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Brief report

Complete genome characterization of *mcr-1*-mediated colistin-resistant *Escherichia coli* from outpatients in Bulgaria

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ABSTRACT

Introduction: This study reports the emergence of *mcr-1*-mediated colistin resistance in human *Escherichia coli* isolates from Bulgaria.
Methods: Three colistin-resistant *E. coli* isolates were obtained from outpatient urine specimens. They were subjected to PCR for detection of *mcr* genes and conjugation experiments. Whole-genome sequencing was employed to analyze the genomic characteristics of the isolates.
Results: PCR identified *mcr-1* in all isolates. In *E. coli* of sequence type (ST) 2067, *mcr-1.1* was found on a self-transmissible IncI2 plasmid, while *mcr-1.32* was chromosomal in the remaining two ST131 *E. coli* isolates. *E. coli* ST2067 co-harbored quinolones resistance mutations (*gyrA*^{D87N}, *gyrA*^{S83L}, *parC*^{S80I}), β-lactam (*bla*$_{TEM-30}$) and aminoglycoside (*aadA1*, *aac(3)-IId*) resistance genes.
Conclusion: This report further confirms the role of IncI2 conjugative plasmids in the dissemination of *mcr* genes. Our findings involving chromosomal *mcr-1* in high-risk ST131 *E. coli* strains from outpatients underscores the need for enhanced surveillance and systematic screening to combat colistin resistance.

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Caracterización completa del genoma de *Escherichia coli* resistente a la colistina mediada por *mcr-1* procedente de pacientes ambulatorios en Bulgaria

Palabras clave:
Resistencia a la colistina
Escherichia coli
ST2067
ST131
mcr-1
Plásmido IncI2
mcr-1.32 localizado cromosómicamente

RESUMEN

Introducción: En este estudio se informa de la aparición de resistencia a la colistina mediada por *mcr-1* en cepas humanas de *Escherichia coli* procedentes de Bulgaria.
Métodos: Se obtuvieron tres aislados de *E. coli* resistentes a la colistina a partir de muestras de orina de pacientes ambulatorios. Se sometieron a PCR para la detección de genes *mcr* y a experimentos de conjugación. Se empleó la secuenciación del genoma completo para analizar las características genómicas de los aislados.
Resultados: La PCR identificó *mcr-1* en todos los aislados. En *E. coli* de tipo de secuencia (ST) 2067, *mcr-1.1* se encontró en un plásmido IncI2 autotransmisible, mientras que *mcr-1.32* era cromosómico en los dos aislados restantes de *E. coli* ST131. *E. coli* ST2067 albergaba mutaciones de resistencia a quinolonas (*gyrA*^{D87N}, *gyrA*^{S83L}, *parC*^{S80I}), a β-lactámicos (*bla*$_{TEM-30}$) y a aminoglucósidos (*aadA1*, *aac(3)-IId*).
Conclusiones: Este informe confirma aún más el papel de los plásmidos conjugativos IncI2 en la diseminación de genes *mcr*. El hallazgo de *mcr-1* cromosómico en cepas de *E. coli* ST131 de alto riesgo procedentes de pacientes ambulatorios subraya la necesidad de mejorar la vigilancia y el cribado sistemático para combatir la resistencia a la colistina.

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Introduction

Colistin has been recognized as a last resort antibiotic for treating infections caused by multi-resistant Gram-negative bacteria. However, in 2016, Liu et al. described the first plasmid-mediated colistin resistance gene (*mcr-1*) found in *Escherichia coli* from animal and human samples, and later its emergence was tracked back to the 1980s. MCR enzymes modify lipid A through phosphoethanolamine transfer blocking the attachment of colistin and conferring resistance or reduced susceptibility.[1]

Because of the increasing resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides, carbapenems have become the preferred antibiotics for the treatment of complicated infections. However, this has led to the emergence and spread of carbapenem-resistant *E. coli*, which often have a multidrug-resistant phenotype, requiring treatment with β-lactam–β-lactamase inhibitor combinations (e.g. ceftazidime–avibactam, meropenem–vaborbactam, imipenem–cilastatin–relebactam), cefiderocol, or combination therapy with other drugs, including colistin.[2,3] The primary obstacle to colistin-based therapy, in addition to its toxic nature, is the rapid surge in colistin resistance. The plethora of chromosomal and often intricate resistance mechanisms have been succeeded by mobile colistin resistance (*mcr*) genes, frequently detected among colistin-resistant *E. coli*.[1] Existing studies have shown that *mcr* genes are usually located on plasmids of IncI2, IncX4, IncP, IncX and IncFII types, and their spread is aided by insertion sequences (IS) such as IS*Apl1* and transposon Tn*6330*.[1,4] Although rare, chromosomally-encoded *mcr* genes have also been reported.[5]

The aim of this study was to investigate the genomic characteristics of three clinical *E. coli* strains harboring the *mcr-1* gene, which was plasmid-encoded in one isolate and chromosomal in two of them. As far as we are aware, this is the first report of *mcr-1*-positive bacteria from human sources in Bulgaria.

Material and methods

Bacterial isolation, identification, antimicrobial susceptibility testing and conjugation experiments

The three isolates (EC707, EC2947, EC1752) were recovered from urine specimens of outpatients presented with community-acquired dysuria in the primary care unit of a cancer hospital. Isolate EC707 was collected in March 2022 from a 38-year-old woman, EC2947 – in October 2022 from a 56-year-old woman, and EC1752 – in May 2023 from a 46-year-old woman.

Isolates were identified by MALDI-TOF Biotyper (Bruker Daltonics GmbH, Bremen, Germany) with MALDI Reference 2022 Library v.4.0.

Colistin resistance was initially screened with SuperPolymyxin medium,[6] which was performed on all 2342 *E. coli* isolates recovered from patients' specimens between January 2017 and December 2023. Clinical specimens were obtained from infected or colonized patients admitted to hospital wards or attending outpatient departments of a 252-bed oncology hospital in Sofia, Bulgaria.

Colistin resistance of the three screened isolates, which represented 0.1% of all isolates tested, was then confirmed by broth microdilution with MIC-strip colistin (Bruker Daltonics GmbH, Bremen, Germany). The minimum inhibitory concentrations (MICs) of a wide range of antimicrobials were also determined using the MicroScan NM-EN52 panel (Beckman Coulter, Inc., Brea, CA, USA) following the manufacturer's protocol. Susceptibility to fosfomycin was determined by the disk diffusion method on Mueller–Hinton agar with disks supplied by Becton Dickinson (BD, Sparks, MD, USA). The results of susceptibility testing were interpreted according to EUCAST clinical breakpoints v14.0. *E. coli* ATCC 25922 and *E. coli* NCTC 13846 were used for quality control.

The transmissibility of colistin resistance was tested by mating with sodium azide-resistant *E. coli* J53 on Luria-Bertani agar plates containing 150 mg/L sodium azide and 2 mg/L colistin sulphate. Transconjugants were confirmed by susceptibility testing and PCR.

Screening for mcr *genes*

Total genomic DNA for PCR and whole-genome sequencing (WGS) was extracted using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Missouri, TX, USA) with all homogenization steps carried out by pipetting.

Multiplex PCR for detection of *mcr* genes was performed as described previously.[7]

Whole-genome sequencing and bioinformatic analysis

Long-read sequencing was performed on MinION Mk1C with the Rapid Barcoding Kit 96 (SQK-RBK110.96) and FLO-MIN106D (R9.4.1) (Oxford Nanopore Technologies, Oxford, UK).

Read filtering was performed with Filtlong v0.2.1 (https://github.com/rrwick/Filtlong, accessed 6.12.23). Assemblies were produced with Trycycler v0.5.3,[8] followed by polishing with MEDAKA v1.7.3 (ONT, https://github.com/nanoporetech/medaka, accessed 21.2.24). Screening for antimicrobial resistance genes was done with AMRFinderPlus v3.11.4.[9] Plasmids were analyzed with Abricate (Seemann T, Abricate, Github https://github.com/tseemann/abricate) using the PlasmidFinder database (v2023-01-18).[10] Multi-locus sequence typing (MLST) was performed using the Achtman scheme with mlst v2.23.0 (Seemann T, mlst Github https://github.com/tseemann/mlst, accessed 21.2.24). Closely related sequences were retrieved by BLAST using the nr/nt database (Update date: 19 March 2024). Gview[11] and Clinker[12] were used for visualization.

Results

PCR revealed a 213 bp band characteristic for *mcr-1* in all *E. coli* isolates. Complete genomes were obtained for all three isolates (European Nucleotide Archive accession number PRJEB70793). EC707 had a genome size of 4.9 Mb, carrying three plasmids of sizes 154 kb (with IncFIB and IncFIC replicons), 110 kb (with IncI1-I(Alpha) replicon), and 64 kb (IncI2 replicon). Similarly, EC1752 and EC2947 had genome sizes of ∼4.97 Mb and ∼4.99 Mb, respectively. Each of them possessed two nearly identical plasmids of sizes 125 kb (with IncFIB and IncFII replicons), and 6.6 kb (untypable).

In silico MLST identified EC707 as sequence type (ST) 2067, while both EC1752 and EC2947 were ST131.

WGS analysis of EC707 revealed mutations in genes associated with resistance to quinolones (*gyrA*^{D87N}, *gyrA*^{S83L}, *parC*^{S80I}), fosfomycin (*glpT*^{E448K}), and colistin (*pmrB*^{Y358N}). Detected resistance genes included *mcr-1.1*, *tet(A)*, *aadA1*, *aac(3)-IId* and the inhibitor-resistant broad-spectrum *bla*$_{TEM-30}$.

EC1752 and EC2947 had a similar resistance profile, characterized by mutations in genes associated with fosfomycin (*glpT*^{E448K}, *uhpT*^{E350Q}), colistin (*pmrB*^{E123D}), and quinolone (*parE*^{I529L}) resistance, along with the *mcr-1.32* gene. Interestingly, all resistance determinants in these two strains were chromosomally encoded.

The *mcr-1.1* variant in EC707 was located on the 64 kb IncI2 plasmid (pNOC-22-mcr-1). The ten genetically closest plasmids were retrieved from NCBI through BLAST analysis, and compared with pNOC-22-mcr-1 (Fig. 1). A 99.9% identity was found between pNOC-

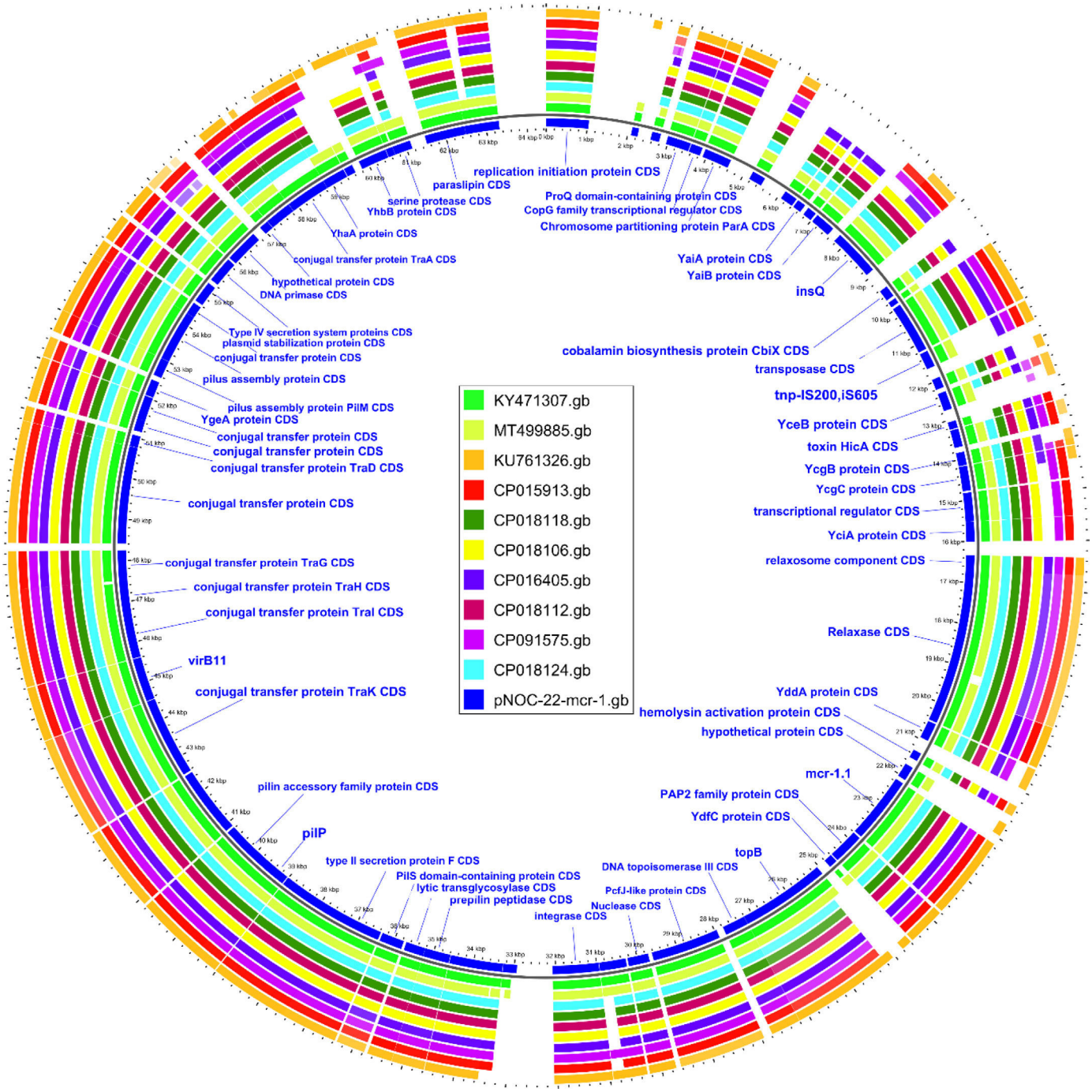

Fig. 1. Comparison of pNOC-22-mcr-1 with the 10 closest plasmids retrieved from BLAST (≥90% identity).

22-mcr-1 and an IncI2 plasmid (pMCR-GN775; KY471307) from a Canadian isolate belonging to ST624.[13]

Analysis of the genetic structures, which surrounded the *mcr-1.1* segment containing the PAP2 gene, revealed the lack of IS*Apl1* similar to that of pMCR-GN775 (KY471307) (Fig. 2), and of two IncI2 plasmids previously described in isolates from Colombian hospitals, and belonging to ST58 and ST46.[14]

In EC1752 and EC2947, the segment containing *mcr-1.32* and PAP2 genes was chromosomally located within a truncated IS*66*. Remnants of this IS were found both upstream and downstream of the *mcr* segment. Besides the IS*66* remnant downstream of PAP2, the region consisted of IS*110* followed by a gene encoding lactate dehydrogenase. Upstream of *mcr-1.32,* the region included the remaining IS*66* remnant, followed by two genes – one encoding a hypothetical protein and the other encoding a GTPase family protein. The same genetic structure was previously described in *mcr-1.13*-positive *E. coli* isolates from turkeys and pigs in Italy.[15]

Consistent with the WGS results, a colistin-resistant transconjugant was obtained from EC707 by conjugal transfer of the IncI2 plasmid carrying only *mcr-1*.

All the three isolates, as well as the transconjugant TC-EC707, exhibited resistance to colistin with MIC of 4 mg/L due to *mcr* genes (Table 1). EC1752 and EC2947 remained susceptible to all other antimicrobials tested, while EC707 showed resistance to penicillins and their inhibitor combinations, to gentamicin and tobramycin,

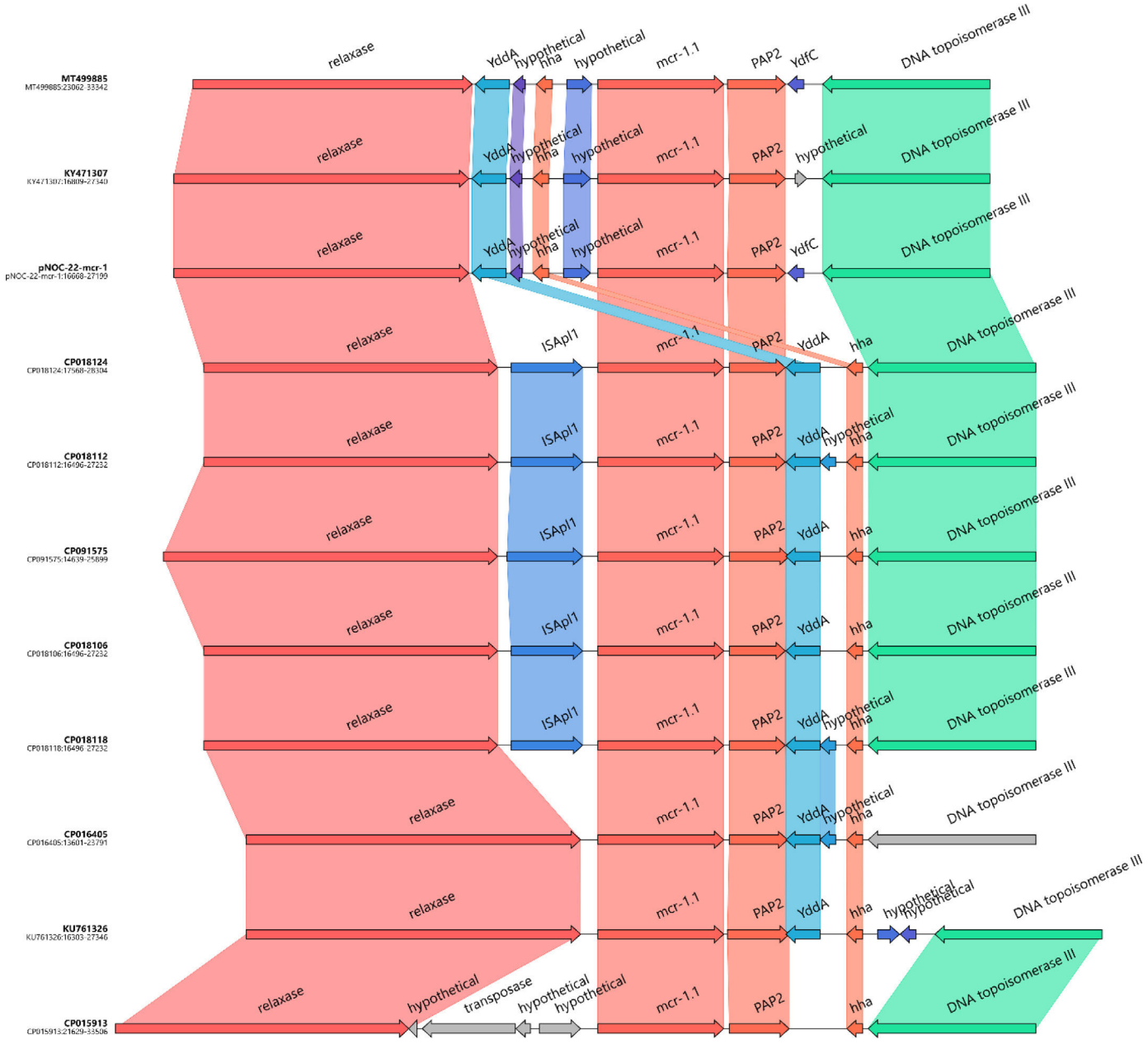

Fig. 2. Comparison of the *mcr-1* segment of pNOC-22-mcr-1 with that of the 10 closest plasmids retrieved by BLAST. Matching genes are shown with the same color.

and to quinolones due to $bla_{\text{TEM-30}}$, to *aac(3)-IId*, and to mutations in *gyrA* and *parC*, respectively. Long-read sequencing of EC707 revealed that $bla_{\text{TEM-30}}$ was located on the IncI1-I(Alpha) plasmid, which also carried the *aac(3)-IId* acetyltransferase gene.

Discussion

The current study describes the first *mcr*-positive *E. coli* isolates of human origin in Bulgaria. Unlike most publications,[4] colistin-resistant isolates in this study were collected only from outpatients. Notably, we observed chromosomal localization of the *mcr-1.32* gene in two isolates, which appears less common compared to the plasmid one. These isolates belonged to the high-risk *E. coli* clone ST131, which was frequently associated with CTX-M-15 production and fluoroquinolone resistance. Fluoroquinolone/cephalosporin-resistant *E. coli* isolates from this clone often carry additional resistance determinants and virulence genes, and are associated with a wide range of infections.[16] Furthermore, we identified *mcr-1.1* in EC707 (ST2067) on a self-transferable IncI2 plasmid, which has been involved in the dissemination of *mcr-1* gene.[13]

Previous studies have associated $pmrB^{\text{Y358N}}$ and $pmrB^{\text{E123D}}$ mutations with colistin resistance. However, their impact remains uncertain.[17] In our study, the presence of *mcr* genes obscured their contribution to the observed colistin MIC.

The detection of *mcr* genes among outpatients raises concerns, considering that colistin susceptibility is not routinely tested in outpatient settings. Therefore, the true extent of *mcr* prevalence in Bulgaria could be underestimated, especially since the zoonotic prevalence is still unidentified. We were able to identify the *mcr*-positive isolates through regular colistin resistance screening performed on all isolates in our laboratory. This emphasizes the importance of systematic surveillance and the incorporation of a targeted screening for colistin resistance for all isolates to mitigate the spread of resistance.

Table 1
Antimicrobial susceptibility of *mcr-1*-positive *Escherichia coli* clinical strains, transconjugant TC-EC707 and *E. coli* J53 recipient strain.

Antibiotic (range tested, mg/L)[a]	EUCAST breakpoints ≤S/>R	*Escherichia coli* clinical strains			Transconjugant TC-EC707	*E. coli* J53
		EC707	EC2947	EC1752		
Amikacin (8–16)	≤8/>8	≤8	≤8	≤8	≤8	≤8
Amoxicillin/clavulanate (4/2–32/16)	≤8/>8	16[c]	≤4	≤4	≤4	≤4
Ampicillin (2–8)	≤8/>8	>8[c]	≤2	≤2	≤2	≤2
Aztreonam (1–4)	≤1/>4	≤1	≤1	≤1	≤1	≤1
Cefepime (1–4)	≤1/>4	≤1	≤1	≤1	≤1	≤1
Cefixime (0.5–1)	≤1/>1	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Cefotaxime (0.5–32)	≤1/>2	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Cefoxitin (8–16)	8 (screen only)	≤8[d]	≤8[d]	≤8[d]	≤8[d]	NA
Cefpodoxime (1)	≤1/>1	≤1	≤1	≤1	≤1	≤1
Ceftazidime (0.5–32)	≤1/>4	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Cefuroxime (4–8)	≤8/>8	≤4	≤4	≤4	≤4	≤4
Ciprofloxacin (0.25–1)	≤0.25/>0.5	>1[c]	≤0.25	≤0.25	≤0.25	≤0.25
Colistin (0.0625–64)[b]	≤2/>2	4[c]	4[c]	4[c]	4[c]	1
Ertapenem (0.12, 0.5–1)	≤0.5/>0.5	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
Gentamicin (2–4)	≤2/>2	>4[c]	≤2	≤2	≤2	≤2
Imipenem (1–8)	≤2/>4	≤1	≤1	≤1	≤1	≤1
Levofloxacin (0.5–1)	≤0.5/>1	>1[c]	≤0.5	≤0.5	≤0.5	≤0.5
Mecillinam (2, 8)	≤8/>8	≤2	≤2	≤2	≤2	≤2
Meropenem (0.12–8)	≤2/>8	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12
Nitrofurantoin (64)	≤64/>64	≤64	≤64	≤64	≤64	≤64
Norfloxacin (0.5–1)	≤0.5/>0.5	>1[c]	≤0.5	≤0.5	≤0.5	≤0.5
Piperacillin (4–16)	≤8/>8	>16[c]	≤8	≤8	≤8	≤8
Piperacillin/tazobactam (4/4–16/4)	≤8/>8	≤4	≤4	≤4	≤4	≤4
Tigecycline (0.5–2)	≤0.5/>0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Tobramycin (2–4)	≤2/>2	>4[c]	≤2	≤2	≤2	≤2
Trimethoprim (2–4)	≤4/>4	≤2	≤2	≤2	≤2	≤2
Trimethoprim/sulfamethoxazole (2/38–4/76)	≤2/>4	≤2	≤2	≤2	≤2	≤2
Fosfomycin[e]	≥24/<24[e]	27[e]	29[e]	28[e]	30[e]	30[e]

[a] MIC values were determined using the MicroScan NM-EN52 panel (Beckman Coulter, Inc., Brea, CA, USA).
[b] MIC of colistin was determined with MIC-strip colistin (Bruker Daltonics GmbH, Bremen, Germany).
[c] MIC values in bold indicate "resistant" categorization.
[d] The obtained value of ≤8 mg/L indicates a negative screening test for AmpC production in the clinical isolates and the transconjugant, respectively; The test is not applicable (NA) for the recipient strain *E. coli* J53, which is designated accordingly.
[e] Susceptibility to fosfomycin was determined by the disk diffusion method. The results obtained in zone diameter (mm) indicate "susceptible" categorization.

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Conflicts of interest

None.

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