



AKADÉMIAI KIADÓ

Resistome analysis of *Escherichia coli* isolates from layers in Hungary

ÁDÁM KEREK^{1,2*}, ÁBEL SZABÓ¹, KRISZTIÁN BÁNYAI^{1,2,4},
ESZTER KASZAB^{2,3,7}, KRISZTINA BALI^{2,7}, MÁRTON PAPP^{2,5},
LÁSZLÓ KOVÁCS⁶ and ÁKOS JERZSELE^{1,2}

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¹ Department of Pharmacology and Toxicology, University of Veterinary Medicine, István street 2, H-1078 Budapest, Hungary

² National Laboratory of Infectious Animal Diseases, Antimicrobial Resistance, Veterinary Public Health and Food Chain Safety, University of Veterinary Medicine Budapest, Hungary

³ One Health Institute, University of Debrecen, Nagyerdei krt. 98., Debrecen, H-4032, Hungary

⁴ Veterinary Medical Research Institute, Hungária krt. 21, H-1143, Budapest, Hungary

⁵ Centre for Bioinformatics, University of Veterinary Medicine, István street 2, H-1078 Budapest, Hungary

⁶ Department of Animal Hygiene, Herd Health and Mobile Clinic, University of Veterinary Medicine, István street 2, H-1078 Budapest, Hungary

⁷ Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, István u 2., Budapest, H-1078, Hungary

RESEARCH ARTICLE



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ABSTRACT

The authors aimed to investigate eight strains of *Escherichia coli* (*E. coli*) strains from Hungarian layer flocks for antimicrobial resistance genes (ARG), using metagenomic methods. The strains were isolated from cloacal swabs of healthy adult layers. This study employed shotgun sequencing-based genetic and bioinformatic analysis along with determining phenotypic minimum inhibitory concentrations. A total of 59 ARGs were identified in the eight *E. coli* isolates, carrying ARGs against 15 groups of antibiotics. Among these, 28 ARGs were identified as transferable. Specifically, four ARGs were plasmid-derived, 18 ARGs were phage-derived and an additional six ARGs were predicted to be mobile, contributing to their mobility and potential spread between bacteria.

KEYWORDS

resistome, antibiotic resistance genes, ARGs, antibiotics, *Escherichia coli*

INTRODUCTION

The discovery of antibiotics made it possible to treat bacterial infectious diseases, but over time, resistance has become widespread (Micoli et al., 2021), and is now one of the most significant global public health issues (von Wintersdorff et al., 2016). Even the most conservative estimates suggest that without any significant intervention, by 2050 nearly 10 million people will die from antimicrobial resistance (AMR) each year (O'Neill, 2014). The resistance mechanisms and genes can be so abundant, that in a single *Escherichia coli* (*E. coli*) strain, almost all known resistance mechanisms can be found, with 68 different resistance genes, making it resistant against 16 widely used antibiotics (Zhang et al., 2019). To reduce the possibilities of resistance development, a number of antibiotic alternatives have emerged, including the use of vaccines as a preventive measure (Micoli et al., 2021), which can prevent bacteria from multiplying to the point where resistance mutations are developed

*Corresponding author.

E-mail: kerek.adam@univet.hu



(Rappuoli et al., 2017). It should also be mentioned that the use of antibiotic resistance genes as marker genes in live bacterial vaccines is strongly discouraged, as these genes can be transferred to humans (El-Attar et al., 2012).

It is also important to note that correlations have been found between several resistance genes and virulence genes, which may lead to the selection of bacteria with different virulence genes (Boerlin et al., 2005). However, in case of some intestinal pathogenic *E. coli* strains it has been observed that the more resistance genes a bacterium has, the less virulence genes it carries (Nagy et al., 2015).

Second-generation sequencing (NGS, next generation sequencing) is a fast parallel sequencing technique, also known as deep or shallow sequencing (Marguerat et al., 2008). New algorithms are continuously being developed to align huge amounts of data, in the form of short reads, identify operons and recombinant variants and build phylogenetic trees based on single nucleotide polymorphisms (SNP), among others (Chan, 2009). At first, sequencing was used for the comprehensive detection of ARGs of *E. coli* by several authors (Veenemans et al., 2014; Hernández-Fillor et al., 2021; Alvarez Narvaez et al., 2022). More recently, the possibility of genome sequencing has also enabled the development of safe vaccines (Prachi et al., 2013) to test strains for efficacy, primarily for virulence factors (Bidmos et al., 2018), but without putting an emphasis on transferable antimicrobial resistance. We aimed to investigate the resistome (complete ARG carriage) of different *E. coli* strains isolated from cloacal swabs of healthy adult poultry using NGS technique and to explore the risks of transmissible antimicrobial resistances for human and for animal health, including the theoretical possibility of using such bacteria for the development of live vaccine candidates.

MATERIALS AND METHODS

Strains

Eight commensal *E. coli* isolates were used in this study, isolated from cloacal swab samples from healthy layer hens in 2020, using Coliform Selective Agar (Biolab Zrt., Budapest, Hungary). These samples were collected from different large laying flocks in different geographical regions of Hungary. They were chosen from among strains collected from flocks based on preliminary phenotypic susceptibility

testing. The selection focused on strains demonstrating the highest resistance to antibiotic agents commonly used in poultry.

Sequencing

DNA from bacterial suspension was isolated using the QIAmp DNA kit (Qiagen, Germany), following the manufacturer's protocol. DNA extraction was performed in a Qiagen Tissue Lyzer LT at 50 Hz for 10 min, with each sample eluting at 50 µg mL⁻¹. Finally, fluorometric quantification was performed using Qubit[®] dsDNA BR Assay kit (Thermo Fisher SSC, Budapest, Hungary).

DNA libraries were prepared using Illumina[®] Nextera XT DNA Library Preparation Kit (Illumina, San Diego, USA). Indexes were used to label DNA fragments using the Nextera XT Index Kit v2 Set C (Illumina, San Diego, USA). The resulting indexed DNA library was purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, Xinpei, Taiwan), following the column purification protocol; and then Qubit[®] dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA) was used for fluorometric quantification for quality control. Paired-end reads generated from the DNA were determined using the Illumina NextSeq 500 sequencer (Sahin-Tóth et al., 2021).

Bioinformatics data processing

Initial quality control of the raw sequences was conducted using FastQC v0.11.9 software (Andrews, 2012), followed by the elimination of sequences with subpar quality using TrimGalore v0.6.7 (Krueger et al., 2021). The read sequences were assembled into longer contigs using MEGAHIT v1.2.9 (Li et al., 2015). For contig quality assessment (Table 1), the QUAST software was employed (Gurevich et al., 2013). All conceivable Open Reading Frames (ORFs) were subsequently identified from the resulting contigs utilizing Prodigal v2.6.3 (Hyatt et al., 2010) and deposited (1028257 BioProject ID).

Identification of ARGs within the ORFs was performed using Resistance Gene Identifier (RGI) v5.1.0 against the CARD database (downloaded on 23/04/2021) (Alcock et al., 2020). Only genes meeting the STRICT threshold criteria established by the CARD database were considered. Furthermore, these genes were only taken into account if they exhibited sequence identity and coverage of at least 95%.

To assess the potential mobility of identified resistance genes, MobileElementFinder (v1.0.3) (Johansson et al., 2021)

Table 1. Quality characteristics of contigs

| Strain | No of trimmed reads | No of contigs | Coverage | NG ₅₀ | NG ₇₅ | LG ₅₀ | LG ₇₅ |
|--------|---------------------|---------------|----------|------------------|------------------|------------------|------------------|
| 1 | 7,783,363 | 306 | 91.973 | 41,186 | 22,355 | 37 | 75 |
| 2 | 3,603,219 | 249 | 86.087 | 77,510 | 43,464 | 20 | 39 |
| 3 | 3,840,473 | 239 | 85.88 | 98,058 | 52,321 | 19 | 34 |
| 4 | 3,412,510 | 221 | 85.25 | 85,768 | 52,888 | 16 | 33 |
| 5 | 3,632,208 | 215 | 83.254 | 100,786 | 52,218 | 13 | 29 |
| 6 | 3,406,337 | 203 | 88.6 | 83,902 | 55,553 | 17 | 34 |
| 7 | 3,440,737 | 138 | 82.821 | 100,453 | 62,485 | 14 | 28 |
| 8 | 3,815,334 | 312 | 83.328 | 88,200 | 44,242 | 19 | 37 |



was employed, predicting Mobile Genetic Elements (MGEs) on the contigs. For evaluating mobility, only ARGs within the range of the longest composite transposon (Tn1681, 24,488 nucleotides in length), typical for *E. coli* in the database, were considered potentially mobile. Additionally, contigs' plasmid origin was investigated using PlasFlow v1.1 software (Krawczyk et al., 2018), while the presence of phage genomes on the contigs was determined using VirSorter v2.2.2 (Roux et al., 2015) software.

As a quality control, we looked at the assembly size (i.e. how many nucleotides there are in the assembly). LG_{50} (length genome metrics) is the least number of contigs that are 50% of the genome length, if we sort the contigs by size. NG_{50} (number genome metrics) is the length of the shortest of these contigs (i.e. those that reach 50% of the genome length). The NG_{75} and LG_{75} values are relative to 75% of the genome. The abbreviations LG_{50} and LG_{75} signify that the shortest contigs reaching 50% and 75% of the genome length are determined when contigs are sorted by size. NG_{50} is always greater than or equal to NG_{75} and LG_{50} is always less than or equal to LG_{75} .

Minimum inhibitory concentration determination

The phenotypic manifestation of AMR was evaluated by determining the minimum inhibitory concentration (MIC) value of each bacterial strain against specific antibiotics. The testing protocol adhered to the methodology outlined by the CLSI (Clinical and Laboratory Standards Institute, 2018).

Breakpoints for interpretation were established using criteria from both the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015) (Table 4).

The active substances used for testing (Merck KGaA, Darmstadt, Germany) included amoxicillin and amoxicillin-clavulanic acid (in a 2:1 ratio) in phosphate buffer solution at pH 6 (0.1 mol L^{-1}); cefquinome, cefotaxime, oxytetracycline, doxycycline, gentamicin, and colistin dissolved in distilled water; imipenem in phosphate buffer solution at pH 7.2 (0.01 mol L^{-1}); sulfamethoxazole dissolved in hot water with a few drops of 2.5 mol L^{-1} NaOH; trimethoprim dissolved with 0.05 mol L^{-1} HCl; and a potent sulphonamide (sulfamethoxazole and trimethoprim in a 20:1 ratio) dissolved as previously described. Enrofloxacin dissolution was prepared with a few drops of 1 mol L^{-1} NaOH solution in distilled water.

The MIC determination range was as follows: $32\text{-}0.06 \mu\text{g mL}^{-1}$ for amoxicillin, amoxicillin-clavulanic acid, gentamicin, oxytetracycline and doxycycline; $16\text{-}0.03 \mu\text{g mL}^{-1}$ for cefquinome, colistin, cefotaxime, imipenem and enrofloxacin; $128\text{-}0.25 \mu\text{g mL}^{-1}$ for sulfamethoxazole, trimethoprim and the potent sulphonamide.

RESULTS

The frequency of each resistance mechanism within each antibiotic group is presented in Fig. 1, while the identified ARGs are comprehensively listed in Table 2. In this analysis,

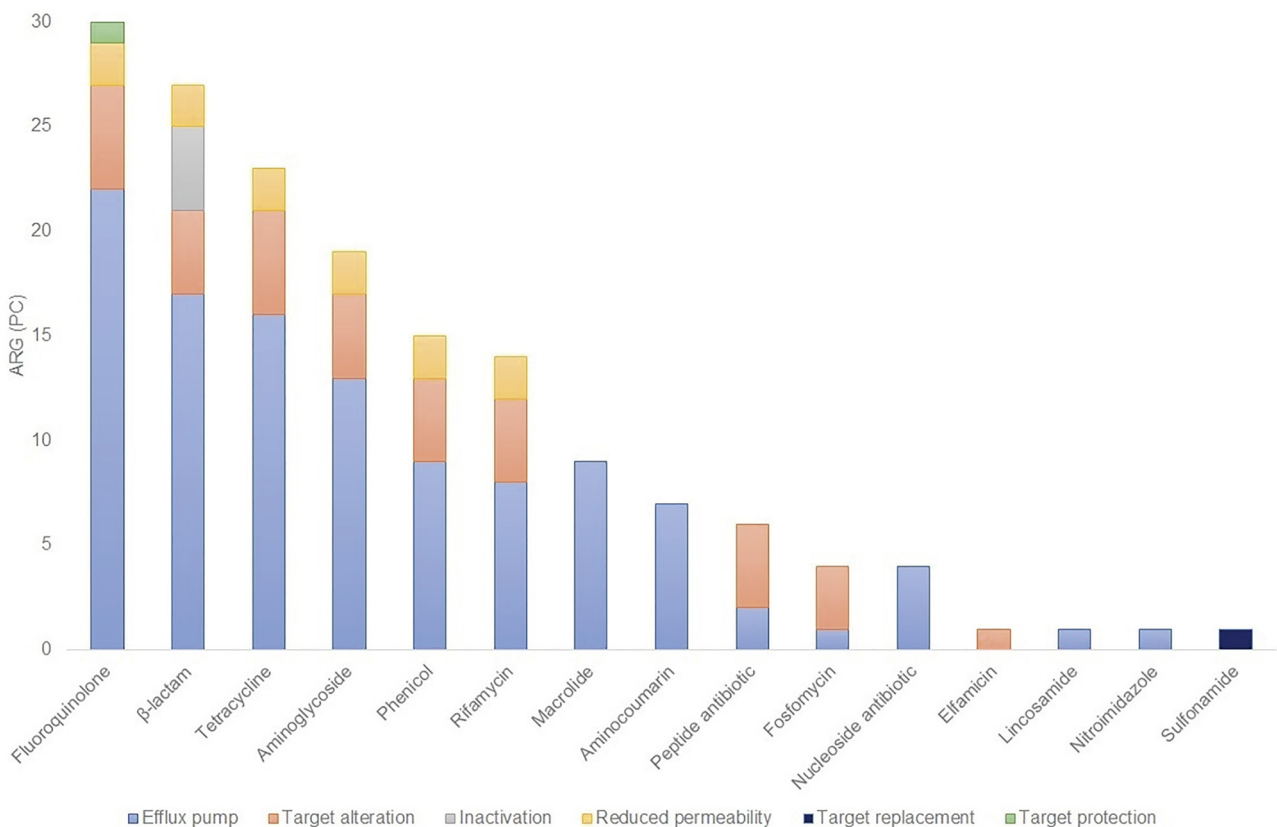


Fig. 1. Frequency of genes encoding major antimicrobial resistance mechanisms by antibiotic group



Table 2. List of antimicrobial resistance genes and gene products identified in the eight *E. coli* isolates investigated. (The number of isolates with the respective genes identified are in parentheses)

| Antimicrobial resistance genes and gene products | Coverage % | Sequence identity % | M ¹ | Ph ² | Pl ³ | Resistance class * | Antibiotic mechanism * |
|--|------------|---------------------|----------------|-----------------|-----------------|---|---|
| <i>acrB</i> (8) | 100 | 100 | | | | fluoroquinolone, cephalosporin, glycylicycline, penam, tetracycline, rifamycin, phenicol, triclosan | antibiotic efflux |
| <i>acrD</i> (8) | 100 | 99.81 | | + | | aminoglycoside | antibiotic efflux |
| <i>acrE</i> (8) | 100 | 100 | | + | | fluoroquinolone, cephalosporin, cephamycin, penam | antibiotic efflux |
| <i>acrF</i> (8) | 100 | 99.61 | | + | | fluoroquinolone, cephalosporin, cephamycin, penam | antibiotic efflux |
| <i>acrS</i> (7) | 100 | 100 | | | | fluoroquinolone, cephalosporin, glycylicycline, cephamycin, penam, tetracycline, rifamycin, phenicol, triclosan | antibiotic efflux |
| <i>bacA</i> (8) | 100 | 100 | | | | peptide antibiotic | antibiotic target alteration |
| <i>baeR</i> (8) | 100 | 99.58 | | + | | aminoglycoside, aminocoumarin | antibiotic efflux |
| <i>baeS</i> (8) | 100 | 99.14 | | + | | aminoglycoside, aminocoumarin | antibiotic efflux |
| <i>cpxA</i> (8) | 100 | 100 | | + | | aminoglycoside, aminocoumarin | antibiotic efflux |
| <i>CRP</i> (7) | 100 | 99.52 | | | | macrolide, fluoroquinolone, penam | antibiotic efflux |
| <i>emrA</i> (8) | 100 | 99.74 | | | | fluoroquinolone | antibiotic efflux |
| <i>emrB</i> (8) | 100 | 100 | | | | fluoroquinolone | antibiotic efflux |
| <i>emrK</i> (8) | 100 | 99.72 | | + | | tetracycline | antibiotic efflux |
| <i>emrR</i> (7) | 100 | 100 | | | | fluoroquinolone | antibiotic efflux |
| <i>emrY</i> (8) | 100 | 99.8 | | + | | tetracycline | antibiotic efflux |
| <i>eptA</i> (8) | 100 | 99.63 | | | | peptide antibiotic | antibiotic target alteration |
| <i>acrA</i> (8) | 100 | 100 | | | | fluoroquinolone, cephalosporin, glycylicycline, penam, tetracycline, rifamycin, phenicol, triclosan | antibiotic target alteration, antibiotic efflux |
| <i>acrR</i> (8) | 100 | 100 | | | | fluoroquinolone, cephalosporin, glycylicycline, penam, tetracycline, rifamycin, phenicol, triclosan | antibiotic target alteration, antibiotic efflux |
| <i>ampC</i> (5) | 100 | 97.35 | + | | | cephalosporin, penam | antibiotic inactivation |
| <i>ampC1</i> (3) | 99.77 | 99.08 | | + | | cephalosporin, penam | antibiotic inactivation |
| <i>ampH</i> (7) | 100 | 99.74 | | | | cephalosporin, penam | antibiotic inactivation |
| <i>cyaA</i> (3) | 100 | 99.29 | | | | fosfomycin | antibiotic target alteration |
| EF-Tu (5) | 96.33 | 99.75 | | | | elfamycin | antibiotic target alteration |
| <i>emrE</i> (8) | 100 | 98.18 | | + | | macrolide | antibiotic efflux |
| <i>glpT</i> (8) | 100 | 99.78 | | | | fosfomycin | antibiotic target alteration |
| <i>gyrA</i> (3) | 100 | 99.77 | | | | fluoroquinolone | antibiotic target alteration |
| <i>marR</i> (8) | 100 | 98.61 | | | | fluoroquinolone, cephalosporin, glycylicycline, penam, tetracycline, rifamycin, phenicol, triclosan | antibiotic target alteration, antibiotic efflux |
| <i>mdfA</i> (8) | 100 | 96.59 | | | | tetracycline, benzalkonium chloride, rhodamine | antibiotic efflux |
| <i>soxR</i> (8) | 100 | 100 | | + | | fluoroquinolone, cephalosporin, glycylicycline, penam, tetracycline, rifamycin, phenicol, triclosan | antibiotic target alteration, antibiotic efflux |
| <i>soxS</i> (8) | 100 | 100 | | + | | fluoroquinolone, cephalosporin, glycylicycline, penam, tetracycline, rifamycin, phenicol, triclosan | antibiotic target alteration, antibiotic efflux |

¹M: Mobile genetic elements; ²Ph: located on Phage; ³Pl: located on Plasmid.

* The Comprehensive Antibiotic Resistance Database (Alcock et al., 2023).

| Antibiotic resistance genes (ARG) and gene products | Coverage % | Sequence identity % | M ¹ | Ph ² | Pl ³ | Resistance class* | Antibiotic mechanism* |
|---|------------|---------------------|----------------|-----------------|-----------------|---|------------------------------|
| <i>uhpT</i> (1) | 100 | 99.57 | + | | | fosfomycin | antibiotic target alteration |
| <i>evgA</i> (8) | 100 | 100 | | | | macrolide, fluoroquinolone, penam, tetracycline | antibiotic efflux |
| <i>evgS</i> (8) | 100 | 99.42 | | | | | |
| <i>gadW</i> (7) | 100 | 96.28 | | | | | |
| <i>gadX</i> (1) | 100 | 98.54 | | | | | |

(continued)



Table 2. Continued

| Antibiotic resistance genes (ARG) and gene products | Coverage % | Sequence identity % | M ¹ | Ph ² | Pl ³ | Resistance class* | Antibiotic mechanism* |
|---|------------|---------------------|----------------|-----------------|-----------------|--|---|
| H-NS (8) | 100 | 100 | | | | macrolide, fluoroquinolone, cephalosporin, cephamycin, penam, tetracycline | antibiotic efflux |
| <i>kdpE</i> (7) | 100 | 99.11 | + | | | aminoglycoside | antibiotic efflux |
| <i>marA</i> (8) | 100 | 100 | | | | fluoroquinolone, monobactam, carbapenem, cephalosporin, glycylicline, cephamycin, penam, tetracycline, rifamycin, phenicol, triclosan, penem | antibiotic efflux, reduced permeability to antibiotic |
| <i>mdtA</i> (6) | 100 | 99.28 | | + | | aminocoumarin | antibiotic efflux |
| <i>mdtB</i> (8) | 100 | 100 | | + | | aminocoumarin | antibiotic efflux |
| <i>mdtC</i> (8) | 100 | 99.41 | | + | | aminocoumarin | antibiotic efflux |
| <i>mdtE</i> (8) | 100 | 100 | | | | macrolide, fluoroquinolone, penam | antibiotic efflux |
| <i>mdtF</i> (7) | 100 | 99.81 | | | | macrolide antibiotic; fluoroquinolone antibiotic; penam | antibiotic efflux |
| <i>mdtG</i> (8) | 100 | 100 | + | + | | fospomycin | antibiotic efflux |
| <i>mdtH</i> (8) | 100 | 100 | | + | | fluoroquinolone | antibiotic efflux |
| <i>mdtM</i> (6) | 100 | 97.8 | | + | | fluoroquinolone, lincosamide antibiotic, nucleoside antibiotic, acridine dye, phenicol, disinfecting agents and intercalating dyes | antibiotic efflux |
| <i>mdtN</i> (8) | 100 | 99.42 | | + | | nucleoside antibiotic, | antibiotic efflux |
| <i>mdtO</i> (7) | 95.75 | 98.62 | | + | | acridine dye, disinfecting agents and intercalating dyes | |
| <i>mdtP</i> (8) | 100 | 98.16 | | + | | | |
| <i>msbA</i> (8) | 100 | 100 | + | + | | nitroimidazole antibiotic | antibiotic efflux |
| <i>pmrF</i> (8) | 100 | 100 | | | | peptide antibiotic | antibiotic target alteration |
| <i>qnrS1</i> (1) | 100 | 100 | + | | + | fluoroquinolone | antibiotic target protection |
| <i>sul2</i> (2) | 100 | 100 | + | | + | sulfonamide antibiotic | antibiotic target replacement |
| <i>bla_{TEM-1}</i> (1) | 100 | 100 | + | | + | monobactam, cephalosporin, penam, penem | antibiotic inactivation |
| <i>tetB</i> (2) | 100 | 99.25 | | | | tetracycline | antibiotic efflux |
| <i>tetR</i> (1) | 99.52 | 100 | | | | tetracycline | antibiotic target alteration, antibiotic efflux |
| <i>tolC</i> (8) | 99.6 | 100 | + | | | macrolide, fluoroquinolone, aminoglycoside, carbapenem, cephalosporin, glycylicline, cephamycin, penam, tetracycline, peptide antibiotic, aminocoumarin, rifamycin, phenicol, triclosan, penem | antibiotic efflux |
| <i>ugd</i> (7) | 100 | 99.23 | + | + | | peptide antibiotic | antibiotic target alteration |
| <i>yojI</i> (8) | 100 | 99.82 | | | | peptide antibiotic | antibiotic efflux |

¹M: Mobile genetic elements; ²Ph: located on Phage; ³Pl: located on Plasmid.

* The Comprehensive Antibiotic Resistance Database (Alcock et al., 2023).



a total of eight distinct *E. coli* strains were subjected to examination, with the majority of the resistance genes listed in the table being detected across all strains.

The majority of ARGs were associated with the fluoroquinolone group within The Antimicrobial Advice Ad Hoc Expert Group (AMEG) class B, holding significant implications for both human and animal health. Notably, 73% of these ARGs were attributed to efflux pumps, a critical component of resistance mechanisms. Furthermore, within this group, which encompasses generation 3–4 cephalosporins, 57.8% of β -lactam ARGs were identified as encoding cephalosporin resistance. Within the same category, the occurrence of genes encoding resistance to polymyxins (colistin) reached six, constituting 3.7% of the total genes detected. Collectively, the total count of ARGs targeting drug groups categorized in the AMEG class B amounted to 50, representing 30.9% of all identified genes.

In total, we detected a diverse spectrum of 59 unique ARGs across the eight sequenced bacterial strains. These 59 genes collectively comprised the entirety of the distinct ARGs identified. Notably, some genes were found to be present in multiple strains, contributing to the total count of 162 ARGs detected within the dataset encompassing all eight strains. The predominant resistance mechanism was efflux pumps, accounting for 110 of the identified genes (67.9%). Among these, 22 genes were associated with fluoroquinolones (13.6%), 17 with β -lactams (10.5%) and the third most frequent occurrence was observed for tetracyclines (16, 9.9%). The prevalence of mutation or enzymatic target alteration type resistance mechanisms was also most notable among the aforementioned drug groups. The differences between the individual gene pool of each strain, compared to the total of 59 resistance genes identified are summarised in Table 3.

Table 3. Antimicrobial resistance genes not detected in the individual eight *E. coli* strains studied relative to their total number of AMR genes

| Differences for all 59 genes identified | |
|---|---|
| Strains | Missing genes |
| 1. Total genes: 49 | <i>ampC1</i> , <i>ampH</i> , <i>cyaA</i> , <i>EF-Tu</i> , <i>gyrA</i> , <i>uhpT</i> , <i>gadW</i> , <i>mdtF</i> , <i>qnrS1</i> , <i>bla_{TEM-1}</i> |
| 2. Total genes: 48 | <i>CRP</i> , <i>ampC</i> , <i>gyrA</i> , <i>uhpT</i> , <i>gadX</i> , <i>kdpE</i> , <i>qnrS1</i> , <i>bla_{TEM-1}</i> , <i>sul2</i> , <i>tetB</i> , <i>tetR</i> |
| 3. Total genes: 51 | <i>gyrA</i> , <i>uhpT</i> , <i>gadX</i> , <i>qnrS1</i> , <i>bla_{TEM-1}</i> , <i>sul2</i> , <i>tetB</i> , <i>tetR</i> |
| 4. Total genes: 50 | <i>acrS</i> , <i>ampC</i> , <i>uhpT</i> , <i>gadX</i> , <i>qnrS1</i> , <i>bla_{TEM-1}</i> , <i>sul2</i> , <i>tetB</i> , <i>tetR</i> |
| 5. Total genes: 48 | <i>ampC1</i> , <i>cyaA</i> , <i>EF-Tu</i> , <i>uhpT</i> , <i>gadX</i> , <i>mdtO</i> , <i>qnrS1</i> , <i>bla_{TEM-1}</i> , <i>sul2</i> , <i>tetB</i> , <i>tetR</i> |
| 6. Total genes: 49 | <i>ampC</i> , <i>ampC1</i> , <i>cyaA</i> , <i>gyrA</i> , <i>uhpT</i> , <i>gadX</i> , <i>qnrS1</i> , <i>bla_{TEM-1}</i> , <i>tetB</i> , <i>tetR</i> |
| 7. Total genes: 46 | <i>emrR</i> , <i>ampC1</i> , <i>cyaA</i> , <i>EF-Tu</i> , <i>uhpT</i> , <i>gyrA</i> , <i>gadX</i> , <i>qnrS1</i> , <i>bla_{TEM-1}</i> , <i>mdtM</i> , <i>sul2</i> , <i>tetB</i> , <i>tetR</i> |
| 8. Total genes: 50 | <i>ampC1</i> , <i>cyaA</i> , <i>uhpT</i> , <i>gadX</i> , <i>mdtA</i> , <i>mdtM</i> , <i>sul2</i> , <i>tetR</i> , <i>ugd</i> |

Moreover, we identified ARGs responsible for enzymatic inactivation against specific β -lactam antibiotics. Particularly significant were the two reduced permeability genes, *soxS* and *marA*, which exhibited their role in conferring resistance against fluoroquinolones, β -lactams, tetracyclines, aminoglycosides, phenicol and rifamycin. A single target replacement gene, *sul2*, was associated with sulphonamides. Additionally, a singular instance of target protection was found, specifically the *qnrS1* gene that contributed to fluoroquinolone resistance. Regarding their origin, 10 of the identified genes were associated with MGEs (6.2%), 23 were located on phage (14.2%) and 3 were located on plasmid (1.9%). Interestingly, three genes displayed both MGEs and were located on sequences of phage attributes (1.9%), while another three genes exhibited dual MGEs and were located on sequences of plasmid characteristics (1.9%). In our assessment, matches with a coverage above 98% and sequence identity above 98% were considered for analysis.

Upon comparing the phenotypic test results (Table 4) with the identified resistance genes, a clear alignment emerged between putative resistance genes and their corresponding high MIC values. This suggests that the genes potentially accountable for the observed resistance are closely matched with the individual elevated MIC levels.

For instance, in the case of amoxicillin and amoxicillin-clavulanic acid, the prominence of β -lactamase overproduction, particularly attributed to the *ampC* gene, is indicative of its responsibility. However, no discernible increase in MIC value was observed with cephalosporins. Fortunately, low MIC value was observed with colistin, too. Regarding imipenem resistance, the activation of efflux pumps (*soxS*, *marA*, *tolC*) could be suspected. A similar trend was inferred for resistance to aminoglycosides and tetracyclines, with the *acrD* and *kdpE* genes associated with the former and the *tetB*, *tetR* and *mdfA* genes associated with the latter. For fluoroquinolone resistance observed in a single strain, an efflux pump mechanism - involving the *acrA*, *acrB*, and *tolC* gene - could be presumed.

DISCUSSION

Genes responsible for enzymatic inactivation were found only against β -lactams (*ampC*, *ampC1*, *ampH*, *bla_{TEM-1}* genes). Genes responsible for permeability reduction were found for fluoroquinolones, β -lactams, tetracyclines, aminoglycosides, phenicols and rifamycin (*soxS*, *marA* genes). Target site modification occurred only against sulphonamides by the *sul2* gene that causes resistance of dihydropteroate synthase to sulphonamides and is usually on small plasmids (Sköld, 2001). The *qnrS1* gene is usually a fluoroquinolone resistance gene on a plasmid preventing the binding of the drug by protecting the target of antibiotic action (Hata et al., 2005).

MIC values determined in phenotypic testing reflect the expression of individual resistance genes. In the case of amoxicillin and amoxicillin-clavulanic acid, the observed resistance is due to the *ampC*, *ampH* and *bla_{TEM-1}* genes, of



Table 4. Phenotypic analysis of strains and comparison of results with sequencing results. Data highlighted in red are considered resistant

| Antibiotic | MIC value (µg/ml) per strain | | | | | | | | Break-point µg/ml | Possible genetic background of phenotypic expression | | | |
|--------------------------------|------------------------------|-------|-------|-------|-------|-------|-------|-------|---------------------|--|--------------------|-----------------------------|-------------|
| | 1. | 2. | 3. | 4. | 5. | 6. | 7. | 8. | | Genes | Type of resistance | | |
| 1. amoxicillin | 4 | 2 | 1 | 1 | 2 | 2 | 16 | >32 | ≥8 ⁴ | <i>ampC</i> ³ <i>ampH</i> ¹ <i>bla</i> _{TEM-1} ^{1,3} | β-lactamase | | |
| 2. amoxicillin-clavulanic acid | 8 | 4 | 4 | 4 | 4 | 4 | 8 | >32 | ≥8 ⁴ | | | | |
| 3. cefquinom | <0.06 | <0.06 | <0.06 | <0.06 | <0.06 | 0.06 | <0.06 | <0.06 | ≥0.25 ⁴ | | | | |
| 4. cefotaxime | 0.06 | 0.06 | <0.06 | <0.06 | <0.06 | <0.06 | <0.06 | <0.06 | ≥0.125 ⁴ | | | | |
| 5. imipenem | 0.25 | 0.125 | 0.5 | 0.5 | 0.25 | 1 | 0.125 | 0.5 | ≥0.5 ⁴ | <i>soxS</i> | <i>marA</i> | <i>tolC</i> | efflux pump |
| 6. gentamicin | 32 | 32 | >32 | 16 | 16 | 32 | >32 | 32 | ≥2 ⁴ | <i>acrD</i> ² | | <i>kdpE</i> ³ | efflux pump |
| 7. oxytetracycline | >32 | 1 | 0.5 | 0.5 | 1 | 2 | 1 | >32 | ≥8 ⁴ | <i>tetB</i> | <i>tetR</i> | <i>mdfA</i> ² | efflux pump |
| 8. doxycycline | 32 | 2 | 1 | 1 | 1 | 2 | 2 | 32 | ≥4 ⁴ | | | | |
| 9. sulfamethoxazole | >128 | >128 | >128 | 128 | >128 | >128 | >128 | >128 | >64 ⁴ | <i>sul2</i> ^{1,3} | | target replacement | |
| 10. trimetoprim | 0.5 | 0.5 | <0.25 | <0.25 | <0.25 | <0.25 | <0.25 | <0.25 | ≥2 ⁴ | | | | |
| 11. potent sulphonamide* | 16 | 2 | 4 | 2 | 2 | 8 | 4 | 1 | ≥0.5 ⁴ | <i>sul2</i> ^{1,3} | | target replacement | |
| 12. enrofloxacin | <0.06 | <0.06 | <0.06 | 0.25 | 0.25 | <0.06 | 1 | 4 | ≥2 ⁵ | <i>acrA</i> | <i>acrB</i> | <i>tolC</i> | efflux pump |
| | | | | | | | | | | <i>gyrA</i> | | <i>qnrS1</i> ^{1,3} | |
| 13. colistin | 0.25 | 0.25 | 0.125 | 0.25 | 0.25 | 0.125 | 0.25 | 0.25 | ≥2 ⁴ | <i>ugd</i> ³ | | envelope overexpression | |

* sulfamethoxazole and trimetoprim, ¹plasmid, ²phage, ³MGE (mobile genetic elements), ⁴EUCAST, ⁵CLSI

which *ampH* is on a plasmid, *ampC* is an MGE and the *bla*_{TEM-1} gene is on a plasmid and is also an MGE. The former two are classified by Ambler as Class-C β-lactamase, the *bla*_{TEM-1} gene is a broad-spectrum Class-A β-lactamase and able to hydrolyse the first generation cephalosporins (Mittal et al., 2007; Lister et al., 2009). Resistance to cephalosporins is not phenotypically expressed, despite the fact that the *Escherichia coli* bacterial species carry a number of these genes. In the case of imipenem, the *soxS*, *marA* and *tolC* genes may be responsible for the emergence of resistance, of which the *soxS* (Aly et al., 2015) and *marA* (Cohen et al., 1988) genes are regulator genes responsible for the upregulation of the *acrAB* efflux pump system, complemented by the subprotein encoded by the *tolC* gene, thus forming a multidrug efflux pump (Tikhonova et al., 2011). The *acrD* gene expresses a phage-encoded aminoglycoside efflux pump (Rosenberg et al., 2000) and *kdpE* is also an aminoglycoside efflux pump encoding gene (Freeman et al., 2013). The emergence of resistance to tetracyclines is due to *tetB* and *tetR* as MFS-type efflux pump encoding genes and the *mdfA* gene is an efflux pump on a phage (Roberts, 2005). The *sul2* gene is on the plasmid is also an MGE, encoding sulphonamide resistance (Sköld, 2001). In the case of

fluoroquinolones, the emerging resistance is attributed to the multidrug efflux pumps *acrA*, *acrB* and *tolC* and in addition, point mutations in the enzyme encoded by the *gyrA* gene prevent binding of fluoroquinolones to the DNA gyrase alpha subunit (Webber et al., 2017). The *qnrS1* gene is a plasmid-mediated, mobile gene that prevents drug binding (Hata et al., 2005). No phenotypic resistance was observed with colistin, but it should be noted that the *ugd* gene encoding increased arabinose synthesis in the lipid layer was found as an MGE (Gunn et al., 1998).

The study of ARGs entering the human body by any means is a critical One Health concept. In the case of *E. coli* in particular, the diversity of ARGs is well observed. Strains harbouring ARGs are given the opportunity to interact with other non-pathogenic bacteria and horizontal gene transfer occurs. This is particularly likely if the ARG is on a mobile DNA gene sequence (*ampC*, *uhpT*, *mdtG*, *msbA*, *qnrS1*, *sul2*, *bla*_{TEM-1}, *tolC*, *ugd*). This is of relevance from a human health perspective, for genes responsible for broad-spectrum β-lactamase production (*bla*_{TEM-1}) and resistance to AMEG B class antibiotics such as colistin (*ugd*), fluoroquinolones (*qnrS1*) and generation 3–4 cephalosporins (*tolC*). Quinolone resistance has been reported from Hungary several



times, both from human and animal origin, which is an emerging concern in public health (Szabó et al., 2008; Szmolka et al., 2011).

No *mcr1* gene was found, which is important for resistance to colistin. In another study, where the the resistance gene pool of *E. coli* in broiler chickens from Hungary was investigated, this *mcr1* gene was also absent, probably due to strict regulations (Adorján et al., 2020). However, the emergence of *mcr-1* in *E. coli* has recently been reported in Hungary (Szmolka et al., 2023). This gene has also been recently identified in different *E. coli* strains from South Africa (Ramatlal et al., 2023). The *pmrE* gene was exclusively chromosomal, although the *pmrE* (*ugd*) gene may be a problem due to its mobility, but these were not expressed. The *marA* gene should be highlighted, as an important regulator gene in the function of the MDR-type *acrAB* efflux pump system and is also responsible for the down-regulation of porin channels through the regulation of the *ompF* gene. Genes encoding macrolide resistance would only be significant if they were mobile, the same being the case for fosfomycin. The presence of the *gyrA* gene mutation is not advantageous, even though it is not mobile. Due its coding and mobility, the *qnrS1* gene on plasmids and the mobility of the *sul2* gene, as well as the presence of the *ampC* gene may also be the matter of One Health concern.

CONCLUSIONS

Our results suggest that the use of genetic analysis to map bacterial resistome does not necessarily indicate phenotypic expression, despite the identification of individual genes. The results of MIC studies reflect the correlation between the emergence of resistance to certain active substances and the presence of respective ARGs.

The frequency of mobile genetic elements generally increases the likelihood of gene spread. The spread of AMR to livestock and to the different products of livestock, as well as to humans coming into contact with animals, is now proven to determine the structure of similar resistances. The most relevant indicator in this respect is *E. coli* (Luiken et al., 2019; Van Gompel et al., 2020; Tóth et al., 2021). This is exemplified in our study, where we identified the gene *TEM-1*, which is one of the important broad-spectrum β -lactamases in veterinary and public clinical use. The widespread distribution of several of these AMRGs is due to the fact that their gene is often carried on self-transmissible or mobilizable plasmids, making it capable of rapid horizontal spread, also among different enterobacterial species (Szabó et al., 2008; Szmolka et al., 2011; Perilli et al., 2002) and they are detected all around the world (Chotinantakul et al., 2022; Ghenea et al., 2022; Zhang et al., 2022).

For the precise elucidation of the underlying genes responsible for driving each phenotypically manifested resistance, we recommend conducting further transcriptomic studies. It is also necessary to carry out extensive surveys and to identify and minimise potential sources of risks for the spread of AMR.

Data availability: Sequences generated in this study are available under [1028257 BioProject ID](https://doi.org/10.28257/BioProject/1028257).

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