



Experimental evolution of enrofloxacin resistance and cross-resistance in *Escherichia coli* using a MEGA-plate system

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ABSTRACT

Fluoroquinolones are widely used in veterinary medicine, and their extensive application has raised concerns regarding the selection of antimicrobial resistance and cross-resistance. This study investigated the experimental evolution of enrofloxacin resistance and associated cross-resistance in *Escherichia coli* using a Microbial Evolution and Growth Arena (MEGA)-plate system. A reference *E. coli* strain (ATCC 25922) was exposed to stepwise increasing concentrations of enrofloxacin, enabling the spatial separation and isolation of bacterial populations adapted to different levels of selective pressure. Isolates recovered from distinct exposure zones were subjected to phenotypic antimicrobial susceptibility testing, including minimum inhibitory concentration (MIC) determination, and whole-genome sequencing to identify genetic changes associated with resistance development. Progressive exposure to enrofloxacin increased the enrofloxacin MIC from 0.003 µg/mL (parental) to 4 µg/mL in 1000 × –zone isolates (1333-fold). Cross-resistance was observed for selected non-fluoroquinolone agents, with MIC increases ranging from 8 to 33-fold for β-lactams, 4-fold for tetracyclines, and 16-fold for florfenicol. Genomic analysis identified mutations in genes encoding fluoroquinolone target enzymes (*gyrA*, *parC*) as well as regulatory genes linked to multidrug efflux systems (*acrR*, *marR*, *robA*, *emrR*). Together, these findings indicate that enrofloxacin-driven selection can promote cross-resistance through a combination of target-site alterations and changes in the regulation of efflux-associated resistance mechanisms. The results show that the MEGA-plate system is a suitable experimental model for studying stepwise resistance evolution and underscore the need for prudent use of enrofloxacin in veterinary settings.

1. Introduction

The frequency of mutations arising in bacterial populations is strongly influenced by evolutionary and population-dynamic factors and may be further intensified by anthropogenic pressures (Lupo et al., 2012). A comprehensive understanding of the evolution of antimicrobial resistance (AMR) therefore requires investigation of resistance

development under selective conditions relevant to natural and managed environments, including exposure to antimicrobial agents (Aminov, 2011; Tokuda and Shintani, 2024). The use of antimicrobials promotes both the emergence of adaptive mutations conferring resistance and the acquisition of resistance determinants, thereby shaping bacterial evolutionary trajectories (Recacha et al., 2019).

Intrinsic resistance mechanisms are most commonly associated with

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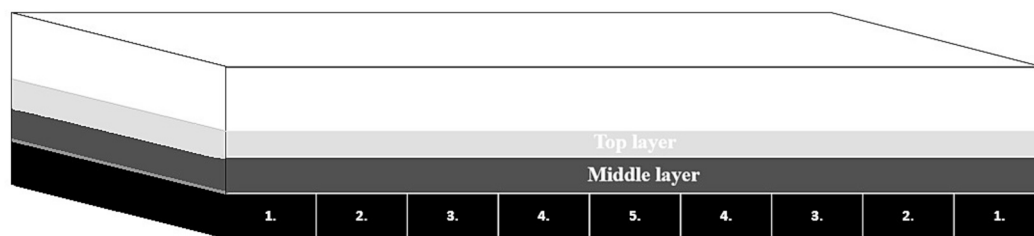


Fig. 1. Schematic representation of the Microbial Evolution and Growth Arena (MEGA)-plate system used in this study. The plate consists of three agar layers: a bottom layer containing nine physically separated compartments with increasing enrofloxacin concentrations arranged symmetrically from the edges toward the center (compartment 1: 0×, compartment 2: 1×, compartment 3: 10×, compartment 4: 100×, and compartment 5: 1000×), a continuous solid agar middle layer preventing mixing between compartments, and a semi-solid top agar layer enabling bacterial diffusion and surface expansion. The mirrored layout of antibiotic concentrations allows the isolation of bacterial populations adapted to successive stages of selective pressure. Layer thicknesses were approximately 12.5 mm (bottom), 12.5 mm (middle), and 5 mm (top), based on poured volumes and chamber dimensions. Antibiotic was confined to the bottom compartments; diffusion into the upper layers establishes the effective surface landscape over the incubation period. We did not directly quantify antibiotic diffusion kinetics in this setup; however, the multi-layer design is intended to minimize inter-compartment mixing while enabling within-zone diffusion toward the growth front.

decreased membrane permeability and the basal activity of efflux pumps, particularly multidrug efflux systems, which represent one of the most prevalent mechanisms contributing to reduced antimicrobial susceptibility (Fajardo et al., 2008). Acquired resistance may arise through the uptake of genetic material via transformation, transposition, or conjugation, processes collectively referred to as horizontal gene transfer (HGT) most frequently mediated by plasmids and, less commonly, bacteriophages (Aminov, 2011; Despotovic et al., 2023). In addition to gene acquisition, environmental stressors such as exposure to chemical agents or ultraviolet radiation can induce genetic mutations, including substitutions and deletions. Although the average bacterial mutation rate is estimated to be approximately one mutation per 10^6 – 10^9 cell divisions, most mutations are neutral or deleterious to cellular fitness (Davies and Davies, 2010), and their persistence may impose a measurable metabolic cost (Reygaert, 2009).

Fluoroquinolones, including enrofloxacin, exert their antibacterial activity by interfering with DNA synthesis and replication through inhibition of DNA gyrase (type II topoisomerase) and DNA topoisomerase IV [8,9]. DNA gyrase introduces negative supercoils to relieve torsional stress during replication, whereas topoisomerase IV resolves intertwined daughter chromosomes following DNA synthesis. Fluoroquinolones bind reversibly to the cleavage–ligation complexes of these enzymes, preventing DNA re-ligation and leading to the accumulation of DNA double-strand breaks and activation of the SOS response, ultimately resulting in a bactericidal effect (Correia et al., 2017; Qin et al., 2015).

Due to incomplete metabolism in both humans and animals, a substantial proportion of fluoroquinolones are excreted in an unchanged form and may enter terrestrial and aquatic ecosystems via fecal discharge (Kristiansson et al., 2011). Such environmental exposure can increase selective pressure on bacterial communities and enhance the likelihood of resistance development and dissemination. Although early assumptions suggested that fluoroquinolone resistance would be unlikely to arise due to the requirement for multiple concurrent mutations, this view was rapidly disproved following the widespread detection of mutations in gyrase genes (Depardieu et al., 2007; Nordmann and Poirel, 2005; Piddock, 2006). Resistance to quinolones has been documented since the clinical use of nalidixic acid, and high levels of fluoroquinolone resistance have since been reported in *Escherichia coli* isolates from both clinical and public health settings (Azimi et al., 2021; Neuhauser et al., 2003; Qin et al., 2015).

Anthropogenic pollution, including the release of large quantities of xenobiotics into the environment, further contributes to the spread of antimicrobial resistance. A notable example is the reported discharge of substantial amounts of ciprofloxacin into aquatic environments in regions associated with pharmaceutical manufacturing, illustrating the scale at which selective pressures may occur (Fick et al., 2009). To preserve the long-term efficacy of antimicrobial agents, responsible use, implementation of alternative control strategies (Essösy et al., 2020;

Hetényi et al., 2024; Jerzsele et al., 2020; Jócsák et al., 2025; Kovács et al., 2021, 2023; Olasz et al., 2023; Sebök et al., 2024), and adherence to strict biosecurity and infection control measures are essential (Farkas et al., 2024; Kovács et al., 2024).

Recent work has further highlighted that mobile genetic elements (plasmids, integrons, transposons and phages) are central to the acquisition and dissemination of antimicrobial resistance across Enterobacterales and other clinically relevant taxa (Partridge et al., 2018).

To counteract the effects of fluoroquinolones, bacteria have evolved multiple resistance mechanisms, predominantly involving chromosomal mutations, reduced intracellular drug accumulation, and plasmid-borne protective genes (Ruiz, 2003).

Current understanding emphasizes that fluoroquinolone resistance typically emerges through stepwise accumulation of target-site mutations in the quinolone resistance–determining regions (QRDRs) combined with changes in permeability and efflux regulation, while plasmid-mediated quinolone resistance determinants can further modulate resistance trajectories (Hooper and Jacoby, 2016; Rodríguez-Martínez et al., 2016).

In food-animal production systems, enrofloxacin use has been associated with shifts in *E. coli* MIC distributions at the farm level, supporting the need to understand how selection can shape resistance and collateral susceptibility patterns over time (Havas et al., 2025).

The aim of this study was to characterize stepwise evolution of enrofloxacin resistance and collateral cross-resistance in a spatially structured antibiotic landscape. Specifically, we quantified changes in MICs for enrofloxacin and a panel of veterinary-relevant antimicrobials across successive exposure zones on a MEGA-plate, identified genomic changes associated with increasing selective pressure using whole-genome sequencing, and evaluated whether cross-resistance patterns were consistent with mutations in established fluoroquinolone targets (QRDR loci) and efflux/permeability regulatory networks, as well as with any evidence of changes in the resistome background. To our knowledge, this is the first study to combine a MEGA-plate spatial selection framework with zone-resolved phenotyping and whole-genome sequencing to map enrofloxacin-driven cross-resistance trajectories in a stepwise manner.

2. Materials and methods

2.1. Bacterial strain

For all experiments, the *E. coli* reference strain ATCC 25922 (LGC Ltd., Teddington, UK), originally isolated in Seattle in 1946, was used. This strain is widely employed as a quality control and reference organism in antimicrobial susceptibility testing and is considered genetically well characterized, with a stable and well-documented baseline resistance profile. Its defined genetic background and absence of

acquired extended-spectrum β -lactamase genes make it a suitable model for investigating de novo resistance development and adaptive genetic changes under controlled selective pressure.

2.2. MEGA-plate preparation

Based on laboratory-specific requirements, a custom polycarbonate chamber (60 × 30 cm, 5 mm thickness; Innotherm Ltd., Budapest, Hungary) was constructed with a design adapted from the original MEGA-plate configuration (Baym et al., 2016) and further modified according to published protocols (Kerek et al., 2022). Prior to use, the chamber was disinfected using a 7.5% hydrogen peroxide solution prepared by diluting 30% hydrogen peroxide (VWR International Ltd., Debrecen, Hungary) with deionized water at a 1:3 ratio, as described previously (Rutala and Weber, 1999; Sattar et al., 1996). The chamber was placed under a laminar flow hood, filled with the disinfectant, and the interior surface of the lid was wiped with 0.9% sodium hypochlorite (NaOCl) solution (Merck KGaA, Darmstadt, Germany). After 15 min of incubation under sterile conditions, the disinfectant was removed using a vacuum pump, followed by a 30-min exposure to ultraviolet light.

The agar medium was prepared and poured into three distinct layers, each serving a specific function within the MEGA-plate system. The bottom layer consisted of nine physically separated compartments arranged symmetrically from the outer edges toward the center of the chamber. These compartments contained increasing concentrations of enrofloxacin (0×, 1×, 10×, 100×, and 1000× relative to the initial MIC). Due to the mirrored layout of the plate, intermediate concentrations (1×, 10× and 100×) were present on both sides of the central high-concentration zones, resulting in a total of nine compartments despite the use of five discrete antibiotic concentrations (Fig. 1).

The middle layer consisted of a continuous solid agar overlay, which served to physically connect the underlying compartments while preventing mixing of antibiotic concentrations between adjacent zones. The top layer was prepared as a semi-solid agar, enabling bacterial diffusion and surface expansion along the established antibiotic gradient during incubation.

A 2% (w/v) BD Bacto Agar (VWR International Ltd., Hungary) was used for all layers except the top layer, which was prepared as a 0.28% (w/v) semi-solid formulation. LB-Lennox capsules (VWR International Ltd., Hungary) were added as a nutrient supplement at a ratio of one capsule per liter; based on a prior pilot experiment, an additional capsule was added to the top layer to support optimal surface growth. To prevent fungal contamination, cycloheximide (Merck KGaA, Darmstadt, Germany) was added at a final concentration of 64 µg/mL. For contrast enhancement, black acrylic paint (Artemie, Budapest, Hungary) was added at 4 mL/L to the media used for the lower and middle layers. The approximate thickness of each agar layer was calculated from the poured volume and chamber dimensions and is reported in the Fig. 1 legend.

The enrofloxacin landscape was scaled to the measured baseline MIC of the parental strain to capture stepwise adaptation across increasing selective pressure. We defined the first selective compartment (1×) as one-quarter of the MIC to allow initial colonization of the low-antibiotic zone while maintaining selection for rare, fitter subpopulations at the advancing front. Higher levels (10×, 100×, and 1000× relative to 1×) were used to impose progressively stronger selection as the population migrated. The nine-compartment layout arises from mirroring five discrete concentrations across the plate (0 × −1 × −10 × −100 × −1000 × −100 × −10 × −1 × −0×), which enables isolation of independently adapted populations from successive selection steps along two symmetric trajectories. This approach follows the established MEGA-plate concept of spatial selection on antibiotic landscapes and its recent extensions (Baym et al., 2016; Gross et al., 2024).

Finally, the *E. coli* strain was inoculated at both outer edges of the plate, and the assembled chamber was incubated at 37 °C.

2.3. Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) of the parental *E. coli* ATCC 25922 strain was determined in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines using the broth microdilution method (Clinical and Laboratory Standards Institute (CLSI), 2024). Briefly, twofold serial dilutions of enrofloxacin were prepared in cation-adjusted Mueller–Hinton broth (CAMHB), inoculated with a standardized bacterial suspension, approximately 5×10^5 colony-forming unit (CFU)/mL, and incubated at 37 °C for 18–24 h. The MIC was defined as the lowest concentration preventing visible bacterial growth.

The baseline MIC of enrofloxacin for the parental strain was 0.003 µg/mL. To facilitate gradual selection and expansion of resistant subpopulations on the MEGA-plate, the initial selective concentration (defined as 1×) was set at one-quarter of the MIC (0.00075 µg/mL). Subsequent antibiotic concentrations used in the MEGA-plate experiment (10×, 100×, and 1000×) were calculated relative to this 1× baseline.

Following a 10-day incubation on the MEGA-plate, during which bacterial growth progressed through zones containing up to the 1000× enrofloxacin concentration, samples were collected from each antibiotic exposure zone. To verify culture purity and exclude contamination, samples were streaked onto ChromoBio Coliform agar (Biolab Zrt., Budapest, Hungary). Colonies derived from a single colony-forming unit were subcultured on tryptic soy agar (Biolab Zrt., Budapest, Hungary) and preserved in Microbank cryovials (Pro-Lab Diagnostics, Richmond Hill, Canada) at −80 °C until further analysis.

For each antibiotic concentration zone, three independent biological replicates were collected. All antimicrobial susceptibility tests were performed in technical triplicates. Prior to testing, frozen isolates were revived in 3 mL CAMHB and incubated at 37 °C for 18–24 h. All MIC determinations were performed in technical triplicate for each isolate, and all triplicates were fully concordant (100% agreement). Therefore, the MIC for each isolate was recorded as the common value across the three technical replicates.

Antimicrobial susceptibility testing was performed using the broth microdilution method in sterile 96-well microtiter plates (VWR International Ltd., Debrecen, Hungary). Stock solutions (1024 µg/mL) of the tested antimicrobial agents, ceftriaxone, cefquinome, cefotaxime, cefotiofur, colistin, enrofloxacin, amoxicillin, neomycin, oxytetracycline, florfenicol, and a potentiated sulfonamide combination were prepared according to CLSI recommendations (Merck KGaA, Darmstadt, Germany). Twofold serial dilutions were prepared in CAMHB to achieve a final volume of 90 µL per well.

Bacterial suspensions were adjusted to a turbidity equivalent to 0.5 McFarland using a nephelometer (Thermo Fisher Scientific, Budapest, Hungary), and 10 µL of each suspension was added to the wells to achieve a final inoculum of approximately 5×10^5 CFU/mL. MIC values were determined using a Sensititre SWIN automatic MIC reader and analyzed with VIZION system software version 3.4 (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Statistical analysis

MIC values were treated as discrete twofold dilution steps and analyzed on a log2 scale. Technical triplicates were used as an internal reproducibility check; because all technical replicates were fully concordant for all isolate–drug combinations, no within-isolate variance was estimated, and the common MIC value was used for each isolate. Statistical comparisons across exposure zones were based on biological replicates (independent isolates) ($n = 3$ per zone). MIC results are reported as median and range across biological replicates. Where applicable, differences across exposure zones were assessed using Kruskal–Wallis tests followed by Dunn's multiple-comparisons procedure. P values <0.05 were considered statistically significant. Where all

Table 1

Effect of increasing enrofloxacin concentrations on the minimum inhibitory concentration (MIC) values of the tested antimicrobial agents. Bold values indicate increased MICs compared to the baseline. Compounds categorized as AMEG category B (critically important for public health) are highlighted.

Sample	ENR	CTX	CFR	CFT	CFQ	COL	AMX	OTC	PSA	FLO	NEO
µg/mL											
0× ENR	0.003	0.03	0.25	0.06	0.06	0.5	8	2	8	16	16
1× ENR	0.03	0.03	0.25	0.06	0.06	0.5	8	2	8	16	16
10× ENR	0.25	0.03	0.25	0.06	0.06	0.5	8	2	16	32	16
100× ENR	0.5	0.03	0.5	0.125	0.06	0.5	8	2	16	32	16
1000× ENR	4	1	1	0.5	0.5	8	32	8	64	256	16

ENR – enrofloxacin, CTX – cefotaxime, CFR – ceftriaxone, CFT – ceftiofur, CFQ – cequinome, COL – colistin, AMX – amoxicillin, OTC – oxytetracycline, PSA – potentiated sulfonamide (trimethoprim and sulfamethoxazole, in ratio 1:19), FLO – florfenicol, NEO – neomycin.

biological replicate MIC values were identical within a zone, results are presented descriptively as dilution-step changes relative to baseline.

2.5. Molecular genetic analysis

Genomic DNA was extracted from bacterial cultures using the Zymo Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions, with mechanical lysis performed by bead beating using a TissueLyzer LT instrument (Qiagen GmbH, Hilden, Germany) at 50 Hz for 5 min. Extracted DNA was stored at −20 °C until library preparation.

Whole-genome sequencing was performed using paired-end sequencing on an Illumina NextSeq 500 platform. Sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit with indexing performed using the Nextera XT Index Kit v2 Set A (Illumina, San Diego, CA, USA). Library preparation, amplification, purification, and quantification were carried out according to the manufacturer's protocols. Individually indexed libraries were pooled and sequenced following standard Illumina procedures.

2.6. Bioinformatic analysis

Raw paired-end sequencing reads were subjected to quality control using FastQC v0.11.9 (Andrews, 2012), fastp v0.23.2 (Chen et al., 2018), and BBDuk (BBTools v38.96) to assess read quality, remove adapter sequences, correct base composition biases, and filter low-quality reads. Additional trimming of residual adapters and low-quality regions was performed using TrimGalore v0.6.6 (Krueger, 2022).

High-quality reads were assembled de novo into contigs using both MEGAHIT v1.2.9 (Dinghua, 2015) and SPAdes v4.0.0 (Vasilinetc et al., 2015). The resulting assemblies were merged to generate an improved draft genome using GAM-NGS v1.1b (Vicedomini et al., 2013). Assembly quality metrics were evaluated using QUAST v5.2 (Gurevich et al., 2013) to ensure suitability for downstream analyses.

Open reading frames were predicted from assembled contigs using Prodigal v2.6.3 (Hyatt et al., 2010). Acquired antimicrobial resistance genes were identified using both the Resistance Gene Identifier (RGI) v5.1.0 and ABRicate (Feldgarden et al., 2019), with reference to the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020). Only resistance genes meeting the CARD *strict* criteria and exhibiting ≥90% sequence identity and coverage were retained.

The genomic context of identified resistance genes was further analyzed to assess their potential association with mobile genetic elements. MobileElementFinder v1.0.3 (Johansson et al., 2021) was used to detect proximity to known transposable elements. Plasmid origin of contigs was predicted using PlasFlow v1.1 (Krawczyk et al., 2018), while potential bacteriophage-associated regions were identified using VirSorter v2.2.2 (Roux et al., 2015). Only mobile element-, plasmid-, or phage-associated regions exceeding 10 kb were considered for further evaluation.

Chromosomal point mutations associated with antimicrobial resistance were identified using ResFinder v4.1 (Bortolaia et al., 2020; Camacho et al., 2009; Zankari et al., 2017) and Snippy v4.6.0 (Seemann,

2022) by comparative analysis against the closest available *E. coli* reference genome (strain SYN8802, RefSeq accession GCF_020995495.1) *Escherichia coli* Genome Assembly ASM2099549v1 [WWW Document], 2025). Detected variants were compared across isolates recovered from different enrofloxacin exposure zones to identify mutations associated with progressive resistance development.

All variant events detected by the variant-calling workflow were retained for reporting. Variants were considered “gene-annotated” when they could be mapped to an annotated coding feature in the reference genome and assigned a gene name and product; variants lacking gene annotation typically reflected intergenic regions or events that could not be reliably assigned to a single coding feature. Because the biological focus of this study was antimicrobial resistance evolution under enrofloxacin selection, downstream interpretation prioritized variants in established resistance determinants and regulators. The complete list of detected variant events and the subset of gene-annotated variants are provided in Supplementary Tables S1–S2.

3. Results

3.1. Growth dynamics on the MEGA-plate

Following inoculation, the *E. coli* population expanded progressively from the antibiotic-free region toward zones containing increasing concentrations of enrofloxacin on the MEGA-plate. Bacterial growth was initially confined to the 0× and 1× concentration zones and subsequently advanced into regions containing higher antibiotic concentrations.

Continuous surface expansion was observed across successive concentration boundaries, resulting in colonization of all defined zones, including those containing the highest enrofloxacin concentration (1000×). Complete overgrowth of the MEGA-plate was achieved after approximately 10 days of incubation.

Distinct bacterial populations were recoverable from each concentration zone, enabling downstream phenotypic and genomic analyses of isolates exposed to different levels of enrofloxacin selection.

3.2. Changes in antimicrobial susceptibility

Phenotypic susceptibility testing shows progressive increases in MIC values associated with increasing enrofloxacin exposure. Complete expansion of the *E. coli* population across the MEGA-plate, including zones containing the highest antibiotic concentration (1000×), was observed after approximately 10 days. At the 1000× enrofloxacin concentration, elevated MIC values were detected for all tested antimicrobial agents. Increases in MIC were already observed at the 100× concentration for ceftriaxone and ceftiofur (Table 1), suggesting reduced susceptibility to selected β-lactam antibiotics at intermediate levels of enrofloxacin exposure.

3.3. Overview of detected genomic mutations

Across all isolates, 9063 variant events were detected. Of these, 4483

Table 2

Baseline resistome of *Escherichia coli* ATCC 25922 isolates recovered from different enrofloxacin exposure zones. The table lists the 44 antimicrobial resistance genes (ARGs) identified by next-generation sequencing and CARD-based annotation (strict criteria; $\geq 90\%$ identity and coverage) that were consistently detected across all sequenced isolates. Genes are reported as a baseline reference to demonstrate resistome stability (no acquisition or loss of ARGs) during enrofloxacin exposure. CARD, Comprehensive Antibiotic Resistance Database; ARG, antimicrobial resistance gene.

Gene	C%	I%	Mechanism	Resistance
<i>acrA</i>	100	99.16	efflux pump	cephalosporins, fluoroquinolones, glycolcyclines, penicillins, phenicols, rifamycins, tetracyclines, triclosan
<i>acrB</i>	100	98.64	efflux pump	aminoglycosides
<i>acrD</i>	100	98.04	efflux pump	cephalosporins, cephamycins, fluoroquinolones, penicillins
<i>acrE</i>	100	98.79	efflux pump	cephalosporins, cephamycins, fluoroquinolones, glycolcyclines, penicillins, phenicols, rifamycins, tetracyclines, triclosan
<i>acrF</i>	100	96.49	efflux pump	
<i>acrS</i>	100	98.34	efflux pump	
<i>ampC</i>	100	98.15	enzymatic inactivation	cephalosporins, penicillins
<i>ampH</i>	100	97.5	enzymatic inactivation	
<i>bacA</i>	99.76	98.17	target mutation	peptide antibiotics
<i>baeR</i>	99.86	96.81	efflux pump	
<i>baeS</i>	100	90.53	efflux pump	aminocoumarins, aminoglycosides
<i>cpxA</i>	100	98.47	efflux pump	
<i>CRP</i>	100	99.21	efflux pump	fluoroquinolones, macrolides, penicillins
<i>emrA</i>	100	98.21	efflux pump	fluoroquinolones
<i>emrB</i>	100	96.95	efflux pump	
<i>emrE</i>	100	92.19	efflux pump	macrolides
<i>emrK</i>	100	97.73	efflux pump	tetracyclines
<i>emrR</i>	100	98.68	efflux pump	fluoroquinolones
<i>emrY</i>	100	97.73	efflux pump	tetracyclines
<i>eptA</i>	100	91.85	target mutation	peptide antibiotics
<i>evgA</i>	100	99.02	efflux pump	fluoroquinolones, macrolides, penicillins, tetracyclines
<i>evgS</i>	100	96.19	efflux pump	fluoroquinolones, macrolides, penicillins
<i>gadW</i>	100	99.86	efflux pump	cephalosporins, cephamycins, fluoroquinolones, macrolides, penicillins, tetracyclines
<i>gadX</i>	100	93.82	efflux pump	aminoglycosides
<i>H-NS</i>	100	99.28	efflux pump	carbapenems, cephalosporins, cephamycins, fluoroquinolones, glycolcyclines, monobactams, penicillins, phenicols, rifamycins, tetracyclines, triclosan
<i>kdpE</i>	99.26	95.84	efflux pump	benzalkonium chloride, rhodamine, tetracyclines
<i>marA</i>	100	98.7	reduce permeability	aminocoumarins
<i>mdfA</i>	100	96.59	efflux pump	fluoroquinolones, macrolides, penicillins
<i>mdtA</i>	100	95.11	efflux pump	fosfomycin
<i>mdtB</i>	100	96.29	efflux pump	fluoroquinolones
<i>mdtC</i>	100	94.15	efflux pump	acridines, fluoroquinolones, lincosamides, nucleosides, phenicols
<i>mdtE</i>	100	98.62	efflux pump	
<i>mdtF</i>	100	97.33	efflux pump	
<i>mdtG</i>	100	98.21	efflux pump	
<i>mdtH</i>	100	98.26	efflux pump	
<i>mdtM</i>	100	95.05	efflux pump	
<i>mdtN</i>	100	95.64	efflux pump	acridines, nucleosides
<i>mdtO</i>	100	97.08	efflux pump	
<i>mdtP</i>	100	97.61	efflux pump	
<i>msbA</i>	100	98.06	efflux pump	nitroimidazoles
<i>pmrF</i>	100	97.63	target mutation	peptide antibiotics
<i>tolC</i>	100	97.98	efflux pump	aminocoumarins, aminoglycosides, carbapenems, cephalosporins, cephamycins, fluoroquinolones, glycolcyclines, macrolides, penicillins, peptide antibiotics,

Table 2 (continued)

Gene	C%	I%	Mechanism	Resistance
<i>ugd</i>	100	96.92	target mutation	phenicols, rifamycins, tetracyclines, triclosan
<i>yojI</i>	100	98.05	efflux pump	peptide antibiotics

C – coverage; I – identity.

could be mapped to annotated genes and assigned a gene name/product (Supplementary Tables S1–S2). Among gene-annotated variants outside the resistance loci discussed in the main text, most were predicted to be synonymous (67.5%) or missense (29.1%), with a smaller fraction of frameshift or stop-gain events. Therefore, detailed interpretation focuses on established resistance determinants and regulators, while the full annotated variant set is provided as Supplementary data.

Preliminary genome quality assessment based on k-mer frequency distributions showed patterns consistent with the expected genome size of *E. coli*. Taxonomic classification using CheckM and Kraken confirmed that all sequenced isolates were assigned to *E. coli* with 100% confidence (Supplementary Figs. 1–5).

Comparative analysis against the Comprehensive Antibiotic Resistance Database (CARD) identified antimicrobial resistance genes (ARGs meeting the database *strict* criteria) in all isolates. All detected ARGs exhibited $>90\%$ sequence identity and coverage. The ARG profiles were identical across all samples and comprised 44 distinct genes predicted to confer resistance to 22 different antimicrobial compounds, consistent with no acquisition or loss of resistance genes occurred during enrofloxacin exposure (Additional data: enrofloxacin).

Notably, multiple multidrug efflux systems were present in all isolates, including the *acrA–acrB–tolC*, *emrA–emrB*, and *mdtA–mdtB–mdtC* gene clusters (Table 2). In addition, the β -lactamase-associated genes *ampC* and *ampH* were detected in all samples. These genes constituted a conserved genetic background across isolates recovered from different enrofloxacin concentration zones. MIC values were identical across technical triplicates for all isolates; therefore, Table 2 reports replicate-concordant MIC values.

Fig. 2 summarizes the distribution of the 44 identified ARGs grouped by antimicrobial compound class and resistance mechanism.

Assembly quality metrics were comparable across all sequenced isolates (Table 3), supporting consistent genome reconstruction for downstream variant comparisons. To confirm that the endpoint isolates remained within the same species background, average nucleotide identity (ANI) comparison comparing the untreated (0 $\times$) and highest-exposure (1000 $\times$) isolates yielded values $>99.9\%$ (Supplementary Fig. 6), consistent with species-level identity. These quality and identity checks were performed to ensure that downstream mutation analyses reflect within-strain evolution under selection rather than contamination or species replacement.

Across all sequenced isolates, a total of 9063 variant calls were detected, of which 4483 could be functionally annotated. The total number of variants per sample ranged from 1631 to 1936, while annotated variants ranged from 765 to 933 per sample. The distribution of mutation types, including single nucleotide polymorphisms, insertions, deletions, and complex variants, is summarized in Table 4.

To enable comparison with the untreated baseline, variants detected in each enrofloxacin-exposed sample were compared against those present in the 0 $\times$ isolate. The majority of variants detected in the 1 $\times$, 10 $\times$, and 100 $\times$ samples overlapped with baseline variants, while the 1000 $\times$ sample exhibited a lower proportion of shared variants, indicating increased divergence at the highest selective pressure.

3.4. Mutations in resistance-associated genes

Analysis of resistance-associated loci identified recurrent mutations

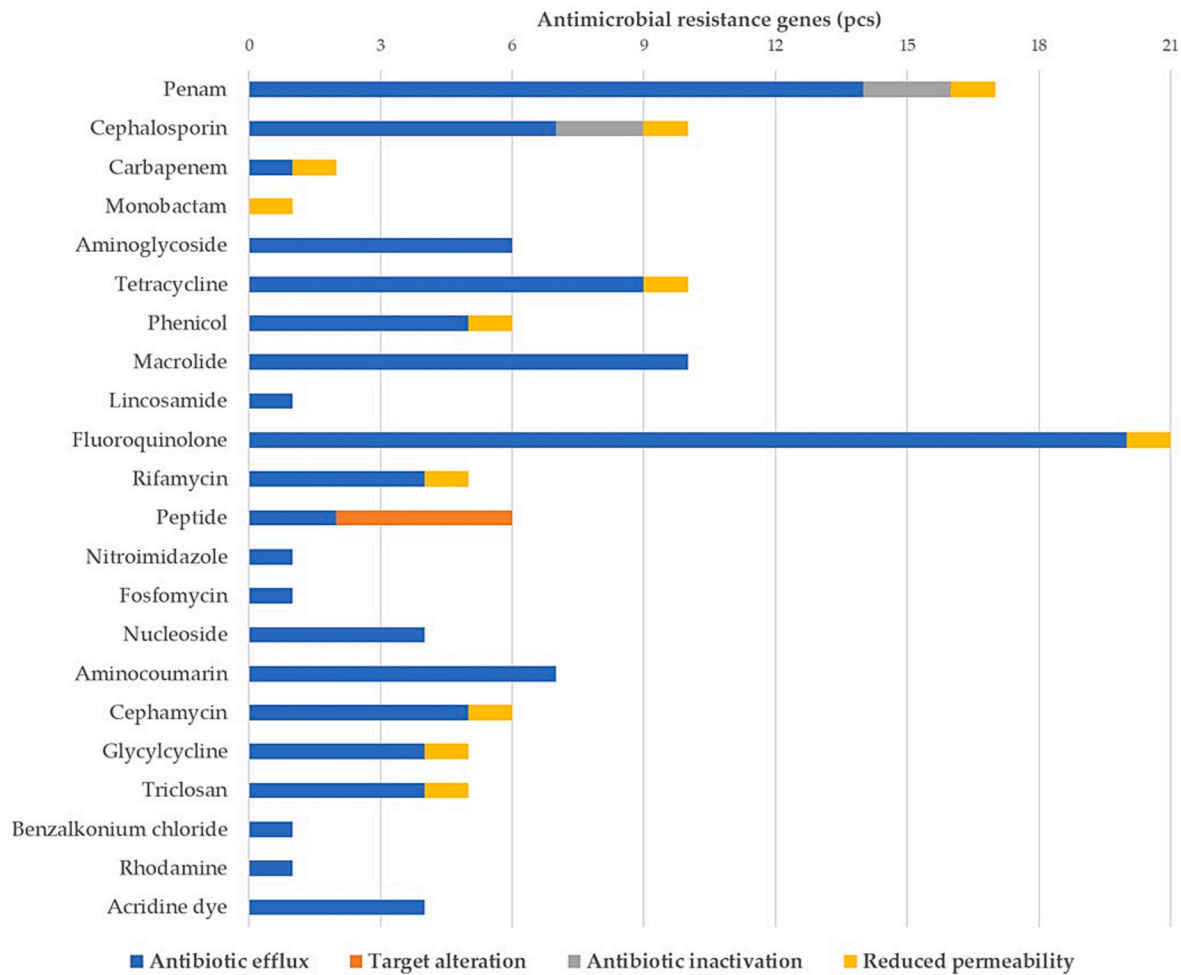


Fig. 2. Classification of identified antimicrobial resistance genes by compound class and resistance mechanism. ARGs were detected using CARD-based annotation (strict criteria; $\geq 90\%$ identity and coverage), and the resistome composition was identical across exposure zones. Bars indicate the number of ARGs assigned to each compound class; shading (or grouping) indicates the predicted resistance mechanism category (e.g., efflux-associated, enzymatic inactivation, or target alteration). Counts reflect gene presence/absence calls and do not represent expression levels.

Table 3
Assembly quality metrics for enrofloxacin-exposed *Escherichia coli* isolates based on QUASt analysis. The table summarizes read counts, contig statistics, estimated coverage, and assembly continuity metrics, supporting comparable assembly quality across samples for downstream comparative analyses. N50/N75 represent the contig length (bp) such that 50%/75% of the assembly is contained in contigs of at least that length; L50/L75 indicate the minimal number of contigs whose cumulative length reaches 50%/75% of the assembly.

Sample	Reads	Contigs	Coverage	N ₅₀	N ₇₅	L ₅₀	L ₇₅
0× ENR	2,644,967	244	64.75	94,103	45,377	16	35
1× ENR	2,439,834	229	59.71	102,465	49,984	16	32
10× ENR	2,330,488	126	57.09	105,213	50,556	14	30
100× ENR	2,280,520	130	55.89	115,841	85,532	14	26
1000× ENR	2,373,082	204	58.67	112,848	58,288	15	29

ENR – enrofloxacin; N50 / N75 – the length of the shortest contig such that 50% / 75% of the total genome length is contained in contigs of this length or longer; L50 / L75 – the minimum number of contigs whose cumulative length accounts for 50% / 75% of the total genome assembly length.

in genes known to be involved in fluoroquinolone resistance. Mutations in *gyrA* and *parC* were detected in isolates recovered from the 10×, 100×, and 1000× enrofloxacin exposure zones (Table 5). A complex mutation affecting *parC*, encoding a subunit of DNA topoisomerase IV, was observed in multiple exposure levels.

In addition, a point mutation in the regulatory gene *uveY* was detected exclusively in the 1000× sample. No resistance-associated point mutations were detected in the untreated (0×) isolate.

3.5. Mobile genetic elements

Analysis of mobile genetic elements revealed no plasmid-associated antimicrobial resistance genes in any of the sequenced isolates. Several resistance-associated genes were identified in proximity to bacteriophage-related sequences or mobile elements, with distinct patterns observed across exposure levels.

The *bacA* gene was consistently associated with phage-related sequences in all samples. In the 10× exposure sample, *emrB* was detected in proximity to phage-associated regions. In the untreated (0×) sample,

Table 4

Summary of detected and gene-annotated variant events by mutation class across enrofloxacin exposure zones. “All” indicates the total number of variant calls per mutation class, including events that could not be mapped to an annotated gene feature. “Identified” indicates the subset of variant calls that could be assigned to an annotated gene and predicted consequence (e.g., synonymous, missense, frameshift) and were retained for downstream interpretation. Values in parentheses denote the number of gene-annotated variants in each exposure zone that were absent from the untreated (0× ENR) baseline within the same mutation class (i.e., variants unique relative to baseline). ENR, enrofloxacin; SNP, single-nucleotide polymorphism; Complex, multi-nucleotide or composite variant event.

Mutation type		0× ENR	1× ENR	10× ENR	100× ENR	1000× ENR
Complex*	Identified	116	119	120 (+2)	122 (+1)	86 (+3)
	All	295	309	321	318	270
Deletion	Identified	19	17	19	22 (+2)	22 (+1)
	All	39	35	38	42	38
Inversion	Identified	4	3	3	5 (+2)	9 (+6)
	All	12	12	12	14	17
SNP**	Identified	788	788 (+6)	791 (+9)	782 (+11)	648 (+32)
	All	1454	1458	1565	1508	1306

* Complex mutation involving multiple insertions, deletions, and substitutions.

** Single nucleotide polymorphism (SNP). ENR – enrofloxacin.

multiple efflux- and stress-related genes, including *acrA*, *gadW*, *gadX*, *mdtE*, *mdtF*, *mdtN*, *mdtO*, *mdtP*, and *tolC*, were classified as phage-associated based on a ≤ 10 kb proximity criterion.

Several genes were associated with mobile genetic elements across exposure levels. *acrE* and *acrF* were mobile genetic element (MGE)-associated in both the 0× and 10× samples, while *ampH* was classified as MGE-associated only in the 10× sample. The *ugd* gene was detected in proximity to mobile elements in the 1×, 100×, and 1000× samples.

4. Discussion

A key contribution of this work is the zone-resolved linkage of phenotype and genotype across successive selection steps on a spatial antibiotic landscape. This enables comparison of resistance emergence and collateral susceptibility shifts at distinct stages of adaptation, rather than only at an endpoint.

Table 5

Key gene-annotated mutations associated with antimicrobial resistance in isolates recovered from increasing enrofloxacin exposure zones. The table reports resistance-relevant variants detected by whole-genome sequencing in established fluoroquinolone target loci and in regulators/components linked to multidrug efflux and stress-response pathways. Presence across exposure zones is indicated in the corresponding columns (e.g., “×” denotes detection in that zone’s sequenced isolate). Nucleotide and amino acid changes are reported relative to the reference genome used for variant calling (see Materials and Methods). Predicted functional effects (e.g., missense, synonymous, frameshift) are provided as annotated consequences. The complete set of gene-annotated variants is provided in Supplementary Table S2.

Gene	Mutation class	Exposure level(s)	Nucleotide change	Protein change	Predicted effect	Product
<i>parC</i>	Complex*	1000×	GCA-ACC	p.Ala108Val	Frameshift	DNA topoisomerase IV subunit A
<i>acrR</i>	Deletion	100×	CA-C	p.Thr144fs	Frameshift	Transcriptional repressor of multidrug efflux pump expression
<i>acrR</i>		1000×	T-TG	p.Glu64fs	Frameshift	
<i>marR</i>	Insertion	1000×	G-GA	p.Val63fs	Frameshift	Transcriptional regulator of multiple antibiotic resistance
<i>ompF</i>		100×, 1000×	A-ATC	p.Ile10fs	Frameshift	Outer membrane porin (OmpF)
			G-GC	p.Ala145fs	Frameshift	
<i>acrA</i>		10×, 100×, 1000×	C-T	p.Leu50Leu	Synonymous	Multidrug efflux RND transporter periplasmic adaptor subunit (<i>acrA</i>)
<i>emrR</i>	SNP**	1000×	T-C	p.Ser160Pro	Missense mutation	<i>emrAB</i> multidrug efflux pump transcriptional repressor
<i>gyrA</i>		10×, 100×, 1000×	T-C	p.Asp87Gly	Missense mutation	DNA topoisomerase (ATP-hydrolyzing) subunit A
<i>robA</i>		10×	C-T	p.Arg156His	Missense mutation	

* Complex mutation involving multiple insertions, deletions, and substitutions.

** Single nucleotide polymorphism.

Adaptation of *E. coli* to agar containing 1000× enrofloxacin required approximately 10 days, consistent with gradual, stepwise selection under increasing antimicrobial pressure. Fluoroquinolone resistance is known to arise primarily through chromosomal alterations affecting target-encoding genes and the overexpression of multidrug efflux pumps, frequently in combination with plasmid-mediated resistance determinants (Dobbyn et al., 2022, pp. 2015–2019). Well-characterized examples include the *aac(6′)-Ib-cr* gene, which encodes an acetyltransferase capable of modifying norfloxacin and ciprofloxacin (Alsharapy et al., 2018; Jones-Dias et al., 2016; Machuca et al., 2016; Robicsek et al., 2006), and the *mdfA* gene, which contributes to fluoroquinolone resistance via efflux activity in *E. coli* (Yang et al., 2003). In addition, MFS-type efflux pumps encoded by *norA*, *norB*, and *norC* have been reported to increase MIC values by 4- to 8-fold (Klein and Raina, 2015).

Target-site modification represents a major mechanism of fluoroquinolone resistance. Mutations in *gyrA*, encoding the A subunit of DNA gyrase, prevent fluoroquinolone binding to the enzyme’s α -subunit and play a pivotal role in resistance development (Azargun et al., 2020; Cheng et al., 2020; Kherroubi et al., 2024; Kim et al., 2021; Nawaz et al., 2015; Reece and Maxwell, 1991; Umeda et al., 2020; Zhao et al., 2021). Similarly, mutations in *parC*, encoding a subunit of topoisomerase IV, impair fluoroquinolone binding and further contribute to resistance, whereas mutations in *gyrB* and *parE* occur less frequently (Dasgupta et al., 2018; Gulyás et al., 2019; Ruiz, 2003; Umeda et al., 2020).

In the present study, a complex mutation was identified in *parC*, a gene involved in DNA decatenation and relaxation, processes essential for transcription and translation. While *parC* mutations alone are typically associated with low-level resistance, high-level resistance commonly emerges when *parC* and *gyrA* mutations co-occur (Drlica and Zhao, 1997). Consistent with this, *gyrA* point mutations were detected in isolates recovered from the 10×, 100×, and 1000× enrofloxacin exposure zones, likely contributing to the markedly elevated enrofloxacin MIC values observed (Nawaz et al., 2015).

Beyond target-site alterations, reduced intracellular antibiotic accumulation represents a key contributor to resistance and cross-resistance. Fluoroquinolones must traverse the outer membrane of Gram-negative bacteria via porin channels and altered expression of porin-regulating genes such as *ompA*, *ompC*, and *ompF* has been associated with resistance in *E. coli* (Ruiz, 2003; Vila, 2005).

In our study, mutations affecting multiple regulators of multidrug efflux systems were detected, suggesting a central role for efflux-mediated resistance. Insertions and deletions in *acrR*, a repressor of

the *acrAB-tolC* efflux system, are known to confer high-level resistance to β -lactams, tetracyclines, phenicols, and fluoroquinolones (Nishino et al., 2021). In addition, exposure to high antibiotic concentrations is known to induce stress responses involving the *marR* regulator, which controls expression of the MarA activator. MarA upregulates the *acrAB* efflux system while downregulating *ompF* porin synthesis (Chubiz, 2023; Delcour, 2009). Point mutations in *marR* observed in this study are therefore likely to contribute to the observed reductions in susceptibility.

The *robA* gene, a positive regulator of *acrAB* expression, exhibited a point mutation in the 10 $\times$ exposure group (Rosenberg et al., 2003; Weston et al., 2018). Furthermore, point mutations in *acrA*, encoding the periplasmic component of the *acrAB-tolC* efflux complex, were detected in isolates recovered from the 10 $\times$, 100 $\times$, and 1000 $\times$ enrofloxacin concentrations (Poole, 2004).

A point mutation in *emrR*, a regulatory gene controlling expression of the *emrAB-tolC* efflux system, was detected exclusively in the 1000 $\times$ enrofloxacin-exposed sample (Lomovskaya et al., 1995). Mutations were also identified in additional efflux-related regulators, including *mdtM* and *robA*, further supporting a broad activation of efflux-based resistance mechanisms.

The upregulation of multidrug efflux systems under antibiotic exposure has been associated with reduced oxidative stress in *E. coli* (Pourahmad Jaktaji and Ghalamfarsa, 2021). In parallel, the SOS response plays a central role in stress-induced adaptation. LexA and RecA are key regulators of the SOS network, which controls the expression of more than 50 downstream genes (Maslowska et al., 2019). In this study, both *lexA* and *recA*, along with the majority of SOS box-regulated genes, were detected in all isolates, suggesting that enrofloxacin exposure may engage conserved stress response pathways.

Taken together, the increased MIC values observed for antimicrobial compounds not directly applied during selection are most plausibly explained by the activation of multiple multidrug efflux pump systems, including *acrAB-tolC*, *mdtABC-tolC*, and *emrAB-tolC*. These systems are regulated by repressor and activator genes such as *acrR*, *emrR*, *marR*, *mdtM*, and *robA*, all of which exhibited mutations in enrofloxacin-exposed isolates.

While the present genomic analysis provides strong evidence for efflux-mediated cross-resistance, transcriptomic analyses will be required to confirm the phenotypic expression levels of the affected genes and to further delineate the regulatory dynamics underlying resistance evolution.

The observed collateral shifts in susceptibility under enrofloxacin selection support antimicrobial stewardship principles that aim to preserve fluoroquinolone efficacy. In veterinary practice, this includes reserving fluoroquinolones for situations where first-line agents are unsuitable, basing treatment on culture and susceptibility testing whenever feasible, and avoiding unnecessary metaphylactic or prolonged exposure that may expand selection for multidrug efflux-associated phenotypes. In parallel, strengthening prevention (biosecurity, vaccination where applicable, and husbandry measures) can reduce overall antimicrobial use and thereby reduce selection pressure for cross-resistance.

This study used a single reference strain in a controlled in vitro MEGA-plate environment. Therefore, the generalizability of the observed evolutionary trajectories to diverse clinical or farm isolates remains to be established. In addition, spatial selection on agar does not fully recapitulate in vivo microenvironments in animals (e.g., host factors, complex microbiota, and pharmacokinetic gradients), which may influence resistance evolution and opportunities for horizontal gene transfer. Finally, our genetic findings point to efflux-related regulatory changes; however, transcriptomic or functional assays would be required to quantify efflux expression and confirm causal links between specific mutations and the observed phenotypes.

5. Conclusions

This study demonstrates that exposure to enrofloxacin under stepwise selective pressure induces rapid genetic adaptation in *E. coli*, resulting in reduced susceptibility to multiple antimicrobial agents. The observed adaptive changes were primarily associated with mutations in genes encoding fluoroquinolone target enzymes (*gyrA*, *parC*) and in regulatory genes controlling multidrug efflux systems (*acrR*, *marR*, *robA*, *emrR*).

Despite pronounced phenotypic shifts in antimicrobial susceptibility, the overall resistance gene repertoire remained conserved across exposure levels, indicating that resistance evolution in this model was driven predominantly by chromosomal mutations rather than by the acquisition of new resistance determinants. These findings highlight the role of efflux-mediated mechanisms in antibiotic-induced cross-resistance.

The MEGA-plate system served as a suitable experimental platform for tracking stepwise resistance development and associated genetic changes under controlled antimicrobial pressure. Together, these results underscore the importance of prudent fluoroquinolone use in veterinary practice and provide mechanistic insights into the emergence of cross-resistance under sustained antibiotic exposure.

CRediT authorship contribution statement

Ádám Kerek: Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Bence Török:** Writing – review & editing, Project administration. **Levente Laczkó:** Formal analysis, Data curation. **Gábor Kardos:** Writing – review & editing, Conceptualization. **Krisztián Bányai:** Resources, Data curation. **Eszter Kaszab:** Writing – review & editing, Formal analysis. **Krisztina Bali:** Methodology, Formal analysis, Conceptualization. **Ákos Jerzsele:** Investigation, Funding acquisition.

Ethics statement

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rvsc.2026.106237>.

Data availability

Sequencing data generated in this study are available in NCBI under

BioProject accession PRJNA1262432. Other datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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