem consumption in India has increased from 7.4 million standard units in 2010 to 18.9 million standard units in 2014 and now has surpassed total carbapenems consumption [3].

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Faropenem consumption is also increasing in China [4]; however, its availability and consumption levels in other low and middle income countries are unknown. Faropenem is not Food and Drug Administration (FDA)-approved for clinical use in the United States [1]. Although faropenem is used for various indications in India and China, there are limited data on its clinical efficacy [4]. In addition, susceptibility testing against faropenem is not routinely performed in clinical microbiology laboratories because of a lack of Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive breakpoints [3].

There is a direct association of antimicrobial consumption with rise in resistance among bacteria, and this is particularly true for enteric Gram-negative bacteria [5]. Low antimicrobial concentrations, both in the environment or in the human body during therapeutic use, can enrich for resistant mutants [6]. Previous studies have shown that induced ertapenem resistance among ESBL-producing *Escherichia coli* isolates resulted in reduced susceptibility to other carbapenems [7,8]. Thus, the structural similarity of penem antibiotics to carbapenems raises a concern for cross-resistance. Additionally, the oral formulation used in India is faropenem sodium, which is poorly absorbed and has low bioavailability [4]. With increasing prevalence of carbapenem resistance in India, the extensive use of faropenem for multiple clinical indications coupled with sub-optimal dosing may result in further development and spread of carbapenem-resistant bacteria [9]. Development of resistance to faropenem could also result in carbapenem (doripenem, ertapenem, meropenem, and imipenem) cross-resistance because of their structural similarity. To test this hypothesis, an in vitro system was developed to induce faropenem resistance in *E. coli* isolates and then antimicrobial susceptibility testing was performed to determine if carbapenem resistance had been induced by faropenem exposure.

2. Methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

Four well-characterized *E. coli* isolates were obtained from the US Centers for Disease Control and Prevention (CDC) antibiotic resistance (AR) isolate bank (Enterobacteriales Carbapenem Breakpoint panel - AR#0011 [NSF1]; AR#0014 [NSF2]; AR#0015 [NSF3]; and AR#0017 [NSF4]) [10]. Three of the isolates (NSF1, NSF2 and NSF3) are ESBL enzyme producers, and the fourth isolate (NSF4) is a pan-susceptible isolate with no known genetic mechanism of resistance (Supplementary Table 1). This panel was selected because these *E. coli* isolates have most common ESBL enzymes (CTX-M-15, OXA-1 and TEM) encountered in *E. coli* isolates in India [11,12]. All experiments were conducted in triplicate.

Faropenem sodium hydrate (Sigma-Aldrich, St. Louis, MO) was used for the experiments. Faropenem minimum inhibitory concentrations (MICs) for each isolate were determined using the standard macro-tube dilution methods (CLSI) with *E. coli* ATCC 25922 used for quality control (faropenem MIC 0.5–1 mg/L) [13,14]. A 2 mg/L susceptibility breakpoint was selected for faropenem as previously reported [15]. Antimicrobial susceptibility testing for ertapenem, meropenem, imipenem-cilastatin and doripenem was performed using broth microdilution (Sensititre, ThermoFisher Scientific, Waltham, MA) using cation-adjusted Mueller Hilton-Broth (MHB) media (ThermoFisher Scientific, Waltham, MA).

2.2. Inducing resistance by serial passage

Antimicrobial resistance to faropenem was induced by serial passage as described previously [16,17]. Briefly, for each isolate, tubes containing 2 mL of MHB with 2-fold increasing concentrations of faropenem (0.125–256 mg/L) were inoculated with 5×10^5 cfu/mL bacteria. Following overnight incubation at 35-37 °C, with no shaking before reading, the faropenem MIC was determined. The tube with the highest drug concentration that permitted growth was selected for transfer. Bacterial counts from this tube were then adjusted to a starting concentration of 5×10^5 cfu/mL, and these cells were used to inoculate a fresh 2-fold dilution series of faropenem, followed by overnight incubation. This process was repeated until MIC reached 64 mg/L for each isolate. The inoculating cultures for each of the passages (exposures) were plated and stored.

Faropenem inhibitory disc zone sizes were also examined, and correlated with the rise in faropenem MIC. Using a 512 mg/L faropenem antibiotic stock solution, 10.2 µg faropenem discs were prepared by adding 20 µL of the stock solution to blank paper discs [18]. The disc was placed on the subculture plates to ensure that antibiotic pressure was maintained during the entire experimental process. The susceptible *E. coli* ATCC 25922 was used as control for the faropenem discs.

2.3. Stability of the faropenem resistance

To assess the stability of "induced" faropenem resistance, isolates with the highest faropenem MIC were serially passaged in antimicrobial-free MHB (2-mL cultures adjusted to a starting concentration of 5×10^5cfu/mL) daily for 10 days. Faropenem MICs and susceptibility to carbapenems testing were repeated after 10 days of passage as described above.

2.4. Whole-genome sequencing analysis

To study the molecular mechanism of faropenem and carbapenem resistance, genomic DNA was extracted from single colonies of all four strains before and after induction of faropenem resistance as described previously using the Masterpure DNA extraction kit (Epicenter®, Madison, WI) and following manufacturer's instructions [19]. Genomic DNA was prepared for shotgun metagenome sequencing using a Nextera XT DNA Library Preparation Kit (Illumina, FC-131-1096, San Diego, CA) and Nextera XT Index Kit v2 Set D for 96 Indexes (Illumina, FC-131-2004, San Diego, CA). Sequencing was performed using an Illumina NextSeq500 System using Mid Output Kit, 300 Cycles (Illumina, FC-420-1004, San Diego, CA) with 1% PhiX control in the pool (Illumina, FC-110-3001, San Diego, CA). Library preparation and sequencing were performed at the University of Illinois Sequencing Core (UICSQC). The raw short-read sequence data were checked for quality with FastQC [20], trimmed with Trimmomatic [21], assembled de novo into contigs with SPAdes [22], and annotated using Prokka [23]. Mutations within strains were identified using breseq [24], a computational pipeline for reference-based alignment of short sequencing reads from microbial genomes. Through this program, reads from the resistant isolate were aligned against the assembly of its corresponding susceptible isolate, enabling comprehensive annotation and comparison of faropenem-susceptible and -resistant isolate genomes.

3. Data Archive

Raw sequence data files were submitted in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI). The BioProject identifier of the samples is PRJNA522790.

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Table 1
Carbapenem susceptibility results for *E. coli* isolates before and after induction of faropenem resistance

	NSF1		NSF2		NSF3		NSF4	
Antibiotic	MIC mg/L Before	MIC mg/L After	MIC mg/L Before	MIC mg/L After	MIC mg/L Before	MIC mg/L After	MIC mg/L Before	MIC mg/L After
Days to reach faropenem MIC of 64 mg/L	-	7	-	7	-	5	-	10
Faropenem	2	64	1	64	2	64	1	64
Doripenem	≤0.5	>4	≤0.5	2	≤0.5	4	≤0.5	≤0.5
Ertapenem	≤0.25	>8	≤0.25	8	≤0.25	>8	≤0.25	≤0.25
Imipenem	≤0.5	4	≤0.5	1	≤0.5	4	1	1
Meropenem	≤0.5	>8	≤0.5	2	≤0.5	8	≤0.5	≤0.5
Faropenem and carbapenems MIC changes after 10 days of passage in antibiotic-free media								
	NSF1		NSF2		NSF3		NSF4	
	MIC mg/L Before	MIC mg/L After	MIC mg/L Before	MIC mg/L After	MIC mg/L Before	MIC mg/L After	MIC mg/L Before	MIC mg/L After
Faropenem	64	32	64	8	64	64	64	64
Doripenem	>4	>4	2	≤0.5	4	2	≤0.5	≤0.5
Ertapenem	>8	>8	8	4	>8	>8	≤0.25	≤0.25
Imipenem	4	4	1	≤0.5	4	1	1	1
Meropenem	>8	>8	2	1	8	4	≤0.5	≤0.5

MIC, minimum inhibitory concentration

4. Results

4.1. Induction of faropenem resistance and carbapenem cross-resistance among E. coli isolates containing ESBL enzymes

Before exposure to faropenem, the *E. coli* isolates had MICs of 1 mg/L (NSF2, NSF4) and 2 mg/L (NSF1, NSF3). Within 5-10 serial passages, faropenem MIC increased to 64 mg/L for all isolates (Table 1). Faropenem resistance was confirmed by the lack of inhibitory zones around the 10.2 µg faropenem discs for all four isolates.

MIC testing for carbapenem antimicrobials was performed both before and after induction of faropenem resistance by serial passage. Although faropenem resistance was successfully induced in all four *E. coli* isolates, only isolates containing ESBL enzymes (NSF1, NSF2, and NSF3) developed cross-resistance to carbapenems. MIC levels varied between the three resistant isolates: 2 to >4 mg/L for doripenem; 8 to >8 mg/L for ertapenem; 1 to 4 mg/L for imipenem; and 2 to >8 mg/L for meropenem (Table 1). The pan-susceptible NSF4 remained susceptible to carbapenems despite developing resistance to faropenem after serial passage.

4.2. Stability of induced faropenem resistance

After 10 days of passage in antimicrobial-free media, all four faropenem-resistant isolates retained elevated faropenem MICs of ≥8 mg/L (Table 2). Isolates NSF3 and NSF4 retained MICs for faropenem of 64 mg/L. Susceptibility to carbapenems was altered after 10 days of passage in antimicrobial-free medium, although the effect varied among isolates. Carbapenem resistance for NSF1 was not altered by passage in antimicrobial-free medium. NSF2 and NSF3 showed a loss of resistance to several carbapenems tested, although MICs to some carbapenems remained higher than prior to faropenem exposure (Table 1). Susceptibility to ertapenem decreased the least after passage, remaining at MICs of ≥4 mg/L in NSF1, NSF2, and NSF3 isolates.

4.3. Genomic changes among faropenem-resistant E. coli isolates

Draft genome assemblies were generated for eight *E. coli* isolates: the initial four faropenem-susceptible *E. coli* isolates and four matched isolates following induction of faropenem resistance (Supplementary Table 2). Alignment of the faropenem-resistant isolate reads against the susceptible assemblies indicated several changes in the genome, comprising mostly single nucleotide polymorphisms (SNPs). Significant genomic changes and corresponding antibiotic susceptibilities for faropenem-susceptible and -resistant isolates are displayed in Table 2. A comprehensive list of all coding mutations is provided in Supplementary Table 3.

4.3.1. OmpC *mutations in ESBL-producing* E. coli *isolates*

All three strains containing ESBL enzymes (NSF1, NSF2 and NSF3) consistently displayed mutations in *ompC*, which is a gene encoding for outer membrane protein C (Fig. 1a). These mutations resulted in a premature stop codon in the extracellular domain (Fig. 1b), a 105-bp deletion (Fig. 1c), and an amino acid change in the beta-stranded domain (Fig. 1d), respectively.

Other mutations observed in NSF1, NSF2 and NSF3 included *acrB* (efflux transporter), *marR* (multiple antibiotic resistance protein), *wecA* (undecaprenyl–phosphate alpha–N–acetylglucosaminyl 1–phosphate transferase), and *aroK* (Shikimate kinase 1).

4.3.2. envZ *mutations in NSF2 and NSF4*

For the pan-susceptible isolate (NSF4), only two coding SNPs residing within annotated genes were detected, and these were located in *galU* (UTP–alpha-D–glucose–1–phosphate uridylyltransferase) and the HAMP domain of *envZ* (osmolarity sensor protein) genes. A non-synonymous *envZ* SNP was also present in the ESBL-encoding NSF2 isolate.

WGS analysis also indicated that isolate NSF3 lost its *CTX-M-15* and *OXA-1* genes but retained *TEM-1B* after serial faropenem passage. The NSF1 and NSF2 strains retained their *CTX-M-15* genes, while the original NSF4 lacked ESBL genes. In the case of retainment, no mutations in the gene sequences were observed.

5. Discussion

These study results show that exposure to increasing concentrations of faropenem can lead to high-level faropenem resistance and, in the presence of ESBL enzymes, cause cross-resistance to ertapenem, meropenem, doripenem and imipenem. Clinical resistance for all carbapenems was observed in two of three ESBL-producing *E. coli* isolates. Development of cross-resistance did not occur in the pan-susceptible *E. coli* isolate, which showed inducible faropenem resistance but remained susceptible to carbapenem antimicrobials. To our knowledge, this is the first report indicating

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Table 2
Genotypic and phenotypic changes before and after inducing faropenem resistance* among *E. coli* isolates

Isolate	CTXM-15	OXA-1	TEM-1B	ompC mutations/ changes	Mutations in other genes	Faropenem susceptibility	Ceftriaxone susceptibility	Doripenem susceptibility	Ertapenem susceptibiltiy	Imipenem susceptibility	Meropenem susceptibility
NSF1	+	+	-	-	-	S	R	S	S	S	S
NSF1^	+	+	-	Q196*	*wecA* *mipA*	R	R	R	R	R	R
NSF2	+	+	-	-	-	S	R	S	S	S	S
NSF2^	+	+	-	Δ105 bp	*envZ* *acrB* *marR*	R	R	I	R	S	I
NSF3	+	+	+	-	-	S	R	S	S	S	S
NSF3^	-	-	+	L358Q	*aroK* *mipA* *ldrA* *rseA*	R	S	R	R	R	R
NSF4	-	-	-	-	-	S	S	S	S	S	S
NSF4^	-	-	-	-	*envZ* *galU*	R	S	S	S	S	S

S, susceptible; I, intermediate; R, resistant
* Faropenem susceptibility breakpoint was 2 mg/L [15]
^ Isolates that were rendered resistant to faropenem following induction

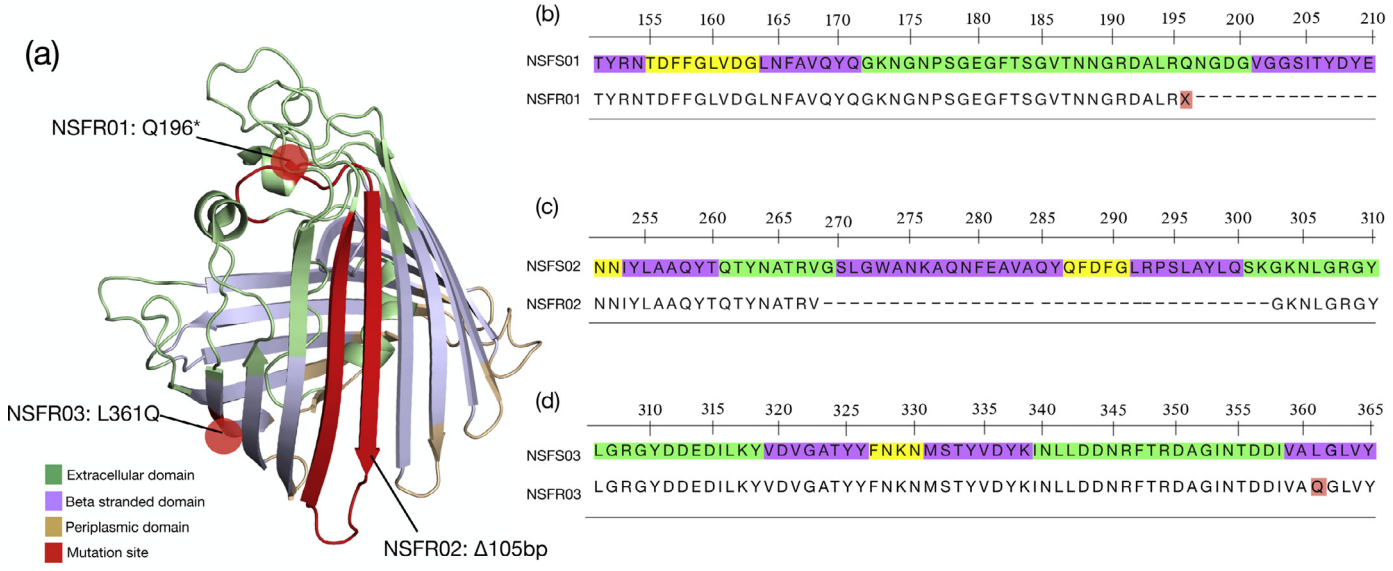

Fig. 1. ESBL-containing isolates NSF1-3 all displayed mutations in *ompC*. (A) Structure of OmpC monomer, with structural domains annotated by color. Mutation sites are indicated in red. (B-C) Aligned OmpC amino acid sequences. S indicates faropenem susceptible isolates and R indicates resistance (2J1N).

that faropenem can induce cross-resistance to carbapenems among *E. coli* isolates that produce CTX-M-15-type ESBL.

Carbapenem resistance has been reported among *E. coli* isolates that produce CTX-M-type ESBL and have a deficient *ompC* [7,8,25]. The current study results are consistent with this finding, as mutations in *ompC* genes were consistently observed among resistant isolates. Mutational changes in *ompC* have been demonstrated to increase resistance to antimicrobials primarily due to structural changes in porin channels and restricted uptake into the bacterial cell [26,27]. In addition to *ompC* mutations, in NSF2 there were also mutations in *envZ, acrB* and *marR*, which have been associated with carbapenem resistance [28,29]. In NSF1 and NSF3, there were mutations in other genes (*wecA, mipA, aroK, ldrA* and *rseA*) that have not been previously reported with carbapenem resistance.

Interestingly, in NSF3 the loss of CTX-M-15 and OXA-1 was observed after faropenem exposure. Consistent with loss of CTX-M-15 ESBL enzyme, the isolate became susceptible to ceftriaxone (Table 2) but developed carbapenem resistance. Carbapenem resistance could be due to the persistence and hyper-production of TEM-1B beta-lactamase. A previous study has demonstrated reduced susceptibility to carbapenems among TEM-1B beta-lactamase-producing *E. coli* isolates in the absence of porin expression, indicating that carbapenem resistance can develop in the absence of ESBL enzymes [8]. However, further molecular studies are needed to confirm this as only the *ompC* SNP as the single porin mutation in the NSF3 isolate was observed in this study.

In the pan-susceptible NSF4 isolate, cross-resistance to carbapenem did not occur despite developing resistance to faropenem. Comparative genomics showed coding mutations in *envZ* and *galU* genes. *envZ* is a gene encoding for the EnvZ protein, which regulates the expression of ompC and ompF through phosphorylation/dephosphorylation of the transcriptional activator OmpR [30]. Previous studies have demonstrated that *envZ* mutations occur among *E. coli* isolates that lack beta-lactamases when exposed to carbapenems [8,29]. In contrast to the high-level faropenem resistance observed in this study, however, mutations in *envZ* alone resulted in low-level carbapenem resistance [8]. Carbapenem resistance in *E. coli* associated with mutations in *galU* genes has not been reported. The presence of mutations in *envZ* and *galU* genes may have resulted in high-level faropenem resistance in the NSF4 isolate. Further molecular studies are needed to confirm this observation.

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The current study findings are of significant concern because faropenem is approved for several community-acquired clinical infection syndromes in India and its consumption is increasing [3]. In addition, faropenem pharmacokinetic/pharmacodynamic studies are crucially lacking, which is a concern for a drug used on such a large scale [4]. The continued use of sub-optimal doses of faropenem could result in selection of carbapenem-resistant Gram-negative organisms in the intestinal microbiota. Carbapenem and faropenem MICs among resistant *E. coli* isolates remained elevated even after passaging for 10 days in an antimicrobial-free environment indicating that resistance resulting from mutation could persist for long periods of time. However, there was reversion of susceptibility to some carbapenems among NSF2 and NSF3 and lowering of faropenem MIC for NSF2. There might be different degrees of changes in porin expression and ESBL enzyme quantities in an antimicrobial-free environment and this will affect antibiotic permeability among the isolates differently. The current study did not evaluate the expression of OmpC or quantify ESBL enzyme production.

The current study results show that the risk of carbapenem resistance is only seen with ESBL- or beta-lactamase-producing *E. coli* isolates; however, the prevalence of asymptomatic intestinal colonization of ESBL-producing Enterobacteriaceae among healthy carriers is high in India. In one study in an urban area in South India, the fecal carriage rate of ESBL-producing Enterobacteriaceae among healthy individuals was 34%, with CTX-M-15 the predominant enzyme [12]. Other studies involving healthy individuals from a remote rural community showed that 19% harbored ESBL-producing *E. coli* and again the predominant enzyme was CTX-M-15 [31]. Thus, the current study results indicate that patients who are prescribed faropenem for treatment of ESBL-producing Gram-negative infections are at high risk for developing faropenem resistance and cross-resistance to carbapenems. The problem is compounded by lack of faropenem drug resistance monitoring due to the absence of routine susceptibility testing in clinical microbiology laboratories.

The current study has limitations. The study utilized *E. coli* isolates from the CDC AR isolate bank belonging to the Enterobacteriales Carbapenem Breakpoint panel and the generalizability of the results to *E. coli* isolates with similar phenotypic and genotypic profiles from other panels is unknown. Observed genomic mutations were not reconstructed to confirm the resistance mechanism and the study did not examine if the resistant isolates have lower replication rates, as previous studies have shown that alterations in porin regulation have a negative effect on bacterial fitness [8]. However, this initial study is significant as it highlights the importance of reducing the use of faropenem in the community to limit the induction of carbapenem resistance among *E. coli* isolates.

In conclusion, these study findings demonstrate that faropenem can cause cross-resistance to carbapenems among *E. coli* isolates that produce CTX-M-15-type ESBL enzyme. Although this is an in vitro study, the results indicate a serious potential threat of carbapenem resistance among Gram-negative organisms in vivo. Alternative antibiotics for treatment of respiratory tract, skin and soft tissue and gynecological infections are available and should be preferred to faropenem. Future studies should focus on understanding the risk of carbapenem resistance selection in Gram-negative bacteria and their persistence among patients who are prescribed faropenem.

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Declarations

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Ethical Approval: Not required

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2020.105902.

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