

Article

Phenotypic Collateral-Susceptibility and Cross-Resistance Signatures among Urinary Escherichia coli Isolates

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Abstract: Collateral effects occur when resistance to one antimicrobial is associated with altered susceptibility to another, creating either collateral susceptibility or cross-resistance. This relationship can provide phenotypic information beyond a conventional antibiogram. Objective to characterize collateral-susceptibility and cross-resistance signatures among urinary Escherichia coli using culture-based antimicrobial susceptibility testing. Eighty non-duplicate E. coli isolate records were evaluated with a nine-agent disk-diffusion panel. Broth microdilution minimum inhibitory concentrations (MICs) were analyzed for ciprofloxacin, ceftriaxone, gentamicin, and nitrofurantoin. Multidrug resistance (MDR), multiple-antibiotic-resistance (MAR) index, pairwise log₂ MIC shifts, Mann-Whitney tests, Benjamini-Hochberg false-discovery-rate correction, Wilson confidence intervals, and Spearman correlations were applied. Resistance was highest to ampicillin (76.3%), trimethoprim-sulfamethoxazole (63.8%), ciprofloxacin (61.3%), and ceftriaxone (56.3%), whereas nitrofurantoin (7.5%) and meropenem (3.8%) retained high activity. Fifty-six isolates (70.0%) met the MDR definition. Ciprofloxacin-resistant isolates had a median nitrofurantoin MIC of 8 µg/mL compared with 16 µg/mL among ciprofloxacin-susceptible isolates (median shift -1 log₂ dilution; FDR q<0.001). Ceftriaxone resistance showed the same one-dilution downward shift in nitrofurantoin MIC (q<0.001). In contrast, ciprofloxacin and ceftriaxone displayed strong bidirectional cross-resistance signatures. MAR index correlated positively with ciprofloxacin, ceftriaxone, and gentamicin MICs but inversely with nitrofurantoin MIC. A phenotypic analysis combining categorical susceptibility with quantitative MIC distributions can reveal resistance-associated susceptibility shifts that are not apparent from routine categorical reporting alone. Such associations should be interpreted as candidate collateral effects unless confirmed by longitudinal, isogenic, or mechanistic studies.

Keywords: Antimicrobial Resistance, Collateral Susceptibility, Cross-Resistance, Escherichia Coli, Urinary Tract Infection, Minimum Inhibitory Concentration, MDR

Introduction

Urinary tract infections (UTIs) are commonplace in clinical practice, and *Escherichia coli* is the most prevalent uropathogen. Fluoroquinolones, trimethoprim-sulfamethoxazole, and extended-spectrum cephalosporins are some of the antimicrobials most commonly used for empirical therapy; however, increasing resistance to these antimicrobials makes choice of appropriate therapy more difficult. Recent reviews and surveillance studies have documented significant variations in UTI resistance across different geographic regions, with high levels of concern in Iraq and other low- and middle-income countries (LMICs) [1-6]. It is prudent to conduct local susceptibility studies and analyze resistance in more detail, rather than relying on data from single-drug resistance tests.

Nitrofurantoin has maintained good activity against urinary *E. coli* for numerous decades. A global meta-analysis published recently reported the resistance of *Escherichia coli* (UPEC) to nitrofurantoin at 6.9%. Additional recent studies have reported low levels of resistance to nitrofurantoin compared to other agents used for the treatment of UTIs [6-10]. Nitrofurantoin is thought to maintain its activity against *E. coli* due to the requirement of multiple targets for resistance and fitness costs associated with some nitroreductase defects [8-10]. Resistance to nitrofurantoin has been reported, and the presence of other multidrug-resistant alleles has been observed.

Resistance to one antimicrobial can affect the susceptibility to another antimicrobial. When the susceptibility of a pathogen to an antimicrobial increases due to resistance to a different antimicrobial, it is referred to as collateral sensitivity or collateral susceptibility. Conversely, when a pathogen's susceptibility to an antimicrobial decreases due to resistance to a different antimicrobial, it is referred to as cross-resistance.

Taken together, recent studies in chemical genetics, large-scale surveillance, and experiments confirm that both directions of interaction can occur, although the magnitude, reproducibility, and clinical occurrence of these interactions depend on the organism, the type of resistance, the pair of drugs, and the organism's evolutionary history [11-14]. Clinical data are of particular importance, as the effects of molecular evolution in a laboratory setting are not necessarily applicable to the heterogeneous isolates found in clinical practice [13,14]. Uropathogenic *E. coli* is a relevant organism to study if there are directional relationships between drug phenotypes that can be found in routine susceptibility testing. Recent studies on UPEC have demonstrated that prior antimicrobial selection can alter the susceptibility of UPEC to other drugs used in the treatment of UTIs, and that collateral effects can be strain background dependent [15,16]. The goal of the present study was to determine the prevalence of resistance to certain antibiotics, as well as the burden and MAR index of multiple antibiotic resistances, and to determine pairwise MIC shifts of the antibiotics ciprofloxacin, ceftriaxone, gentamicin, and nitrofurantoin, among other things, to detect directional MIC shifts and collateral-susceptibility signatures among the urinary *E. coli* isolates, avoiding making any evolutionary causation assumptions based on the cross-sectional isolates collected [17]-[20].

Materials and Methods

Study design and isolate panel

A cross-sectional phenotypic analysis included 80 non-duplicate urinary *E. coli* isolates, with one isolate representing one patient episode. The laboratory analysis used conventional culture and biochemical identification followed by disk diffusion and manual broth microdilution.

Culture and identification

Isolates were streaked on MacConkey agar and incubated aerobically at 35–37°C for 18–24 h. A well-isolated lactose-fermenting colony was subcultured on tryptone soya agar to obtain a pure culture. Identification was performed using API 20 E strips. A single pure colony was suspended in 5 mL of 0.85% sodium chloride medium, the API 20 E cupules were inoculated according to the manufacturer's instructions, and strips were incubated at 36±2°C for 18–24 h. Reactions requiring

post-incubation reagents were developed with the API 20 E reagent kit and interpreted manually using the seven-digit biochemical profile.

Disk-diffusion antimicrobial susceptibility testing

A 0.5 McFarland suspension was prepared from an 18–24 h pure culture. Within 15 min of suspension preparation, a sterile swab was used to inoculate the entire surface of Mueller-Hinton agar in three directions to produce a confluent lawn. Agar depth was maintained at approximately 4 mm and the medium pH was within the manufacturer's specified range. Nine antimicrobial discs were applied: ampicillin (10 µg), amoxicillin/clavulanate (20/10 µg), ceftriaxone (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), trimethoprim/sulfamethoxazole (25 µg), nitrofurantoin (300 µg), and meropenem (10 µg). Plates were incubated in ambient air at 35±2°C for 16–18 h. Inhibition-zone diameters were measured manually to the nearest millimeter using CLSI disk-diffusion procedures [21] and interpreted according to CLSI M100, 36th edition (2026), using organism- and drug-appropriate criteria [23].

Broth microdilution MIC determination

Sterile 96-well plates were set up with cation-adjusted Mueller-Hinton broth and clinical laboratory standards institute (CLSI) dilution methodology was followed for the microdilution method [22], [22]. Stock solutions were prepared using analytical grade powders and diluted to correct for labeled potency. Ceftriaxone, ciprofloxacin, gentamicin, and nitrofurantoin working solutions were prepared with a twofold dilution and final concentrations of 0.0156–16 µg/mL, 0.0625–64 µg/mL, 0.125–128 µg/mL, and 1–256 µg/mL, respectively. Gentamicin and ceftriaxone working solutions were prepared in reagent water, and the ciprofloxacin working solution was prepared in an aqueous diluent with CLSI dilution procedures. Nitrofurantoin was dissolved in N,N-dimethylformamide and added to the broth to keep the volume of solvent used to a minimum and to include an appropriate solvent control.

The working inoculum was prepared from a fresh culture, adjusted to 0.5 McFarland, and was then diluted to provide a final well inoculum of approximately 5×10^5 CFU/mL. A volume of 100 µL was added to each well to obtain a final volume of 100 µL in each well. Growth-control wells contained inoculated broth without antimicrobial, and sterility-control wells contained uninoculated broth. Plates were incubated at 35±2°C for 16–20 h in ambient air. Endpoints were read visually; the MIC was the lowest antimicrobial concentration showing no visible growth compared with the growth control. MIC interpretation followed CLSI M100 criteria for *E. coli*/Enterobacterales and, for nitrofurantoin, the applicable urinary-isolate interpretation [23].

MDR definition and MAR index

Resistance was grouped into eight antimicrobial categories: aminopenicillins, penicillin/β-lactamase-inhibitor combinations, extended-spectrum cephalosporins, fluoroquinolones, aminoglycosides, folate-pathway inhibitors, nitrofurans, and carbapenems. MDR was defined as non-susceptibility to at least one agent in three or more antimicrobial categories, consistent with the widely used international definition [24]. The MAR index for each isolate was calculated as the number of tested agents to which the isolate was resistant divided by the total number of tested agents (nine).

Pairwise collateral-susceptibility and cross-resistance analysis

MICs were transformed to log₂ concentration values. For each focal index drug (ciprofloxacin, ceftriaxone, gentamicin, or nitrofurantoin), isolates categorized as intermediate to that index drug were excluded from the pairwise comparison. The MIC distribution of each other focal target drug was compared between index-drug-resistant and index-drug-susceptible isolates. The directional effect was summarized as $\Delta \log_2 \text{MIC} = \text{median } \log_2(\text{target MIC in index-resistant isolates}) - \text{median } \log_2(\text{target MIC in index-susceptible isolates})$. A $\Delta \log_2 \text{MIC}$ of –1 therefore represents a one twofold-dilution reduction, whereas +1 represents a one-dilution increase. A candidate collateral-susceptibility signature required $\Delta \log_2 \leq -1$ with FDR-adjusted $q < 0.05$; a cross-resistance signature required $\Delta \log_2 \geq +1$ with $q < 0.05$. These criteria describe phenotypic associations and do not by

themselves establish an evolutionary mechanism.

Statistical analysis

Resistance proportions were reported with Wilson 95% confidence intervals. MIC data were summarized using medians, interquartile ranges, MIC50, and MIC90. Pairwise MIC distributions were compared using the two-sided Mann–Whitney U test. The Benjamini–Hochberg procedure controlled the false discovery rate across the 12 directional pairwise comparisons. Spearman rank correlation tested associations between MAR index and log2 MIC. Two-sided $P < 0.05$ and FDR $q < 0.05$ were considered statistically significant.

Results

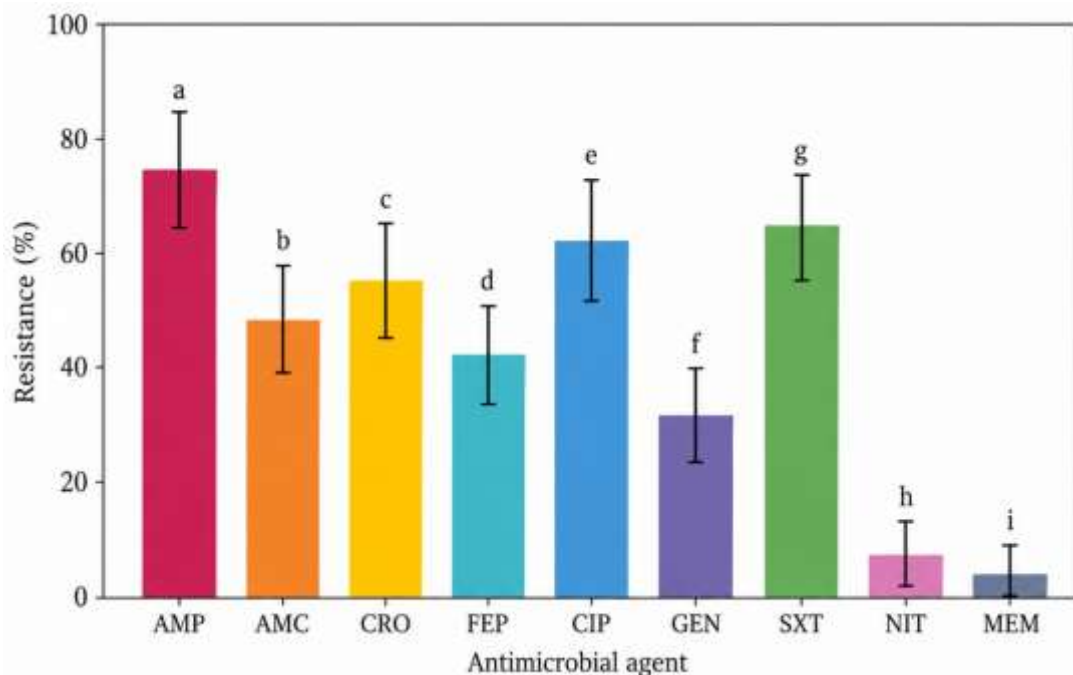
Antimicrobial resistance profile

The highest resistance frequencies were observed for ampicillin (61/80, 76.3%), trimethoprim–sulfamethoxazole (51/80, 63.8%), ciprofloxacin (49/80, 61.3%), and ceftriaxone (45/80, 56.3%). Resistance to cefepime and amoxicillin/clavulanate was 41.3% and 48.8%, respectively. Gentamicin resistance occurred in 31.3% of isolates. In contrast, nitrofurantoin and meropenem retained high activity, with resistance rates of only 7.5% and 3.8%, respectively (Table 1; Figure 1).

Table 1. Resistance prevalence among the 80 urinary *E. coli* isolates.

Code	Antimicrobial	Resistant, n/N	Resistance (%)	95% CI (%)
AMP	Ampicillin	61/80	76.3	65.9–84.2
AMC	Amoxicillin-clavulanate	39/80	48.8	38.1–59.5
CRO	Ceftriaxone	45/80	56.3	45.3–66.6
FEP	Cefepime	33/80	41.3	31.1–52.2
CIP	Ciprofloxacin	49/80	61.3	50.3–71.2
GEN	Gentamicin	25/80	31.3	22.2–42.1
SXT	Trimethoprim-sulfamethoxazole	51/80	63.8	52.8–73.4
NIT	Nitrofurantoin	6/80	7.5	3.5–15.4
MEM	Meropenem	3/80	3.8	1.3–10.5

Figure 1. Resistance prevalence with Wilson 95% confidence intervals for the nine tested antimicrobial agents.



Multidrug resistance and MAR index

Fifty-six of 80 isolates (70.0%) met the MDR definition. The median MAR index was 0.444 (IQR 0.222–0.667; range 0.000–0.778). The largest subgroup was resistant in five antimicrobial categories (18/80, 22.5%), followed by four categories (16/80, 20.0%) and six categories (12/80, 15.0%). Eight isolates showed no resistance to the tested agents (Figure 2).

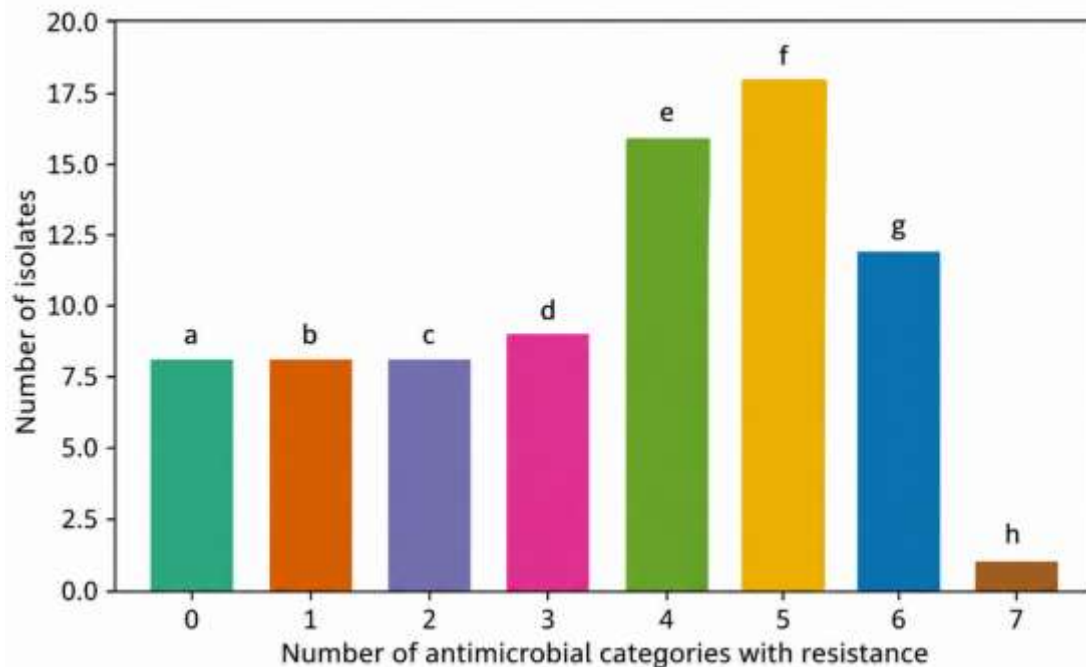


Figure 2. Distribution of isolates according to the number of antimicrobial categories showing resistance.

MIC distributions of focal antimicrobial agents

Table 2. MIC distribution for the four focal antimicrobial agents.

Drug	Median MIC	IQR	MIC50	MIC90	S/I/R (n)
CIP	3	0.25–8	4	16	26/5/49
CRO	8	1–32	8	64	31/4/45
GEN	2	1–32	2	64	53/2/25
NIT	16	8–16	16	64	71/3/6

Ciprofloxacin had an MIC50 of 4 µg/mL and MIC90 of 16 µg/mL, while ceftriaxone had an MIC50 of 8 µg/mL and MIC90 of 64 µg/mL. Gentamicin MIC50 and MIC90 values were 2 and 64 µg/mL, respectively. Nitrofurantoin showed the most favorable distribution, with a median and MIC50 of 16 µg/mL and an MIC90 of 64 µg/mL.

Collateral-susceptibility and cross-resistance signatures

The clearest collateral-susceptibility signal involved nitrofurantoin. Among ciprofloxacin-resistant isolates, the median nitrofurantoin MIC was 8 µg/mL (IQR 8–16) compared with 16 µg/mL (IQR 16–32) among ciprofloxacin-susceptible isolates, corresponding to a –1 log2 dilution shift ($P=6.78 \times 10^{-6}$; FDR $q=2.71 \times 10^{-5}$; Figure 3). Ceftriaxone resistance produced the same one-dilution reduction in nitrofurantoin MIC (8 versus 16 µg/mL; $P=4.11 \times 10^{-6}$; $q=2.47 \times 10^{-5}$).

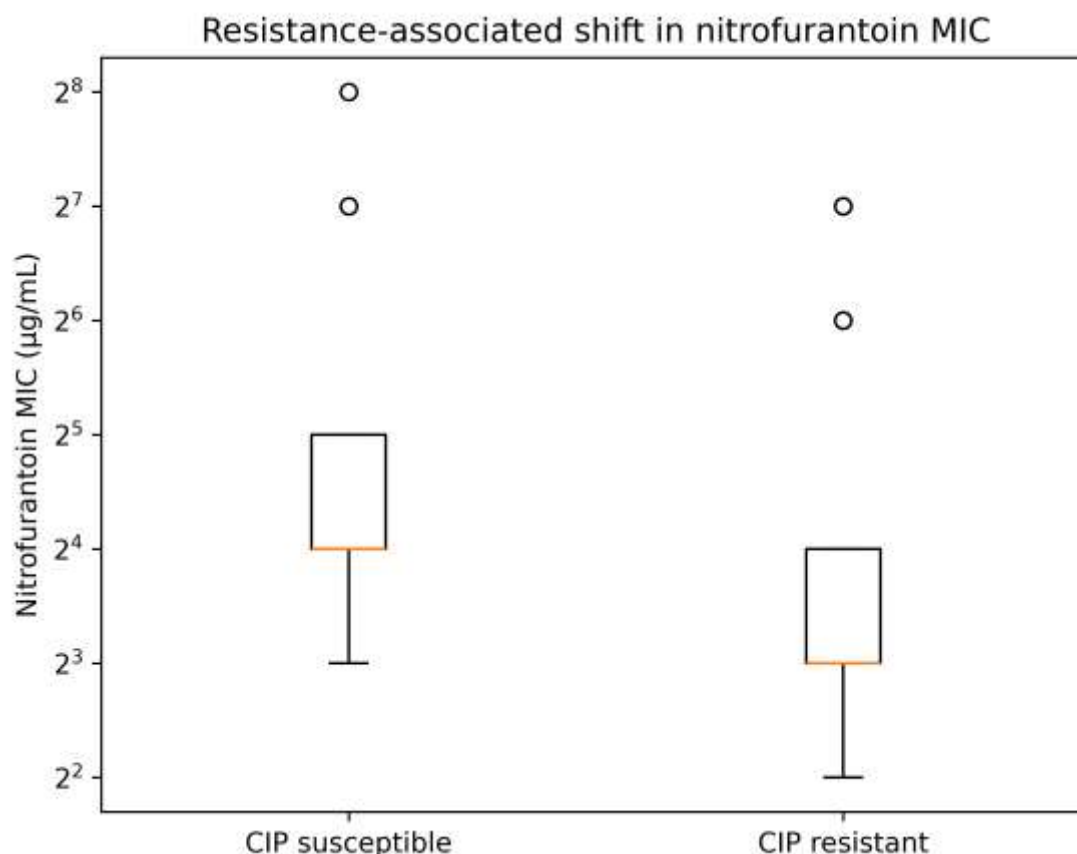


Figure 3. Nitrofurantoin MIC distribution according to ciprofloxacin phenotype. Intermediate ciprofloxacin isolates were excluded from this comparison.

In contrast, strong cross-resistance was evident between ciprofloxacin and ceftriaxone. Ceftriaxone-resistant isolates had a four-log₂ higher median ciprofloxacin MIC than ceftriaxone-susceptible isolates ($q < 0.001$), while ciprofloxacin-resistant isolates had a five-log₂ higher median ceftriaxone MIC ($q < 0.001$). Gentamicin resistance was also associated with increased ceftriaxone and ciprofloxacin MICs. The complete directional effect matrix is shown in Figure 4 and Table 3.

Table 3. Directional pairwise MIC shifts among focal antimicrobials.

Index drug	Target drug	$\Delta \log_2 \text{MIC}$	P	FDR q	Interpretation
CIP	CRO	+5.0	2.99e-05	8.97e-05	Cross-resistance association
CIP	GEN	+1.0	5.89e-02	7.07e-02	No FDR-significant directional effect
CIP	NIT	-1.0	6.78e-06	2.71e-05	Candidate collateral susceptibility
CRO	CIP	+4.0	4.49e-07	5.38e-06	Cross-resistance association
CRO	GEN	+1.0	9.50e-03	1.63e-02	Cross-resistance association
CRO	NIT	-1.0	4.11e-06	2.47e-05	Candidate collateral susceptibility
GEN	CIP	+1.0	4.35e-03	8.70e-03	Cross-resistance association
GEN	CRO	+5.0	7.69e-04	1.85e-03	Cross-resistance association
GEN	NIT	-1.0	1.70e-02	2.55e-02	Candidate collateral susceptibility
NIT	CIP	-3.5	2.30e-01	2.30e-01	No FDR-significant directional effect
NIT	CRO	-5.0	4.43e-02	5.90e-02	No FDR-significant directional effect
NIT	GEN	-0.5	9.89e-02	1.08e-01	No FDR-significant directional effect

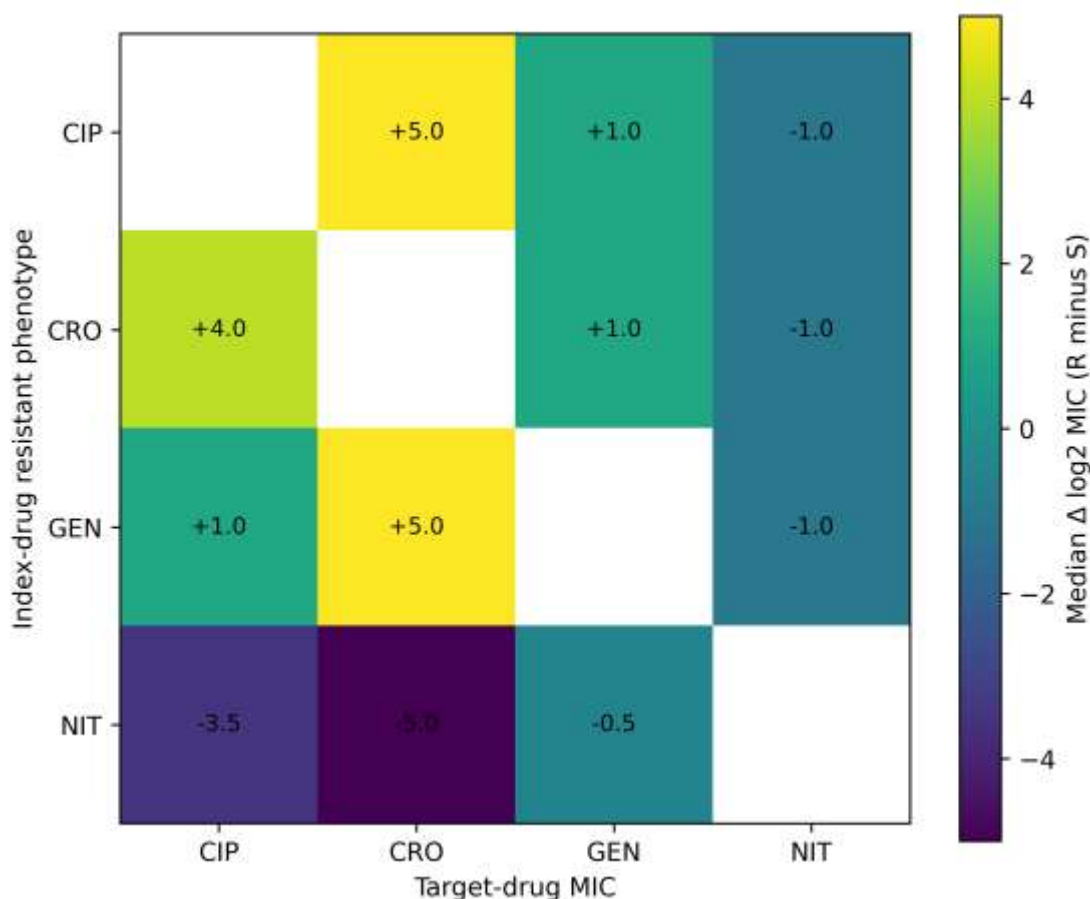


Figure 4. Pairwise collateral-effect matrix. Negative values indicate lower target-drug MICs in index-resistant isolates; positive values indicate higher target-drug MICs. Diagonal cells are not analyzed.

Relationship between resistance burden and focal-drug MIC

The MAR index correlated positively with ciprofloxacin MIC ($\rho=0.639$, $P<0.001$), ceftriaxone MIC ($\rho=0.725$, $P<0.001$), and gentamicin MIC ($\rho=0.453$, $P<0.001$). In contrast, MAR index correlated inversely with nitrofurantoin MIC ($\rho=-0.453$, $P<0.001$), providing an independent quantitative signal consistent with the nitrofurantoin collateral-susceptibility pattern (Figure 5).

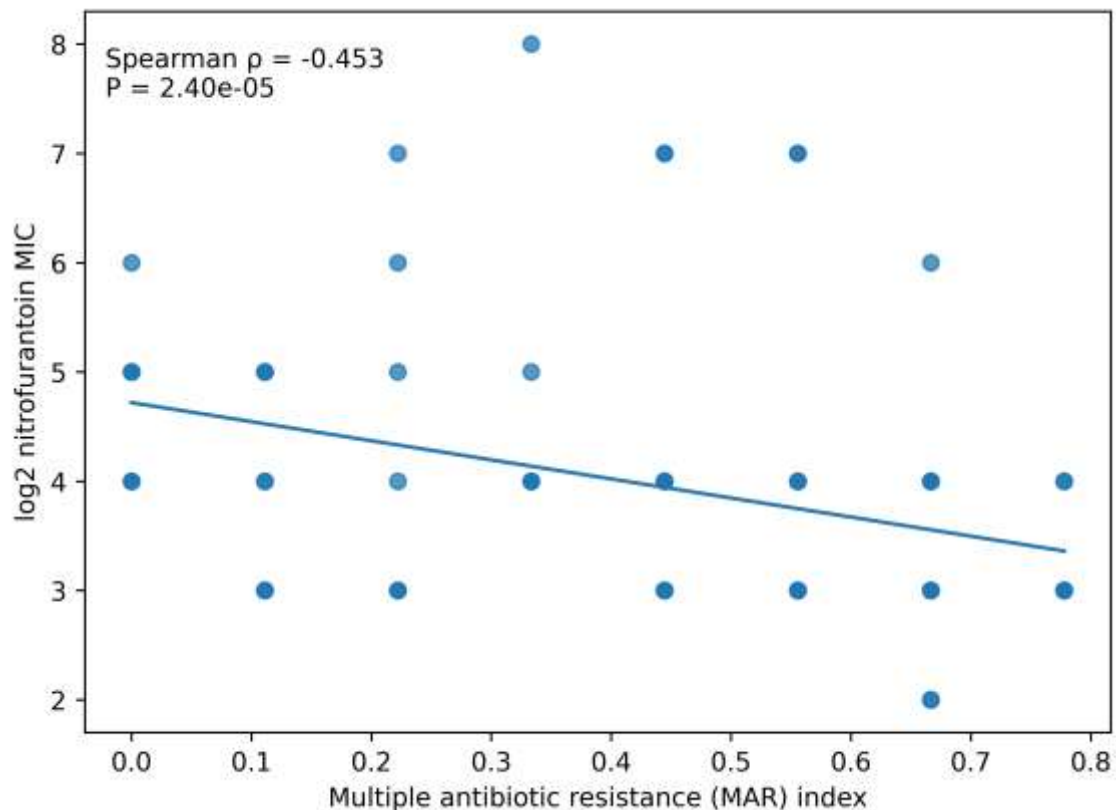


Figure 5. Relationship between MAR index and log2-transformed nitrofurantoin MIC.

Discussion

The resistance profile observed in this isolate collection is consistent with the broader concern surrounding antimicrobial-resistant UPEC. Ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, and ceftriaxone showed the highest levels of resistance in our samples. Nitrofurantoin and meropenem were active against the majority of our isolates. Increased resistance to the β -lactams, fluoroquinolones, and trimethoprim-sulfamethoxazole has been reported in recent studies from Iraq, but the specific frequencies varied with the location and population studied. Internationally, there is the same level of variability, with high pooled MDR estimates in some regions and a better preservation of the activity of nitrofurantoin in contemporary cystitis surveillance. Taking into account sampling and referral patterns, the 70.0% MDR in our sample can be considered a high resistance burden. Nitrofurantoin had the best overall susceptibility with only 7.5% of the isolates showing categorical resistance. This value is close to the global pooled estimate of 6.9% reported in a 2025 meta-analysis of UPEC and is compatible with recent evidence that nitrofurantoin remains comparatively durable against urinary *E. coli* [7–10]. Its continued activity is clinically important because resistance to several alternative oral agents is substantially more common in many regions. At the same time, recent mechanistic studies confirm that nitrofurantoin resistance can arise through alterations affecting nitroreductase pathways and that resistant UPEC isolates may occur in MDR backgrounds [9,10].

The main analytical finding was a one-log₂ lower nitrofurantoin MIC among ciprofloxacin-resistant and ceftriaxone-resistant isolates compared with their susceptible counterparts. MIC-level analysis is useful in this setting because a directional shift can occur within the categorical susceptible range and therefore remain invisible in a conventional S/I/R summary. Large-scale clinical surveillance has recently shown that collateral-sensitivity signals can be detected in clinical MIC datasets, although they are much less common than collateral resistance and are not uniformly conserved across pathogens or antimicrobial classes [13]. Experimental studies similarly show that collateral effects are strongly dependent on resistance trajectory and strain background [11,12,14–16]. For this reason, the nitrofurantoin shifts observed here are best described as candidate collateral-

susceptibility associations rather than proof of evolutionary collateral sensitivity.

The direction of the nitrofurantoin association should also be interpreted against evidence that collateral responses are not universal. In UPEC, trimethoprim selection has been reported to reduce nitrofurantoin susceptibility in some resistant derivatives, illustrating that even clinically relevant UTI drug pairs can show an opposite collateral direction depending on the selecting drug and genetic background [15]. Conversely, large-scale laboratory evolution and clinical comparison studies have identified reproducible collateral relationships in *E. coli* under other resistance trajectories [16]. Together, these findings support the value of phenotype-specific analysis but argue against assuming that a collateral relationship identified for one resistance background will generalize to all UPEC isolates.

Strong positive MIC shifts between ciprofloxacin and ceftriaxone, together with associations involving gentamicin, indicate that cross-resistance was the dominant pattern among several focal-drug pairs. This is consistent with recent work showing that cross-resistance and collateral susceptibility can coexist within the same bacterial species and that the direction of an interaction may depend on the underlying resistance mechanism [12,14,17,18]. The observed ciprofloxacin-ceftriaxone association should not be interpreted as evidence that resistance to one drug directly caused resistance to the other; in a cross-sectional clinical collection, co-selection, linked resistance determinants, clonal background, and prior antimicrobial exposure can all produce correlated MIC phenotypes.

The relationship between MAR index and nitrofurantoin MIC provides an additional descriptive pattern, but it is not statistically independent from susceptibility data because the MAR index contains the focal agents in its nine components. Therefore, when interpreting MAR-MIC correlations, we should be pleased rather than surprised. Some steps the authors took to strengthen their study were performing quantitative MICs, stating the direction of the effect size, correcting for multiple comparisons, and separating potential collateral susceptibility from cross-resistance. The authors acknowledge several limitations, such as their small single-species sample, four-drug MIC panel, cross-sectional design, and lack of resistance genotype or clonal background data. The authors also state that the absence of an independent validation cohort substantiates the coupling that may have existed in their MAR analyses. The authors encourage phenotypic MIC testing of nitrofurantoin in conjunction with resistance genotyping and other resources permitting.

Conclusion

This study demonstrated a high antimicrobial resistance burden among urinary *E. coli* isolates, with 70.0% meeting the MDR definition. Ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, and ceftriaxone showed the highest resistance rates, whereas nitrofurantoin and meropenem retained high activity. A significant one-log₂ reduction in nitrofurantoin MIC was observed among ciprofloxacin- and ceftriaxone-resistant isolates, indicating candidate collateral-susceptibility associations, while cross-resistance was observed among several other antimicrobial pairs. These findings highlight the value of quantitative MIC analysis for identifying directional susceptibility patterns and support further studies incorporating larger isolate collections, resistance genotyping, and clonal background analysis to validate the observed associations.

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