



One-year multicenter surveillance of Fosfomycin resistance Enterobacterales: the rise of FosA3-producing *P. mirabilis*

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ABSTRACT

The global rise of antimicrobial resistance has renewed interest in fosfomycin (FOS), an old antibiotic with activity against multidrug-resistant *Enterobacterales*. However, resistance to FOS is increasing, driven by impaired drug uptake, target modification, and by *fosA*-encoded enzymatic inactivation. This study assessed the prevalence and molecular basis of FOS resistance among *Enterobacterales* collected in Czech tertiary care hospitals in 2024. A total of 211 preliminary FOS-resistant isolates were obtained from nine hospitals across the Czech Republic, predominantly *Proteus mirabilis* (n=149) and *Escherichia coli* (n=57). All isolates showed elevated FOS MICs, and the PPF test identified FosA activity in 34/211 isolates. Carbon-source growth testing demonstrated widespread impairment of GlpT and UhpT transporters (93.8 % affecting both). PCR confirmed *fosA* genes in 14 isolates (9 *P. mirabilis*, 5 *E. coli*). WGS revealed *fosA3* as the dominant variant (85.7 %), followed by *fosA4* (14.3 %). *P. mirabilis* isolates primarily belonged to ST185 and ST135, forming two Czech-specific *fosA3* clusters with limited relatedness to international genomes. FosA-producing *E. coli* displayed broader diversity (ST69, ST58, ST550, ST1308). *FosA4* was detected exclusively in *E. coli*. Most *fosA*-positive strains co-harbored ESBP genes, predominantly *bla_{CTX-M-65}*. SNP-based phylogenies indicated local clonal circulation of *fosA3*-positive *P. mirabilis* ST185, whereas *E. coli* isolates showed heterogeneous international linkages. Analysis of GlpT/UhpT/MurA identified numerous amino acid substitutions, though only a minority were predicted to affect protein function. This study documents the first broader emergence of plasmid-mediated FOS resistance in Czech *Enterobacterales* and underscores the importance of continuous genomic surveillance of *fosA*-mediated resistance.

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1. Introduction

The global rise in antimicrobial resistance continues to undermine the effectiveness of standard therapies, with multidrug-resistant (MDR) Gram-negative pathogens increasingly implicated in severe, difficult-to-treat infections. As treatment options narrow, attention has turned not only to novel antibiotics but also to older antimicrobials with *in vitro* activity against some MDR pathogens (Paranos et al., 2022).

One of these newly rediscovered antibiotics is fosfomycin (FOS). Its broad-spectrum activity is explicated by irreversible inhibition of MurA (UDP-N-acetylglucosamine enolpyruvyl transferase), involved in peptidoglycan synthesis. For this reason, FOS is a valuable treatment against MDR organisms, including, among others, ESBL-producers, AmpC (hyper)producers, and some carbapenemase-producing *Enterobacterales* (Falagas et al., 2019; Putensen et al., 2019). Clinically, FOS plays an increasingly important role not only in the treatment of uncomplicated and complicated urinary tract infections (Rouphael et al., 2023), but also in life-threatening infections caused by MDR organisms, mainly as part of combination therapy (Antonello et al., 2020). Unfortunately, with the more frequent use of FOS in clinical practice, the FOS resistance level is also increasing.

FOS resistance arises via several well-characterized molecular mechanisms. The first is reduced drug uptake: mutations or deletions of FOS transporters, glycerol-3-phosphate transporter (GlpT) and/or the glucose-6-phosphate transporter (UhpT), as well as in their regulators (e. g., *uhpA*, *uhpB*, *cyaA*, *ptsI*) (Ohkoshi et al., 2017; Nilsson et al., 2003). These alterations of transporter/regulators lead to reduced cell permeability, resulting in FOS minimum inhibitory concentrations (MICs) increase. Second, target modification can be detected: although rare in clinical isolates, point mutations of the active site Cys115 of MurA can reduce FOS binding and thus its efficacy (Cao et al., 2019). The third is enzymatic inactivation mediated by FosA enzymes. FosA is a glutathione-S-transferase that catalyzes the opening of the FOS epoxide ring, leading to FOS inactivation (Zurfluh et al., 2020). Currently, 14 genetically related *fosA* variants on chromosomes and plasmids in members of the genus *Enterobacterales* have been reported in the GenBank database.

The prevalence of *fosA*-type genes mediated by plasmids is increasing globally. Findlay et al., in a comprehensive study, demonstrated a high prevalence of *fosA3* localized on conjugative plasmids of various *Inc*-types in an international collection of *E. coli* isolates. Less frequently detected *fosA*-types in this study were four *fosA4* (Findlay et al., 2023). Similar conclusions were reached in Portuguese national study, where Grilo et al. also described the dominance of the transferable *fosA3* followed by *fosA4* in *E. coli* (Grilo et al., 2023). There are also reports of *fosA3* in *Citrobacter freundii*: a recent Italian clinical carbapenemase producer (KPC-2, VIM-1) with the presence of the *fosA3* gene, carried on a large conjugative plasmid (Mattioni Marchetti et al., 2024). Many of these plasmids carrying *fosA* also harbored ESBL or carbapenemase genes, underscoring the mobility and clinical risk of *fosA*-mediated resistance, as well as the potential for spreading resistance to additional antibiotics.

National surveillances on FOS resistant strains are scarce in Europe due to the limited diagnostic approaches for MICs and for rapid FosA enzymes' identification. In the Czech Republic, FOS has not been available until 2014 and only one multicentric national surveillance on *fosA* genes incidence among carbapenemase-producing *Enterobacterales* dated back to 2023 (Mattioni Marchetti et al., 2023). This study aims to assess the prevalence of FOS resistance among *Enterobacterales* in Czech health-care settings and to provide a comprehensive characterization of the dominant molecular resistance mechanisms in these isolates.

2. Materials and methods

2.1. Study design, bacterial collection, susceptibility determination and FosA detection

In this study nine different catchment tertiary care hospitals, spanning throughout the Czech Republic, have been included: University Hospital Pilsen (FNP), General University Hospital in Prague (VFN), University Hospital Hradec Kralove (HK), University Hospital Brno (FNB), St. Anne's University Hospital in Brno (FNUSA), University Hospital Ostrava (OST), University Hospital Olomouc (OL), Motol University Hospital in Prague (MOT), and Na Homolce Hospital in Prague (HOM). Participating hospitals were requested to collect FOS-resistant isolates of *E. coli*, *Proteus* spp., and *Citrobacter* spp. identified during the year 2024. Resistance to FOS was defined according to the EUCAST breakpoint criteria (version 14.0). Other *Enterobacterales* species, as *Serratia* spp. and *Klebsiella* spp., were excluded due to the intrinsic chromosomal presence of the *fosA* gene. To avoid duplicate and clonally related isolates, only one isolate per patient was included. In addition, isolates originating from the same hospital department and sharing identical antibiograms were excluded to minimize the inclusion of potential clonal strains. FOS susceptibility testing was performed using the EUCAST-recommended agar dilution method. Alternatively, gradient diffusion (E-test) or disk diffusion methods were accepted as screening approaches when performed and interpreted according to EUCAST standards.

Identification of strains was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) with MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). FOS MICs were confirmed using Agar-dilution method (ADM) and interpreted according to EUCAST clinical breakpoints v 15.0, whereas the production of FosA-like and FosC2 enzymes was detected by the PPF test (Nakamura et al., 2014). All FosA producers' strains underwent *fosA*-like gene amplification via Polymerase-chain reaction (PCR), as reported elsewhere (Freire et al., 2023).

2.2. Carbon-source growth test (CSG)

The activity of GlpT and UhpT transporters was investigated by carbon-source growth test (CSG), as reported elsewhere (Huang et al., 2021). Each strain was investigated in duplicate and *E. coli* ATCC 25922 was used as positive control. Absence of growth in the presence of G3P and/or G6P is related to functional deficiency of the transporters GlpT and UhpT, respectively (Huang et al., 2021).

2.3. Whole-genome sequencing

Genomic DNA was extracted from the 14 *fosA*-positive isolates using NucleoSpin Microbial DNA kit (Macherey-Nagel, Germany). Whole genome sequencing (WGS) was performed on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA). Initial paired-end reads were quality trimmed using the Trimmomatic tool v0.40 (Bolger et al., 2014) and then assembled by use of the de Bruijn graph-based *de novo* assembler SPAdes v4.2.0 (<https://github.com/ablab/spades>). Specification on the reads' quality are available on Supplementary table S1. Assembled sequences were annotated using the RAST (Rapid Annotation using Subsystems Technology) server. *In-silico* multilocus sequence typing (MLST) evaluation of the strains, when applicable, was accomplished through PubMLST database (<https://pubmlst.org>). Reconstruction of the resistome, plasmidome, and virulome of the isolates was accomplished using ResFinder v4.7.2, PlasmidFinder v2.1, and the Virulence Factors Database (VFDB) via ABRicate (github.com/tseemann/ABRicate).

2.4. Phylogenetic analysis

In order to track strains transmission path and, thus, to understand their microevolution, SNP-based core genome phylogenies of each STs were accomplished on the FosA-producing strains using parsnp v1.2 (<https://github.com/marbl/parsnp>). Genomes were retrieved from Enterobase (<https://enterobase.warwick.ac.uk>) for *E. coli* and from PubMLST (<https://pubmlst.org/organisms/proteus-spp>) for *Proteus* spp. In detail, a total of 127 *P. mirabilis* ST135 and 27 *P. mirabilis* ST185 were compared with fosA3-harboring *P. mirabilis* from this study using *P. mirabilis* HI4320 (AM942759.1) as reference. Referring to *E. coli*, a total of 169 *E. coli* ST550 and 220 *E. coli* ST1308 genomes were compared, using *E. coli* TUM9803 (CP135671.1) as reference genome. Considering *E. coli* ST69 and ST58, due to large datasets, a genome selection was applied, including all genomes harboring fosA-like genes or genomes from the Czech Republic, obtaining the following datasets: n=80 *E. coli* ST58 and n=131 *E. coli* ST69. For both *E. coli* and *Proteus* spp., a SNP cutoff of ≤ 20 was used for defining close clonal relationship, as reported elsewhere (de Been et al., 2015). The built datasets were additionally evaluated for the resistome and plasmidome. A graphic illustration of the trees was built with the Interactive Tree Of Life (iTOL) (<https://itol.embl.de/>).

2.5. Protein mutations

To molecularly investigate the presence of amino acid mutation in MurA, GlpT and UhpT transporters, BLAST alignment was conducted using the following amino acid reference sequences:

MurA *E. coli* (AAA24187.1), GlpT *E. coli* (CAA68598.1), UhpT *E. coli* (AAA24723.1), MurA *P. mirabilis* (WP_004245292.1), GlpT *P. mirabilis* (WP_004245406.1). The effects of amino acid alterations on the biological function were predicted using the online Sorting Intolerant from Tolerant tool (SIFT) (<https://sift.bii.a-star.edu.sg>). The SIFT involves searching for similar protein sequences, creating an alignment, and

calculating a score for a given substitution. A score below 0.05 indicates a "deleterious" substitution that likely affects protein function, while a score of 0.05 or higher suggests the change is "tolerated".

3. Results

A total of 211 preliminary FOS-resistant *Enterobacterales* strains were collected in the period January-December 2024 among the nine enrolled hospitals (Fig. 1). The isolates originated from different departments, of which the General Medicine (17.5 %), Urology (15.2 %), Intensive Unit Care (ICU) (10 %) and Follow-up Care (8.5 %) were the most numerically represented (Fig. 2A). Urine was the most represented biological sample (84.4 %), followed by soft tissue (5.2 %), respiratory tract (4.3 %), blood (2.8 %), abdominal cavity (1.4 %), rectal and vaginal swab (0.9 %, respectively) (Fig. 2B).

The collection included 149/211 *P. mirabilis*, 57/211 *E. coli*, 3/211 *P. vulgaris*, 1/211 *P. hauseri*, and 1/211 *C. amalonaticus* (Fig. 2C). All species strains were confirmed through MALDI-TOF MS. Increased FOS MICs were highlighted in all *Proteus* spp. (FOS MIC > 64 mg/L), while the FOS resistance phenotype was confirmed in all *E. coli* strains using ADM (FOS MIC > 8 mg/L). The PPF test pointed out the probable production of FosA enzymes in 34/211 strains (*P. mirabilis* n = 29, *E. coli* n = 5). Moreover, all 211 isolates were subjected to a carbon-source growth test (CSG test), which revealed impairment in the FOS transporters' systems. Among *Proteus* spp. (n = 153), 93.46 % exhibited reduced growth in the presence of G3P, indicating impaired GlpT transporter function. In the remaining 10 isolates (6.54 %), no alteration in GlpT activity was detected; notably, five of these isolates harbored the fosA gene, which may explain the observed FOS resistance (Fig. 2D). The activity of the UhpT transporter was not assessed in *Proteus* spp. due to the natural absence of the corresponding gene. Among *E. coli* and *C. amalonaticus* isolates, 98.28 % demonstrated impaired GlpT activity. In addition, all isolates (100 %) showed reduced growth in the presence

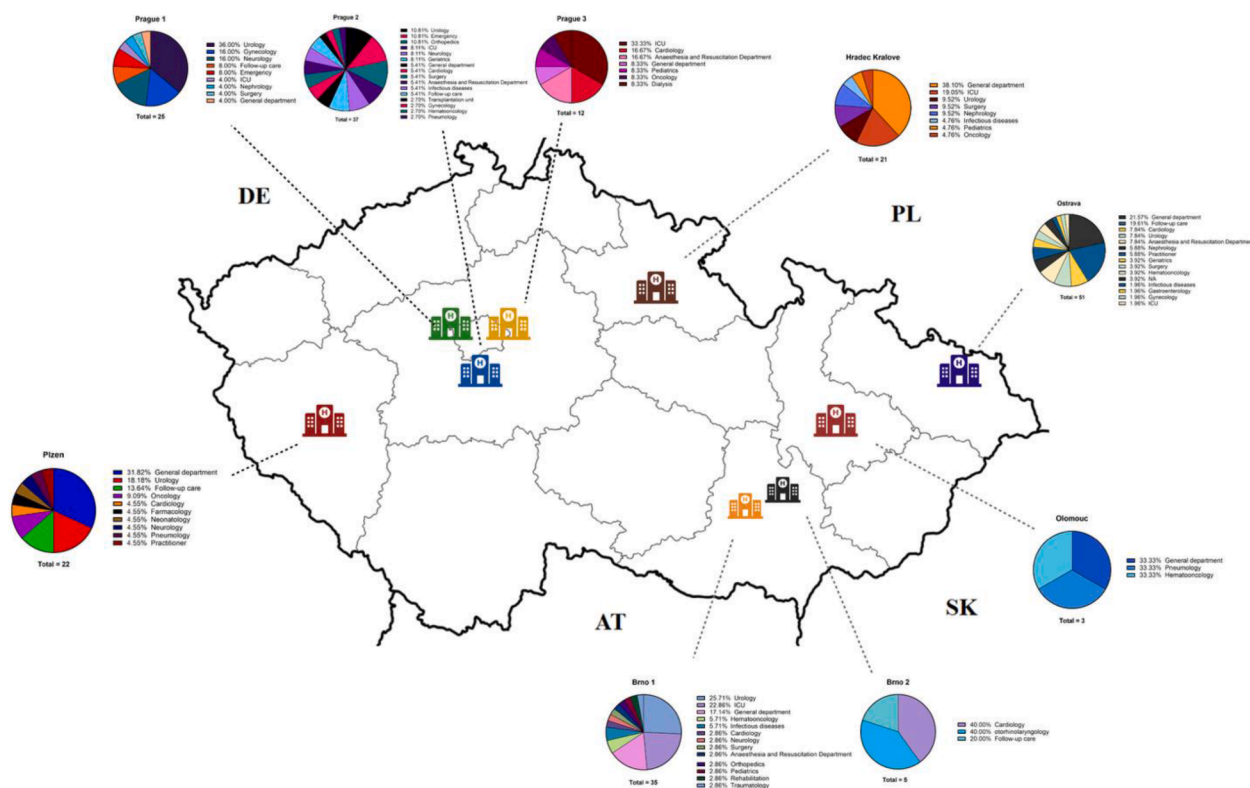


Fig. 1. Maps of clinical settings included in the one-year study. The pie chart reflects the percentage of FOSR strains isolated from different wards. Prague 1 = General University Hospital in Prague; Prague 2 = Motol University Hospital in Prague; Prague 3 = Na Homolce Hospital in Prague; Brno 1 = University Hospital Brno; Brno 2 = St. Anne's University Hospital in Brno.

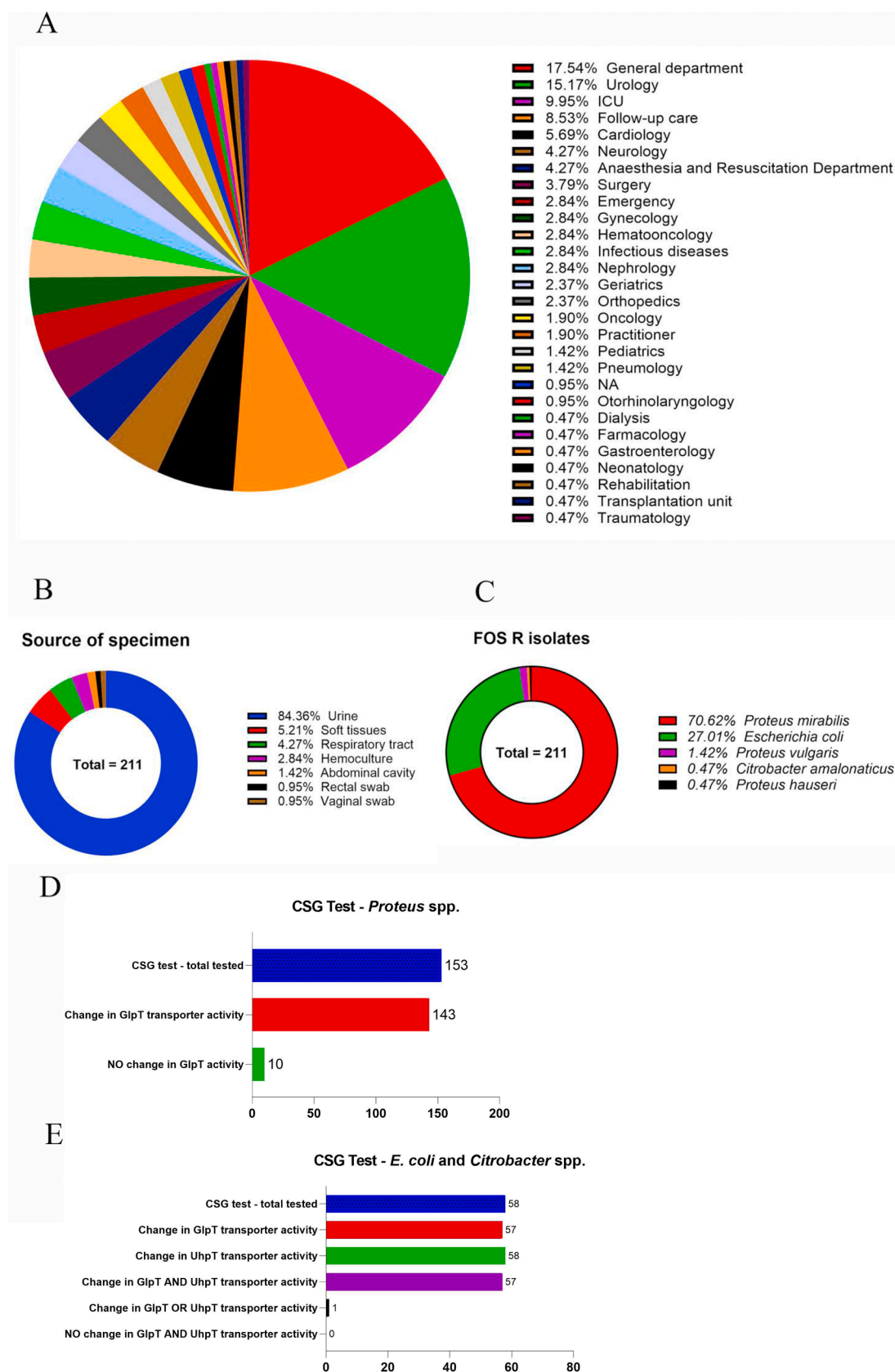


Fig. 2. A: Pie chart of the total amount of strains collected from different wards (expressed in percentage). B: Source of specimens. C: species identification and composition expressed in percentage. D: Carbon source test results for *P. mirabilis*. E: Carbon source test results for *E. coli*.

of G6P, consistent with impaired UhpT transporter activity. Impairment of both GlpT and UhpT transporters was observed in 98.28 % of these isolates, while in 1.72 %, only one transporter impairment was implied (Fig. 2E). These transporter impairments were associated with increased FOS MICs in most of the tested strains.

3.1. Molecular and WGS analysis

All strains with a positive PPF test were subsequently investigated through PCR for the presence of *fosA* genes, confirming in 14/34 strains (9/14 *P. mirabilis* and 5/14 *E. coli*). All 14 *fosA*-carrying strains underwent WGS and displayed substantial genomic diversity. In detail, seven

Table 1
WGS and SIFT results.

Sample ID	Strain	ST	<i>fosA</i> allelic variant	BL genes	Plasmid Types	MurA	GlpT	UhpT
FNP2	<i>P. mirabilis</i>	135	<i>fosA3</i>	<i>bla</i> CTX-M-65, <i>bla</i> OXA-1	ND	ND	ND	NE
VFN10	<i>P. mirabilis</i>	135	<i>fosA3</i>	<i>bla</i> CTX-M-65, <i>bla</i> OXA-1	ND	ND	ND	NE
VFN13	<i>E. coli</i>	550	<i>fosA3</i>	<i>bla</i> CTX-M-3, <i>bla</i> TEM	Col(BS512) IncFII	ND	E448K	E350Q
VFN18	<i>P. mirabilis</i>	185	<i>fosA3</i>	<i>bla</i> CTX-M-65	ND	ND	ND	NE
VFN24	<i>E. coli</i>	69	<i>fosA4</i>	<i>bla</i> TEM-215	Col156 IncFIB(AP001918) IncFIC(FII)	ND	L297F, T348N, E443Q, Q444E, E448K	ND
VFN27	<i>P. mirabilis</i>	185	<i>fosA3</i>	<i>bla</i> CTX-M-65	ND	ND	ND	NE
VFN28	<i>E. coli</i>	69	<i>fosA4</i>	<i>bla</i> TEM-215	IncFII	ND	L297F, E299K, T348N, E443Q, Q444E, E448K	ND
HK4	<i>P. mirabilis</i>	185	<i>fosA3</i>	<i>bla</i> CTX-M-65	ND	ND	ND	NE
HK6	<i>P. mirabilis</i>	185	<i>fosA3</i>	<i>bla</i> CTX-M-65	ND	ND	ND	NE
HK10	<i>P. mirabilis</i>	185	<i>fosA3</i>	<i>bla</i> CTX-M-65	ND	ND	ND	NE
HK11	<i>P. mirabilis</i>	185	<i>fosA3</i>	<i>bla</i> CTX-M-65	ND	ND	ND	NE
OL3	<i>P. mirabilis</i>	185	<i>fosA3</i>	<i>bla</i> CTX-M-65	ND	ND	ND	NE
MOT4	<i>E. coli</i>	58	<i>fosA4</i>	<i>bla</i> TEM-1	IncFIB(AP001918) IncFIC(FII)	F322L	E448K	Y60F
HOM20	<i>E. coli</i>	1308	<i>fosA3</i>	<i>bla</i> CTX-M-55, <i>bla</i> LAP-2, <i>bla</i> TEM	IncFIB(pB171) IncFII(pCoo) IncHI2 IncHI2A	ND	E448K	S122N

ND = not detected; NE = not evaluated

RED = deleterious mutations – changing the protein functionality

GREEN = not deleterious mutations – NOT changing/minimal change of the protein functionality

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out of nine *P. mirabilis* belonged to ST185 and the remaining two to ST135. This variety increased among the five FosA-producing *E. coli*, where ST69 (n=2), ST58 (n=1), ST1308 (n=1) and ST550 (n=1) were identified (Table 1). *FosA3* emerged as the leading allelic variant (85.7 %), followed by *fosA4* (14.3 %). Interestingly, *fosA4* was uniquely detected in *E. coli* strains (n=2 ST 69 and n=1 ST58), while *fosA3* reached both *E. coli* and *P. mirabilis* (Table 1). Moreover, except for *fosA4*, all strains were co-harboring *bla*CTX-M-like genes (n= 8 *bla*CTX-M-65, n= 1 *bla*CTX-M-55 and n=1 *bla*CTX-M-3). All *fosA4*-carrying *E. coli* co-harbored *bla*TEM-215 ESβL. Differently, plasmidome analysis depicted a more homogeneous distribution of replicons. In fact, IncF replicons emerged in five out of 12, Col type in two and IncHI2 in one (Table 1). No replicon types were spotted among *P. mirabilis* strains, speculating a chromosomal location for *fosA3* (Table 1).

3.2. *P. mirabilis* ST185

Seven FosA-producing strains carried the allelic variant *fosA3* (Table 1, Fig. 3A). These seven strains all belong to ST185. Based on the SNP-based core genome phylogeny, all seven ST185 *P. mirabilis* clustered together, revealing high-level relatedness (SNPs ≤ 11). Interestingly, this Czech cluster included strains obtained from three different Czech regions: Hradec Kralove (n= 4/7), Olomouc (n=1/7) and Prague (n=2/7). Despite the clonal relation, strains from Prague (VFN27 and VFN18) presented more SNPs difference compared to OL3 (Olomouc) (SNPs= 7) and Hradec Kralove strains (HK4, HK6, HK10 and HK11) (SNPs= 9-11)(Fig. 3B). VFN18 and VFN27 were clonally identical (SNPs = 0), although collected from different patients on 20th May and 2nd September 2024 (Supplementary Table S2). The Czech cluster did not show similar genomic link with other *fosA3*-harboring and no-*fosA3*-harboring *P. mirabilis* ST185, revealing the closest join with “791” and “559” (SNPs = 772-776), collected in China (Fig. 3B), suggesting a remote connection with Chinese food-chains animals.

3.3. *P. mirabilis* ST135

Two FosA3-producing *P. mirabilis* (FNP2 and VFN10) were assigned as ST135. The strains were obtained from different patients: FNP2 was collected on 12th January 2024 at the University Hospital Pilsen, while VFN10 on 22nd March 2023 at the General University Hospital of Prague. Despite the different date and place of origin, FNP2 and VFN10 manifested a close genomic link (SNPs = 15), clustering in the same branch (Fig. 3C). As well as *P. mirabilis* ST185, FNP2 and VFN10 were the only representative strains from the Czech Republic and showed a distant evolution path to other ST135 genomes (SNPs ≥ 1,000).

3.4. *E. coli* ST550

The only case of *E. coli* ST550 (VFN13), an emerging MDR Extra-intestinal Pathogenic *E. coli*, belonged to phylogroup B2, harbored the *fosA3* variant and was collected at the Neurology ward of General University Hospital in Prague on 22nd April 2024. The core genome phylogeny highlighted a more homogeneous relation with other European *E. coli* ST550 genomes. In detail, VFN13 clustered with a wide branch including several North European *E. coli* ST550 of clinical origin, as “RC_6272AA” from Norway (SNPs = 58) and “XB_5121AA” from Denmark (SNPs = 59) (Fig. 4A). Interestingly, based on the metadata analysis, none of the included genomes owned any *fosA* variants. These data not only certified the consistent circulation of *E. coli* ST550 but the eventual entry of such ST within Czech Republic from the North European side as well.

3.5. *E. coli* ST1308

HOM20 was obtained from the Cardiology ward of Na Homolce Hospital in Prague on 15th July 2024. Based on the WGS analysis,

HOM20 was assigned to ST1308 and carried the *fosA3* variant. The core genome phylogeny and the available metadata revealed a worldwide circulation of ST1308 mainly in the animal ecosystem (Fig. 3B). Interestingly, HOM20 disclosed relevant linkage with a USA cluster, collected from livestock animals (Fig. 4B). No genomic relatedness was indicated with other *fosA3*-carrying *E. coli* ST1308. This evidence supports the hypothesis of a link of diffusion with farm animals of US origin and of a subsequent plasmid-mediated acquisition of the *fosA3* gene.

3.6. *E. coli* ST58

MOT4 was identified on 3rd February 2024 from the Gynecology department of Motol University Hospital in Prague. WGS analysis recognized the presence of *fosA4* variant and the association with ST58 (clonal complex 155). From a phylogenetic perspective, MOT4 is evolutionarily distant from other *fosA*-like-carrying ST58 isolates circulating in different ecological niches within the Czech Republic (Fig. 5A). Interestingly, MOT4 showed the highest genomic similarity with ZC_3535AA, collected in the USA from an unknown source (Fig. 5A). As the least widespread and studied gene of the *fosA* family, the presence of *fosA4* in a high-risk clone such as ST58 in the Czech Republic could indicate the beginning of an epidemiologic variation of FOS-resistant phenotypes.

3.7. *E. coli* ST69

The remaining FosA-producing *E. coli*, VFN24 and VFN28, were both obtained from the Gynecology ward of General University Hospital in Prague. Moreover, VFN24 and VFN28 shared the *fosA4* variant and belonged to ST69. Unexpectedly, this strain resulted genomically distant, carrying more than 2,600 SNPs differences, suggesting different evolution and diffusion path. This divergence was reflected in similarities with the compared genomes. In fact, VFN24 manifested close linkage with ZC_6749AA (SNPs= 22), obtained from human niches in the USA, and other relevant likeness (SNPs = 40-55) with ST69 of human origin from China. VFN28 exhibited a different evolutionary path, showing similarities with human isolates from China, but with a higher phylogenetic distance (SNPs = 170-181) (Fig. 5B). Interestingly, in both clusters VFN24 and VFN28 were the sole representatives of *fosA4*-carrying. The data further suggest connections with different ecological niches in the United States and China but also highlight a difference in the acquisition of FOS resistance genes, favoring the stabilization of *fosA4*.

3.8. Single amino acid mutations

Among the 14 sequenced strains, GlpT disclosed the wider pattern of single-point mutations not affecting protein function (Table 1). Among *E. coli*, E448K was the predominant, followed by L297F, T348N, E443Q and Q444E. Interestingly, VFN28 presented the mutation E299K, predicted as affecting protein function. No altering mutations were detected in GlpT among *P. mirabilis* strains. No mutations with a SIFT score <0.05 were identified within MurA. These findings show a multifactorial involvement of FOS resistance, which may contribute to a reduced sensitivity to the drug also following loss of *fosA* genes.

4. Discussion

The use of FOS has gained optimistic interest in the treatment of serious infections caused by MDR and carbapenemase-producing *Enterobacterales*. However, its recent reuse in clinical practice has highlighted a series of limitations that make difficult not only to evaluate it but also to study the resistance phenomena associated with it. Both EUCAST and CLSI recognize ADM as the reference method for FOS MIC evaluation (Chudejova et al., 2024). Faster alternatives can be found in disc diffusion methods but applicable on *E. coli* only (Chudejova

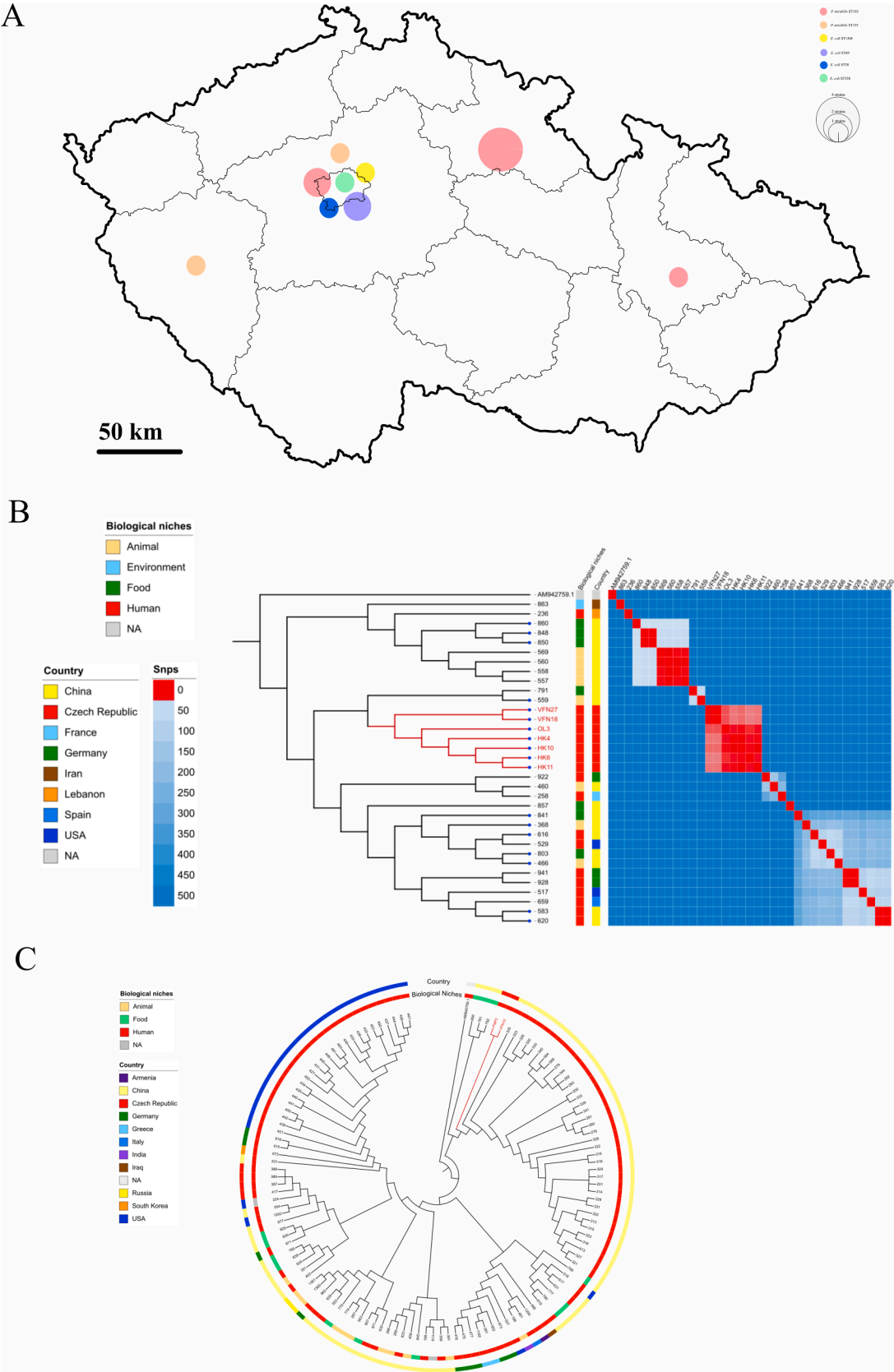


Fig. 3. A: epidemiological map of the FosA enzymes circulating within the Czech Republic. B: SNP-based phylogeny pictured using iTOL v6 of the available *P. mirabilis* ST135. C: SNP-based phylogeny pictured using iTOL v6 of the available *P. mirabilis* ST185.

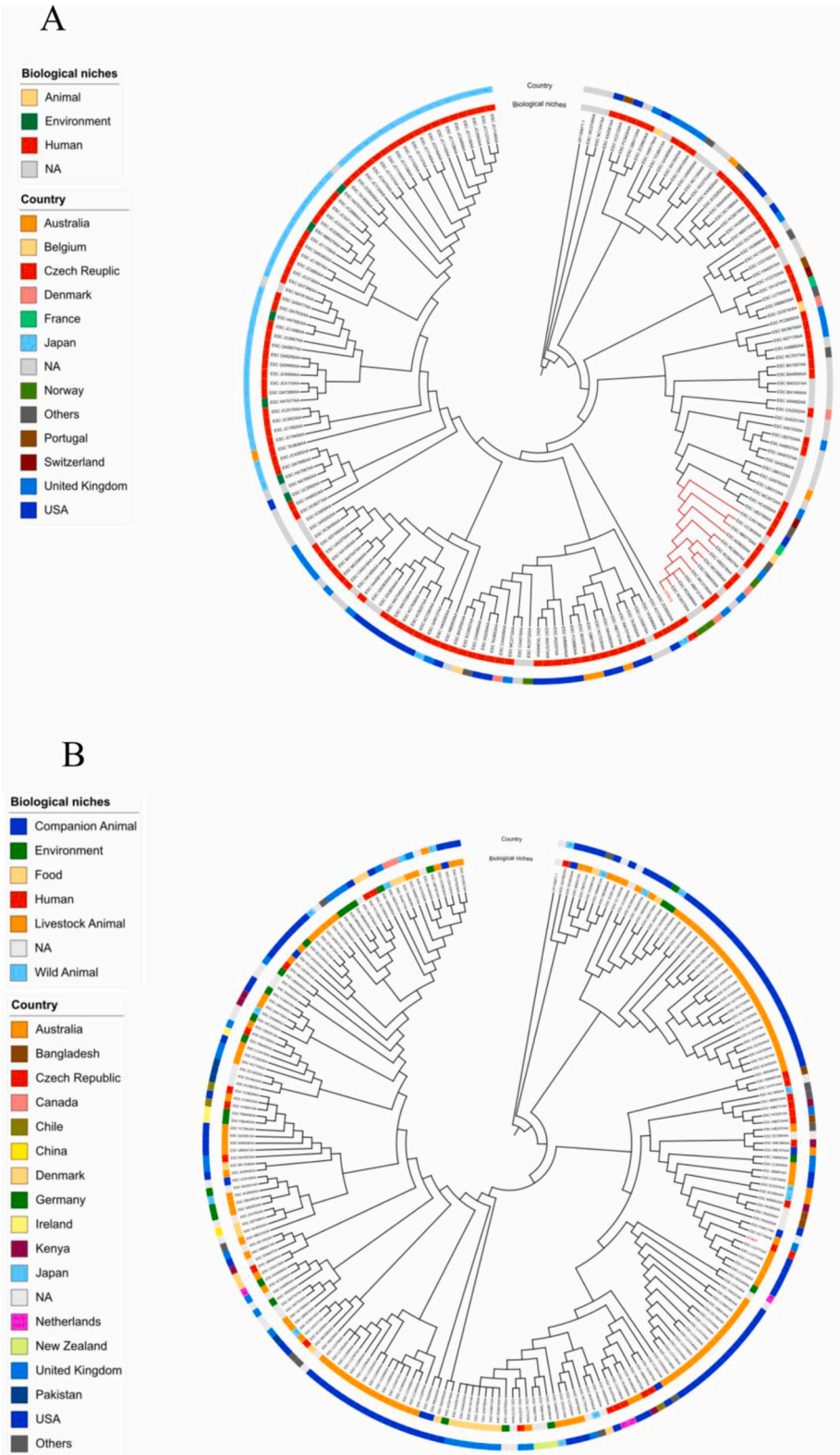


Fig. 4. A: SNP-based phylogeny pictured using iTOL v6 of the available *E. coli* ST550. B: SNP-based phylogeny pictured using iTOL v6 of the available *E. coli* ST1308.

et al., 2024).

In this study PPF test revealed a positivity percentage of 16.11 %, against a 6.63 % of detected *fosA* genes, with a 58.82 % of false positive results. Based on our results is PPF test applicable on *E. coli* only (100 % specificity), but with limited use for other *Enterobacterales* due to reduced specificity. This phenotypic performance is in line with

literature results, where a reduction in sensitivity for species as *Proteus* spp. can occur due to naturally lack of UhpT transporter (O'May et al., 2009). For this evidence, the molecular approach may provide the most reliable results.

Contrary to what was reported in the literature so far, FOS-resistant *P. mirabilis* was the leading encountered species in our study and the one

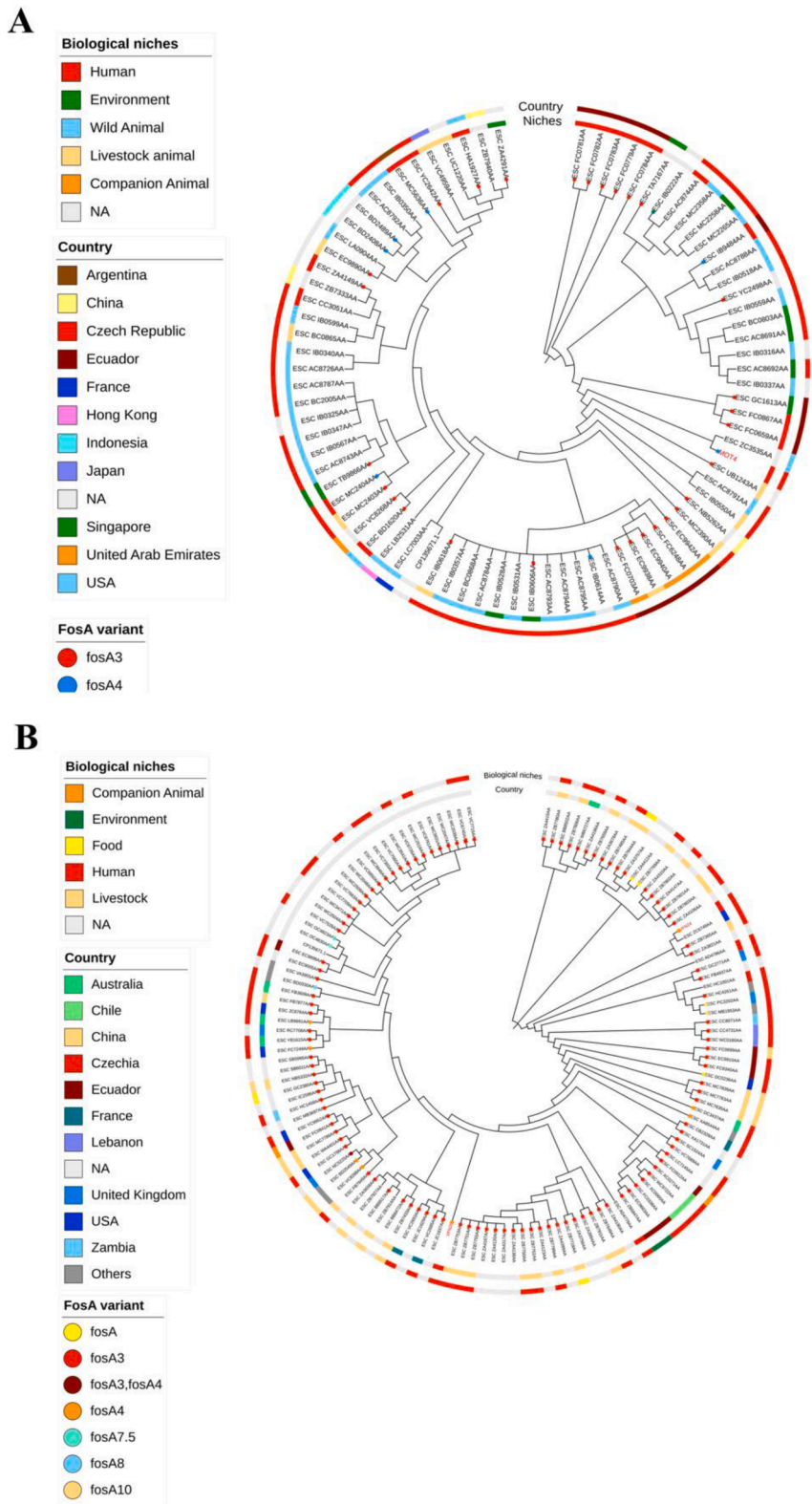


Fig. 5. A: SNP-based phylogeny pictured using iTOL v6 of the available *E. coli* ST58. B: SNP-based phylogeny pictured using iTOL v6 of the available *E. coli* ST69.

most associated with *fosA3* genes. *P. mirabilis* is an opportunistic pathogen that commonly causes catheter-associated urinary tract infections, wound infections, gastroenteritis and, in some cases, bacteremia. In recent years, unusual cases of *Proteus* spp. infections have emerged with increasing regularity on a large scale (Su et al., 2025). However, the literature is still scarce on this phenomenon in Europe. Loras and

co-authors evidence the presence of only two FosA3-producing strains among 623 FOS-resistant *P. mirabilis*, marking the low incidence of *fosA3* in *P. mirabilis* (Loras et al., 2023). The evident epidemiological gap of FOS-resistant *P. mirabilis* requires greater attention at the clinical level, using both phenotypic and molecular approaches, in order to avoid misidentifications.

The ST135 lineage includes international clones that are emerging in Asian continent due to its potential in acquiring various carbapenemase genes, as *bla*OXA-48, *bla*VIM-78, and *bla*NDM-1 (Mavroidi et al., 2025; Guo et al., 2025). The association *P. mirabilis* ST135 and *FosA3* is rare but already pointed out in China by Guo and co-authors from human urine and feces (Guo et al., 2025). Similarly, Su and colleagues highlight the circulation of *bla*CTX-M-65 + *fosA3* *P. mirabilis* ST135 in the Chinese veterinary niche (Su et al., 2025). In our study both ST135s showed clonal relatedness, while no significant similarities have been identified with other ST135s, suggesting the emergence of a clonal ST135 group uniquely within the Czech Republic.

The ST185 is a not-well characterized *P. mirabilis* group that is rarely reported on a global level. Among Chinese isolates it has been associated with *bla*CTX-M-65 and *fosA3* in clinical health-care settings (Guo et al., 2025). In our study the ST185 represented the largest ST and the most widespread within the Czech hospitals. As well as ST135, the seven *P. mirabilis* ST185 exhibited the *bla*CTX-M-65 and *fosA3* co-harboring, suggesting the implication of horizontal transferability. The seven ST135 emerged in three different locations and times: in Hradec Kralove in February-March 2024, in Olomouc in March 2024, and in Prague from May to September 2024. This geographical diversity seems also reflected in genetic variability, with the Prague clones being genetically more distant from those of Hradec Kralove and Olomouc.

E. coli ST69 is a notorious extraintestinal pathogenic strain and the leading lineage causing urinary tract infections (Mattioni Marchetti et al., 2021). It has been implicated in horizontal acquisition of several hydrolyzing enzymes, such as CTX-M and KPC (Giacobbe et al., 2015). The occurrence of *fosA4* within *E. coli* ST69 is a rare event that has been reported only in Pakistan from wild birds' feces and in Australia from humans (Munir et al., 2025; Mowlaboccus et al., 2020).

In this study, we highlighted the occurrence of the *fosA3* in *E. coli* ST1308, a strictly veterinary clone, genomically connected with livestock animals from the USA (Elias et al., 2019). The use of FOS is worldwide but heterogeneous, with a restricted use to human niches in European countries and an expanded usage in both human and animal niches in Latin America countries (Juscelle et al., 2025). According to the Food and Agriculture Organization of the United States, the use of FOS is allowed in treating *E. coli*-related diarrhoea and salmonellosis in cattle, pseudotuberculosis in Perciformes, and other various bacterial infections in broiler chickens and pigs. For this reason, FOS is classified as a highly important veterinary antimicrobial agent (<https://www.fao.org/faoterm/viewentry/en/?entryId=175085>).

From December 2018 to February 2022 a genomic surveillance on FOS resistance among carbapenemase-producing *Enterobacterales* was conducted in the Czech Republic. In fact, the overall rate of FOS resistance *Enterobacterales* was around 29 %, with impairment in transporters as leading mechanisms (97.6 %) against *FosA* production (2.4 %) (Mattioni Marchetti et al., 2023). In this study, the share of *FosA* enzyme-producing strains rose to 6.6 %, underlining an increase in the trend that is approaching the global estimated share (Seok et al., 2020). This increase can be explained by several factors. Firstly, the Czech Republic has witnessed an increased use of the FOS molecule in clinical practice and, in combination, against severe infections due to the increased rates of general antibiotic resistance (Mattioni Marchetti et al., 2023; Fajfr et al., 2017). Secondly, during the COVID-19 pandemic and post-pandemic period, the Czech Republic experienced an increase in tourism and international travel, especially in Pilsen and Prague, with an annual increase of 4.4 % (<https://www.czechtourism.cz>). Moreover, as reported by the Czech National Bank (CNB), the Czech Republic population has increased significantly due to an influx of refugees from Ukraine following the 2022 invasion, with Ukrainians making up the largest group of the more than one million foreign nationals now living in the country (<https://www.cnb.cz>).

According to Research Institute for Labour and Social Affairs (RILSA), the largest population increases in the Czech Republic after the start of the Ukraine war have been concentrated in Prague, followed by

Central Bohemia, South Moravia, and Pilsen and Hradec Kralove regions (<https://www.rilsa.cz/>). In our study, those regions were the most affected by *fosA*-like genes circulation. In this historical period characterized by an ever-increasing number of intracontinental movements, it is essential to strengthen molecular surveillance systems for those molecules that were previously considered minor, such as FOS.

5. Conclusion

A depiction of the current circulation of FOS-resistant *Enterobacterales* in clinical settings in the Czech Republic has been provided. The results raise concerns about the increase in strains harboring acquired *fosA* genes and the limitations of culture-based methods available in clinical practice. The molecular approach needs to be implemented and complemented with culture-based methods, to monitor and contain the phenomenon of FOS resistance. Multifactorial approaches in FOS-resistant phenomenon are essential for the timely and specific detection of FOS resistance and for the optimal management of antimicrobial stewardship programs, which aim to preserve the effectiveness of FOS.

Data availability

The nucleotide sequences of the 14 genomes were deposited and are available in GenBank under the Bioproject ID [PRJNA1373516](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1373516).

Ethical statement

The work described herein is an observational study performed on bacterial isolates from human samples that were obtained as part of routine hospital care and used anonymously. Consent to participate was not required, as samples were collected as part of standard patient care.

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CRedit authorship contribution statement

Katerina Chudejova: Conceptualization, Investigation, Validation, Writing – original draft, Writing – review & editing. **Vittoria Mattioni Marchetti:** Conceptualization, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Vita Zaccaria:** Investigation, Writing – review & editing. **Stepanka Kanova:** Resources, Writing – review & editing. **Anna Sramkova:** Resources, Writing – review & editing. **Marcela Krutova:** Resources, Writing – review & editing. **Lenka Ryskova:** Resources, Writing – review & editing. **Gabriela Kroneislova:** Resources, Writing – review & editing. **Beata Horvathova:** Resources, Writing – review & editing. **Renata Tejkalova:** Resources, Writing – review & editing. **Eva Krejci:** Resources, Writing – review & editing. **Iva Vagnerova:** Resources, Writing – review & editing. **Zuzana Zemanova:** Resources, Writing – review & editing. **Ibrahim Bitar:** Data curation, Formal analysis, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

I finally confirm that there are no conflict of interest by all the authors.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.crmicr.2026.100582](https://doi.org/10.1016/j.crmicr.2026.100582).

References

- Antonello, R.M., Principe, L., Maraolo, A.E., Viaggi, V., Pol, R., Fabbiani, M., Montagnani, F., Lovecchio, A., Luzzati, R., Di Bella, S., 2020. Fosfomycin as partner drug for systemic infection management. A systematic review of its synergistic properties from *In vitro* and *In Vivo* Stud. *Antibiot.* 9 (8), 500. <https://doi.org/10.3390/antibiotics9080500>. Aug 10.
- Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30 (15), 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
- Cao, Y., Peng, Q., Li, S., Deng, Z., Gao, J., 2019. The intriguing biology and chemistry of fosfomycin: the only marketed phosphonate antibiotic. *RSC Adv.* 9 (72), 42204–42218. <https://doi.org/10.1039/c9ra08299a>. Dec 19.
- Chudejova, K., Caltagirone, M.S., Mattioni Marchetti, V., Rezzani, A., Navarra, A., Bitar, I., 2024. FosA8-producing *E. coli* ST131: clinical cases in Italy, February 2023. *Euro. Surveill.* 29 (21), 2400276. <https://doi.org/10.2807/1560-7917.ES.2024.29.21.2400276>. May.
- de Been, M., Pinholt, M., Top, J., Bletz, S., Mellmann, A., van Schaik, W., Brouwer, E., Rogers, M., Kraat, Y., Bonten, M., Corander, J., Westh, H., Harmsen, D., Willems, R.J., 2015. Core genome multilocus sequence typing scheme for high-resolution typing of *Enterococcus faecium*. *J. Clin. Microbiol.* 53 (12), 3788–3797. <https://doi.org/10.1128/JCM.01946-15>. Dec.
- Elias, L., Gillis, D.C., Gurrola-Rodriguez, T., Jeon, J.H., Lee, J.H., Kim, T.Y., Lee, S.H., Murray, S.A., Ohta, N., Scott, H.M., Wu, J., Rogovskyy, A.S., 2019. The occurrence and characterization of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolated from clinical diagnostic specimens of equine origin. *Anim* 10 (1), 28. <https://doi.org/10.3390/ani10010028>. Dec 21.
- Fajfr, M., Louda, M., Paterová, P., Rysková, L., Pacovský, J., Košina, J., Žemličková, H., Brodák, M., 2017. The susceptibility to fosfomycin of Gram-negative bacteria isolates from urinary tract infection in the Czech Republic: data from a unicentric study. *BMC Urol* 17 (1), 33. <https://doi.org/10.1186/s12894-017-0222-6>.
- Falagas, M.E., Athanasaki, F., Voulgaris, G.L., Triarides, N.A., Vardakas, K.Z., 2019. Resistance to fosfomycin: mechanisms, frequency and clinical consequences. *Int. J. Antimicrob. Agents* 53 (1), 22–28. <https://doi.org/10.1016/j.ijantimicag.2018.09.013>.
- Findlay, J., Sierra, R., Raro, O.H.F., Aires-de-Sousa, M., Andrey, D.O., Nordmann, P., 2023. Plasmid-mediated fosfomycin resistance in *Escherichia coli* isolates of worldwide origin. *J. Glob. Antimicrob. Resist.* 35, 137–142. <https://doi.org/10.1016/j.jgar.2023.09.003>. Dec.
- Freire, S., Grilo, T., Nordmann, P., Poirel, L., Aires-de-Sousa, M., 2023. Multiplex PCR for detection of acquired plasmid-borne fosfomycin resistance *fos* genes in *Escherichia coli*. *Diagn. Microbiol. Infect. Dis.* 105 (2), 115864. <https://doi.org/10.1016/j.diagmicrobio.2022.115864>. Feb.
- Giacobbe, D.R., Del Bono, V., Coppo, E., Marchese, A., Viscoli, C., 2015. Emergence of a KPC-3-producing *Escherichia coli* ST69 as a cause of bloodstream infections in Italy. *Microb. Drug Resist.* 21, 342–344. <https://doi.org/10.1089/mdr.2014.0230>.
- Grilo, T., Freire, S., Miguel, B., Martins, L.N., Menezes, M.F., Nordmann, P., Poirel, L., Sousa, M.J.R., 2023. Aires-de-Sousa M. Occurrence of plasmid-mediated fosfomycin resistance (*fos* genes) among *Escherichia coli* isolates, Portugal. *J. Glob. Antimicrob. Resist.* 35, 342–346. <https://doi.org/10.1016/j.jgar.2023.08.001>. Dec.
- Guo, J., Xu, H., Wu, C., Yu, H., Yang, L., Qi, Y., Zuo, X., Yang, H., Zhang, L., Yu, Y., Li, X., 2025. Global phylogeography and genomic characterization of bla_{NDM-1}-positive clinical *Proteus mirabilis* isolates from China. *mSystems* 10 (10), e0075825. <https://doi.org/10.1128/mSystems.00758-25>. Oct 22.
- Huang, L., Cao, M., Hu, Y., Zhang, R., Xiao, Y., Chen, G., 2021. Prevalence and mechanisms of fosfomycin resistance among KPC-producing *Klebsiella pneumoniae* clinical isolates in China. *Int. J. Antimicrob. Agents* 57 (1), 106226. <https://doi.org/10.1016/j.ijantimicag.2020.106226>. Jan.
- Juscelle, F., Saidenberg, A.B.S., Christoffersen, L.E.B., Edslev, S.M., Hallström, S., Nacarato, J.R., Barbosa, F.B., Cunha, M.P., Esposito, F., Lincopan, N.H., Stegger, M., Knöbl, T., 2025. *Escherichia coli* in Brazilian poultry fecal samples: Co-carriage of fosfomycin and ESBL resistance. *Antibiot* 14 (3), 269. <https://doi.org/10.3390/antibiotics14030269>. Mar 6.
- Loras, C., Pérez-Vázquez, M., González Prieto, A., Oteo-Iglesias, J., Alós, J.-I., 2023. Prevalence of *fosA3* gene in Fosfomycin-resistant *Proteus mirabilis* in the Autonomous Community of Madrid (Spain) and characterization of two positive strains. *Microb. Drug Resist.* 29 (10), 444–447. <https://doi.org/10.1089/mdr.2022.0269>.
- Mattioni Marchetti, V., Kraftova, L., Finianos, M., Sourenian, T., Hrabak, J., Bitar, I., 2023. Polyclonal spread of fosfomycin resistance among carbapenemase-producing members of the enterobacterales in the Czech Republic. *Microbiol. Spectr.* 11 (3), e0009523. <https://doi.org/10.1128/spectrum.00095-23>. Jun 15.
- Mattioni Marchetti, V., Venturelli, L., Cassetti, T., Meschiari, M., Migliavacca, R., Bitar, I., 2024. FosA3 emerging in clinical carbapenemase-producing *C. freundii*. *Front Cell Infect. Microbiol.* 14, 1447933. <https://doi.org/10.3389/fcimb.2024.1447933>. Aug 6.
- Mavroidi, A., Froukala, E., Spanakis, N., Michelaki, A., Orfanidou, M., Koumaki, V., Tsakris, A., 2025. Genomic insights of emerging multidrug-resistant OXA-48-producing ST135 *Proteus mirabilis*. *Antibiot* 14 (8), 750. <https://doi.org/10.3390/antibiotics14080750>. Jul 25.
- Mowlaboccus, S., Daley, D., Pang, S., Gottlieb, T., Merlino, J., Nimmo, G.R., George, N., Korman, T.M., Streitberg, R., Robson, J., Peachey, G., Collignon, P., Bradbury, S., Colombi, E., Ramsay, J.P., Rogers, B.A., Coombs, G.W., 2020. Identification and characterisation of fosfomycin resistance in *Escherichia coli* urinary tract infection isolates from Australia. *Int. J. Antimicrob. Agents* 56 (4), 106121. <https://doi.org/10.1016/j.ijantimicag.2020.106121>. Oct.
- Munir, A., Lu, X., Humak, F., Kurekci, C., Mahmood, M.S., Gul, S., Wang, Z., Mohsin, M., Li, R., 2025. Emergence of plasmid-mediated fosfomycin resistance among *Escherichia coli* harboring *fosA4*, *tet(X4)*, and *mcr-1* genes in wild birds. *mSystems* 10 (4), e0167324. <https://doi.org/10.1128/mSystems.01673-24>. Apr 22.
- Nakamura, G., Wachino, J., Sato, N., Kimura, K., Yamada, K., Jin, W., Shibayama, K., Yagi, T., Kawamura, K., Arakawa, Y., 2014. Practical agar-based disk potentiation test for detection of fosfomycin-nonsusceptible *Escherichia coli* clinical isolates producing glutathione S-transferases. *J. Clin. Microbiol.* 52 (9), 3175–3179. <https://doi.org/10.1128/JCM.01094-14>. Sep.
- Nilsson, A.I., Berg, O.G., Aspevall, O., Kahlmeter, G., Andersson, D.I., 2003. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. *Antimicrob. Agents Chemother.* 47 (9), 2850–2858. <https://doi.org/10.1128/AAC.47.9.2850-2858.2003>. Sep.
- O'May, G.A., Jacobsen, S.M., Longwell, M., Stoodley, P., Mobley, H.L.T., Shirtliff, M.E., 2009. The high-affinity phosphate transporter Pst in *Proteus mirabilis* H4320 and its importance in biofilm formation. *Microbiol* 155 (Pt 5), 1523–1535. <https://doi.org/10.1099/mic.0.026500-0>. May/Epub 2009 Apr 16.
- Ohkoshi, Y., Sato, T., Suzuki, Y., Yamamoto, S., Shiraishi, T., Ogasawara, N., Yokota, S.I., 2017. Mechanism of reduced susceptibility to Fosfomycin in *Escherichia coli* clinical isolates. *Biomed. Res. Int.* 2017, 5470241. <https://doi.org/10.1155/2017/5470241>.
- Paranos, P., Vourli, S., Pournaras, S., Meletiadi, J., 2022. Assessing clinical potential of old antibiotics against severe infections by multi-drug-resistant gram-negative bacteria using *In silico* modelling. *Pharm* 15 (12), 1501. <https://doi.org/10.3390/ph15121501>. Nov 30.
- Putensen, C., Ellger, B., Sakka, S.G., Weyland, A., Schmidt, K., Zoller, M., Weiler, N., Kindgen-Milles, D., Jaschinski, U., Weile, J., Lindau, S., Kieninger, M., Falthäuser, A., Jung, N., Teschendorf, P., Adamzik, M., Gründling, M., Wahlers, T., Gerlach, H., Litty, F.A., 2019. Current clinical use of intravenous fosfomycin in ICU patients in two European countries. *Infection* 47 (5), 827–836. <https://doi.org/10.1007/s15010-019-01323-4>. Oct.
- Rouphael, N., Winokur, P., Keefer, M.C., Traenkle, J., Drobeniuc, A., Doi, Y., Munsiff, S., Fowler, V.G., Evans, S., Oler, R.E., Tuyishimire, B., Lee, M., Ghazaryan, V., Chambers, H.F., 2023. DMID 15-0045 study group. Daily fosfomycin versus levofloxacin for complicated urinary tract infections. *mBio* 14 (5), e0167723. <https://doi.org/10.1128/mBio.01677-23>. Oct 31.
- Zurfluh, K., Treier, A., Schmitt, K., Stephan, R., 2020. Mobile fosfomycin resistance genes in Enterobacteriaceae—an increasing threat. *Microbiologyopen* 9 (12), e1135. <https://doi.org/10.1002/mbo3.1135>. Dec.
- Su, H., Yang, L., Liu, M., Pan, X., Li, H., Sha, W., Yin, B., Dong, W., Li, G., 2025. Investigation of genomic islands involved in antimicrobial resistance in *Proteus mirabilis* isolated from swine. *J. Glob. Antimicrob. Resist.* 44, 160–165. <https://doi.org/10.1016/j.jgar.2025.06.003>. Sep.
- Seok, H., Choi, J.Y., Wi, Y.M., Park, D.W., Peck, K.R., Ko, K.S., 2020. Fosfomycin resistance in *Escherichia coli* isolates from South Korea and *in vitro* activity of Fosfomycin alone and in combination with other antibiotics. *Antibiot* 9 (3), 112. <https://doi.org/10.3390/antibiotics9030112>. Mar 6.