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Impact of *AcrAB-TolC* Pump of Efflux on Biofilm-Associated Antibiotic Resistance in *Escherichia coli*

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Abstract

Introduction: Multidrug-resistant (MDR) *Escherichia coli* strains causing urinary tract infections pose therapeutic challenges due to biofilms and efflux-mediated tolerance. The AcrAB-TolC system expels antibiotics and promotes biofilm formation. To investigate the contribution of the AcrAB-TolC pump to biofilm-associated resistance in *E. coli* and evaluate chlorpromazine (CPZ) as an efflux inhibitor to restore antimicrobial efficacy.

Methods: Sixty clinical *E. coli* isolates from UTI patients were identified by phenotypic and molecular testing. Antibiotic susceptibility was assessed using the Kirby–Bauer method, and biofilm formation was quantified using a microtiter plate assay. Efflux activity was phenotypically evaluated using the ethidium bromide (EtBr) cartwheel method. The presence of *acrA*, *acrB*, *tolC*, and *gapA* was confirmed by PCR, and their relative expression levels were quantified by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method.

Results: Of the 60 isolates, 70% were MDR, and 66.6% produced strong or moderate biofilms. Active efflux was observed in 68.3% of the isolates and was strongly correlated with multidrug resistance. PCR confirmed the presence of *acrA*, *acrB*, and *tolC* in all MDR isolates. Combining a sub-inhibitory CPZ concentration with ciprofloxacin reduced the CPZ MIC by two- to fourfold, thereby restoring susceptibility. RT-qPCR revealed significant downregulation of *acrA*, *acrB*, and *tolC* expression following CPZ.

Conclusion: The AcrAB-TolC efflux pump is central to the pathogenesis and persistence of MDR E. coli infections. Integrating targeted efflux inhibitors, such as CPZ, with antibiotics holds significant promise for dismantling biofilm resilience and restoring their efficacy.

Keywords: *Escherichia coli*, Biofilms, Drug resistance, Multiple, Chlorpromazine, Membrane transport proteins

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Introduction

Efflux pump systems are a major factor in the escalating problem of antimicrobial resistance, as they actively expel antibiotics and other toxic substances from the bacterial cells. Among these systems, AcrAB-TolC is the most prominent multidrug resistance (MDR) efflux pump in *Escherichia coli* and other Enterobacteriaceae species. This system is capable of extruding a broad spectrum of antimicrobial agents, as well as a variety of other toxic compounds, such as bile salts and detergents (1).

Several MDR efflux pumps have been implicated in biofilm development, enabling bacterial cells to withstand antibiotic pressure within structured communities. Notably, the transcriptional activation of genes encoding the RND-type AcrAB-TolC efflux system has been observed in E. coli biofilms following exposure to various antimicrobial agents (2). Conversely, genetic disruption of efflux pump components is associated with impaired biofilm formation. For instance, *tolC* deletion in enteroaggregative *E. coli* markedly reduced surface adhesion and biofilm biomass, accompanied by downregulation of aggregative fimbrial expression. Similarly, the loss of other proton motive force-dependent efflux pump genes, including *mdtE*, *acrE*, *acrD*, *emrK*, *emrE*, and *emrD*, resulted in a significantly diminished biofilm-forming capacity compared to that of wild-type strains (3). At the regulatory level, AcrAB-TolC expression integrates environmental cues through global activators such as MarA, SoxS, and Rob, as well as local repressors such as AcrR/EnvR (4,5).

Mechanistically, efflux contributes to biofilm robustness by lowering intracellular antibiotic concentrations and exporting quorum-sensing signals and matrix-modulating metabolites that facilitate biofilm maturation. Inhibiting RND pumps using adjuvants such as phenylalanine-arginine β-naphthylamide (PAβN) or 1-(1-naphthylmethyl)-piperazine (NMP) often reduces biofilm biomass and restores antibiotic susceptibility in *E. coli* and related gram-negative bacteria, underscoring a functional link between efflux activity and biofilm integrity(6). However, strain-specific outcomes and compensatory pathways have been reported, highlighting the necessity of context-aware analyses (7,8).

Clinically, the AcrAB-TolC system dampens the bactericidal effects of multiple antibiotic classes and promotes phenotypes such as fluoroquinolone persistence. These factors complicate the treatment of urinary and device-associated infections, where biofilms predominantly occur (9). Therapeutic combinations that pair antibiotics with efflux pump inhibitors (EPIs) or regulatory modulators

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are being investigated as resistance-modifying approaches against biofilm-embedded *E. coli* (10).

Among potential efflux pump inhibitors, the phenothiazine derivative chlorpromazine (CPZ) has emerged as a promising candidate for repurposing against resistant bacterial infections. Originally developed as an antipsychotic medication, CPZ has been demonstrated to effectively inhibit the AcrAB-TolC efflux system by interfering with substrate binding, thereby restoring the susceptibility of *E. coli* to various antimicrobial agents (11). Furthermore, studies indicate that CPZ not only impairs efflux-mediated resistance but also significantly attenuates quorum-sensing regulated virulence factors and prevents the formation of bacterial biofilms (12,13). The dual action of CPZ, inhibiting both efflux activity and biofilm development, makes it a highly relevant compound for investigating resistance-modifying strategies in device-associated and urinary tract infections.

Precise characterization of efflux pump families and elucidation of their specific contributions to biofilm formation are essential for developing targeted inhibitory strategies to prevent biofilm establishment. Given that multidrug resistance is a defining characteristic of efflux pump systems, examining its association with biofilm development in bacterial populations is of particular importance(14). Although enhanced expression of efflux pump genes and increased antimicrobial tolerance are commonly observed in biofilm-associated cells, reduced expression has also been reported. Such variability is likely attributable to the physiological heterogeneity within biofilm communities (15).

Despite the recognized importance of the AcrAB-TolC efflux system in multidrug resistance, a significant research gap remains regarding its precise mechanistic role in biofilm-associated tolerance among clinical *E. coli* isolates, particularly concerning how efflux inhibition with agents such as CPZ affects both biofilm structural integrity and antibiotic susceptibility. Therefore, this study aimed to investigate the functional contribution of the AcrAB-TolC efflux pump to biofilm-associated antibiotic resistance in *E. coli* and to evaluate the potential of efflux pump inhibition as a therapeutic strategy to enhance antimicrobial efficacy against biofilm infections.

Materials and Methods

Sample Collection and Identification of E. coli

Clinical *E. coli* isolates were collected from patients diagnosed with urinary tract infection (UTIs) at local Iraqi hospitals. Each isolate was cultured on MacConkey agar and Eosin Methylene Blue (EMB) agar at 37°C for 24 h, and typical metallic-green sheen colonies were identified as presumptive *E. coli.* Colonies were confirmed by Gram staining and standard biochemical tests, including indole, methyl red, Voges–Proskauer, and citrate utilization assays. Confirmed isolates were preserved in tryptic soy broth with 20% glycerol at –80°C until further use.

Molecular Identification of E. coli

Genomic DNA was extracted from bacterial cells using a spin-column-based genomic DNA isolation kit (Norgen®, Canada) according to the manufacturer's protocol. Purity and concentration were evaluated using a Qubit 4 fluorometer (Invitrogen®, USA).

Conventional PCR was used for the molecular detection of *gapA* as a bacterial confirmation marker and for the pump of efflux-related genes (*acrA*, *acrB*, and *tolC*). Each 25 µL reaction contained 4 µL HOT FIREPol® Master Mix (Solis®, Spain), 3 µL of DNA template, 0.5 µL of each primer (10 pmol/µL, Macrogen®, Korea), and 17 µL nuclease-free water. Amplification was performed under the following cycling conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 52°C for 45 s, 72°C for 45 s; and a final extension at 72°C for 7 min. PCR products were visualized by electrophoresis in a 1.5% agarose gel stained with RedSafe dye and photographed using a Cleaver® gel documentation system.

Antibiotic Susceptibility Testing

Antibiotic susceptibility profiles were determined by the Kirby–Bauer disc diffusion method on Mueller–Hinton agar according to(16)guidelines. The tested antibiotics included Amikacin (AMK, 30 µg), Gentamicin (GEN, 10 µg), Ampicillin (AMP, 10 µg), Amoxicillin (AMX, 25 µg), Imipenem (IPM, 10 µg), Tetracycline (TET, 30 µg), Ciprofloxacin (CIP, 5 µg), Levofloxacin (LEV, 5 µg), Nalidixic acid (NAL, 30 µg), Nitrofurantoin (NIT, 300 µg), Cefixime (CFM, 5 µg), Cefepime (FEP, 30 µg), Piperacillin–Tazobactam (PIT, 100/10 µg), and Aztreonam (ATM, 30 µg). The diameters of inhibition zones were interpreted as susceptible, intermediate, or resistant. Multidrug resistance (MDR) was defined as resistance to ≥ 3 antimicrobial classes.

Biofilm Formation Assay

Biofilm formation was quantified using a crystal violet microplate assay. Standardized bacterial suspensions (0.5 McFarland) prepared in tryptic soy broth containing 1% glucose were inoculated (200 µL) into 96-well polystyrene plates and incubated at 37 °C for 24 h. After PBS washing, the adherent cells were stained with 0.1% crystal violet and solubilized with 95% ethanol. Biofilm biomass was determined by measuring the optical density at 570 nm, and the isolates were categorized according to the strength of biofilm formation.

Phenotypic Efflux Detection (EtBr Cartwheel)

Phenotypic detection of active efflux pumps was performed using the Ethidium Bromide (EtBr) agar cartwheel method, as described by Martins et al.(17), with minor modifications. Briefly, *E. coli* isolates were initially grown in BHI broth and incubated at 37 °C for 24 h. The overnight cultures were centrifuged, washed, and resuspended in 5 mL of PBS, after which the bacterial density was adjusted to the 0.5 McFarland standard. MHA plates supplemented with graded concentrations of EtBr (0, 0.5, 1.0, 1.5, and 2.0

mg/L) were prepared on the same day of use and shielded from light to prevent degradation. Each plate was aseptically divided into eight radial sectors to form a cartwheel pattern. The Standardized bacterial suspensions were streaked across each sector, and the plates were incubated in the dark at 37 °C for 16–18 h. Following incubation, bacterial growth was visualized under a UV transilluminator. Isolates exhibiting reduced fluorescence at higher EtBr concentrations were interpreted as possessing active efflux pump mechanisms, whereas strong fluorescence indicated diminished efflux activity.

Determination of Minimum Inhibitory Concentration

The MIC of CPZ was determined for selected MDR *E. coli* isolates using the broth microdilution method, in accordance with standard guidelines(18). CPZ was serially diluted two-fold in Mueller–Hinton broth to achieve final concentrations from 2–512 µg/mL. Each well was inoculated with a standardized bacterial suspension diluted to the 0.5 McFarland standard and incubated at 37 °C overnight (18–24 h). The wells were then examined visually, and the MIC was defined as the lowest concentration of CPZ that completely inhibited visible growth. The MIC values obtained were used to assess the inherent antibacterial activity of CPZ and included a sub-inhibitory concentration (0.5 × MIC) to combine assays with ciprofloxacin (19).

MIC of Ciprofloxacin in the Presence of Chlorpromazine

The minimum inhibitory concentration (MIC) of Ciprofloxacin was assessed using the broth microdilution technique, with twofold serial dilutions ranging from 2 to 512 µg/mL. The minimum inhibitory concentration (MIC) was determined as the lowest concentration that effectively inhibited visible bacterial growth following a 24-hour incubation period at 37 °C. A sub-MIC concentration (0.5 × MIC) of CPZ was subsequently employed in conjunction with ciprofloxacin to assess its impact on antibiotic susceptibility.

Gene Expression

Total RNA was extracted from *E. coli* biofilm and planktonic cultures utilizing TRIzol™ reagent (Invitrogen®, USA). In summary, cell pellets were lysed in 0.5 mL of TRIzol, followed by phase separation with chloroform and precipitation with isopropanol. The RNA pellets were washed with 75% ethanol, air-dried, and subsequently resuspended in RNase-free water. The integrity of RNA was validated by agarose gel electrophoresis, and its purity was assessed by measuring the A_{260}/A_{280} ratio with spectrophotometry. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA utilizing the FIREScript® RT cDNA Synthesis Kit (Solis®, Spain) with random primers, in accordance with the manufacturer's guidelines.

Quantitative Real-Time PCR

The gene expression levels of *acrA*, *acrB*, and *tolC* were quantified using HOT FIREPol® SolisGreen qPCR Master Mix (Solis®, Spain) on a Bioer® Real-Time PCR system. All primers utilized in this study were designed by Macrogen® (Korea) (Table 1). The reactions (20 µL) contained 10 µL of 2 × Master Mix, 1 µL of each primer (0.5 µM), 2 µL of cDNA, and 6 µL of nuclease-free water. The thermocycling protocol consisted of an initial denaturation at 95°C for 60 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s (with plate read). Melt-curve analysis (60–95°C). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method(20), normalizing to the gapA (housekeeping gene).

Statistical Analysis

All quantitative data are expressed as mean ± SD. Statistical analyses and graph plotting were conducted using GraphPad Prism v9.0. The relationship between biofilm formation strength, efflux gene expression, and antibiotic resistance patterns was analyzed to elucidate the role of AcrAB-TolC in biofilm-associated antimicrobial tolerance.

Results

A total of 60 isolates were successfully recovered from 100 urine specimens collected from patients diagnosed with UTIs following comprehensive clinical and laboratory examination. All 60 isolates were subsequently subjected to molecular identification to confirm their species. Conventional PCR targeting the *gapA* gene was used as a definitive confirmatory step. Molecular analysis corroborated the phenotypic findings, definitively identifying all 60 isolates as *E. coli* (Figure 1).

Table 1. Primers that were utilized in this study

ID	Sequence (5'-3')	Length (bp)	Tm (°C)	GC%	Product (bp)
Q-*tolC*-F	AACGCCGGAACAGAATGCTA	20	60.04	50	95
Q-*tolC*-R	CGTTACTGGTGGTAGTGCGT	20	60.04	55	
Q-*acrA*-F	AGCGGGATTATCCTGAAGCG	20	59.97	55	117
Q-*acrA*-R	ATCACCTTTCGCACTGTCGT	20	59.97	50	
Q-*acrB*-F	AGCTTCCTGATGGTTGTCGG	20	60.04	55	102
Q-*acrB*-R	ACGGCTGATGGCATCTTTCA	20	60.04	50	
Qh-*gapA*-F	ACTTACGAGCAGATCAAAGC	20	60.2 °C	45 %	170
Qh-*gapA*-R	AGTTTCACGAAGTTGTCGTT	20	60.3 °C	40 %	

The antimicrobial susceptibility patterns of the isolated *E. coli* strains demonstrated marked heterogeneity across the 14 antimicrobial agents tested (Figure 2). Amikacin (AMK) was the most effective, with 100% sensitivity and no resistance. Imipenem (IMP) and nitrofurantoin (NIT) also demonstrated strong activity, with sensitivity rates of 96.6% and 90.0%, respectively. Conversely, piperacillin-tazobactam (PIT) and aztreonam (ATM) exhibited moderate efficacy, with sensitivity rates of 71.6% and 43.3%, respectively. Fluoroquinolones (Ciprofloxacin, Levofloxacin, and Nalidixic acid) and tetracycline demonstrated poor activity, with sensitivity rates ranging from 6.6% to 31.6%. Notably, Ampicillin was entirely ineffective, with 100% of isolates demonstrating complete resistance.

Assessment of biofilm-forming capabilities revealed that a substantial proportion of the 60 E. coli isolates exhibited strong adherence. Specifically, 48.3% (n = 29) of the isolates were classified as strong biofilm producers, representing the largest cohort in the sample. Furthermore, 28.3% (n = 17) of the isolates demonstrated a moderate capacity for biofilm formation. In contrast, the remaining 23.3% (n = 14) were categorized as weak biofilm producers, with over three-quarters (66.6%) exhibiting either strong or moderate biofilm-producing capabilities (Table 2).

Phenotypic assessment of efflux pump activity was conducted across both MDR and non-MDR *E. coli* isolates using the EtBr agar cartwheel method. The findings indicated a widespread prevalence of active efflux mechanisms in the bacterial population, with 41 of 60 isolates (68.33%) demonstrating positive efflux pump activity (Figure 3).

A robust association was observed between the presence

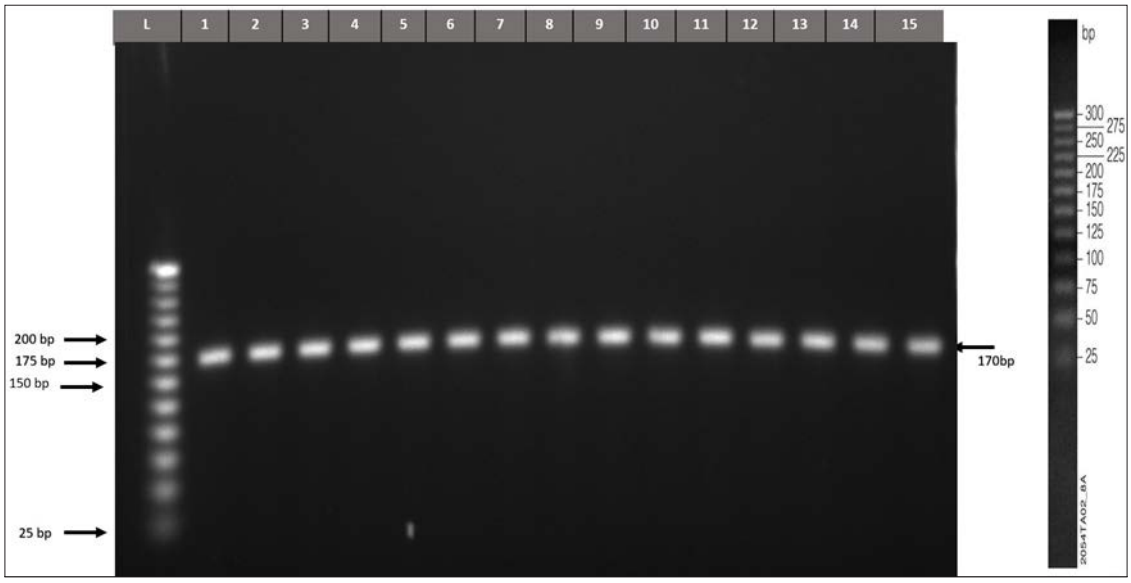
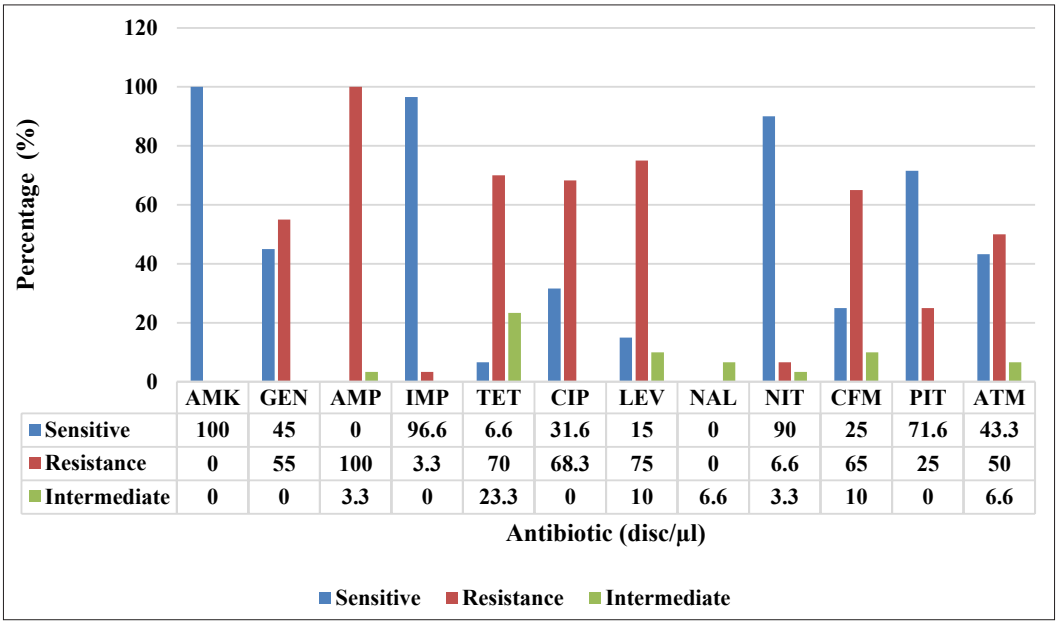


Figure 1. Gel electrophoresis on 2% agarose showing PCR amplification of the *gapA* gene (170 bp) from *E. coli* isolates. All lanes display the expected 170 bp positive band, confirming the presence of the *gapA* gene. Lane L represents the 300 bp DNA Ladder used as a molecular weight marker.



	AMK	GEN	AMP	IMP	TET	CIP	LEV	NAL	NIT	CFM	PIT	ATM
Sensitive	100	45	0	96.6	6.6	31.6	15	0	90	25	71.6	43.3
Resistance	0	55	100	3.3	70	68.3	75	0	6.6	65	25	50
Intermediate	0	0	3.3	0	23.3	0	10	6.6	3.3	10	0	6.6

Figure 2. Antibiotic susceptibility test of *E. coli*

of active efflux pump systems and the multidrug-resistant phenotype in *E. coli* isolates. Of the 60 isolates evaluated, a significant majority (70.0%; n = 42) were classified as MDR, whereas 30.0% (n = 18) were categorized as non-MDR. Within the MDR cohort, 60.0% (n = 36) exhibited positive efflux pump activity, compared to only 10.0% (n = 6) who lacked such activity. In contrast, in the non-MDR cohort, the prevalence of active efflux pumps was markedly lower: only 8.33% (n = 5) were positive, while 21.67% (n = 13) were negative. Overall, 41 isolates (68.33%) exhibiting active efflux mechanisms significantly contributed to the reservoir of antimicrobial resistance(Table 3).

Molecular characterization of the AcrAB-TolC efflux system confirmed the ubiquity of its constituent genes across MDR E. coli isolates. Electrophoretic analysis of the PCR products unequivocally demonstrated the successful amplification of *tolC*, *acrA*, and *acrB* in all MDR isolates. Specifically, the *tolC* gene was consistently identified by the presence of a distinct 95 bp band on a 2% agarose gel (Figure 4). Similarly, the *acrA* gene was successfully amplified in all isolates, yielding an expected 117 bp band (Figure 5). Furthermore, the *acrB* gene was detected in all samples, producing a clear, distinct 102 bp band (Figure 6). The absence of non-specific smearing across all gels indicates the high conservation and structural integrity of these genes in the isolates.

The potential of CPZ to modulate antimicrobial resistance was evaluated by determining the MICs of ciprofloxacin in combination with a sub-inhibitory concentration (0.5 × MIC) of CPZ in the tested *E. coli* isolates. MIC of CPZ was 32 µg/mL. Before CPZ exposure, the ciprofloxacin MICs ranged from 2 to 32 µg/mL, indicating reduced susceptibility or established clinical resistance. However, co-administration of CPZ substantially reduced the ciprofloxacin MICs to ≤ 1-16 µg/mL. This shift represents a two- to four-fold reduction in MIC values across the tested isolates. Notably, isolates 2 and 6 exhibited a pronounced four-fold reduction in their respective ciprofloxacin MICs (from 16 to 4 µg/mL and from 8 to 2 µg/mL, respectively) when challenged with combination therapy (Table 4).

The analysis demonstrated a consistent and significant downregulation of the efflux pump genes *acrA*, *acrB*, and *tolC* following exposure to CPZ. As detailed in Table 5,

Table 2. Biofilm formation ability of *E. coli*

Biofilm formation	n	%
Strong biofilm producers	23	38.3
Moderate-biofilm producers	17	28.3
Weak-biofilm producers	20	33.3
Total	60	100

Table 3. Distribution of the pump of efflux among MDR and non-MDR isolates of *E. coli*

Antibiotic resistance	Efflux +		Efflux -		Total	
	No.	%	No.	%	No.	%
MDR	36	60	6	10	42	70
Non-MDR	5	8.33	13	21.67	18	30
Total	41	68.33	19	31.67	60	100

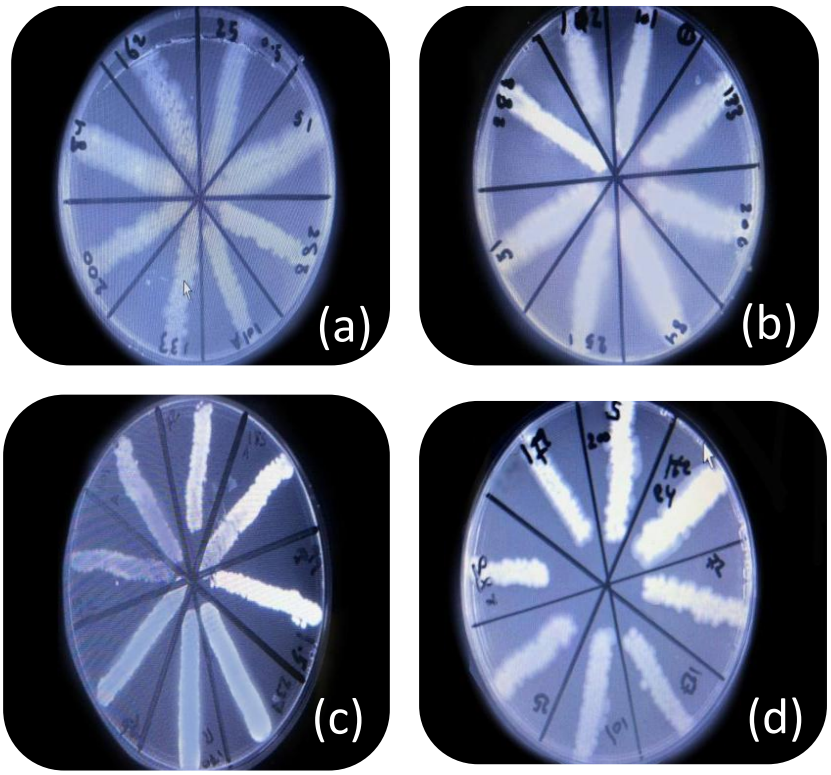


Figure 3. EtBr agar cartwheel assay demonstrating phenotypic detection of active efflux pump systems in multidrug-resistant *E. coli* isolates. Panels (A–D) show bacterial growth patterns at increasing EtBr concentrations: (A) 0.5 µg/mL, (B) 1.0 µg/mL, (C) 1.5 µg/mL, and (D) 2.0 µg/mL. Reduced fluorescence intensity at higher EtBr concentrations indicates the presence of functional efflux mechanisms.

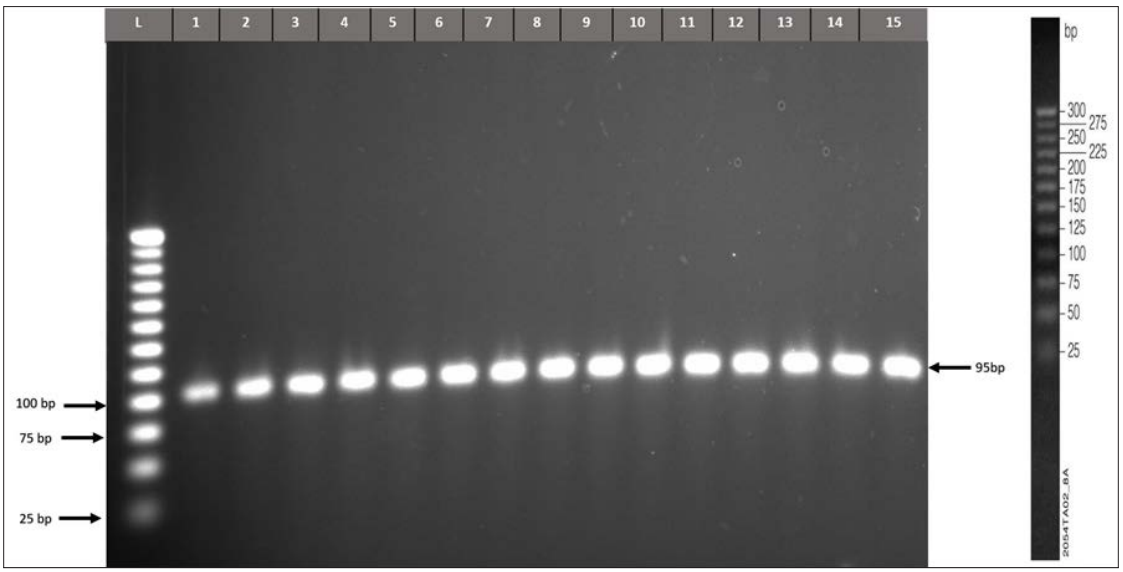


Figure 4. Gel electrophoresis on 2% agarose showing PCR amplification of the *TolC* gene (95 bp) from *E. coli* isolates. All lanes display the expected 95 bp positive band, confirming the presence of the *TolC* gene. Lane L represents the 300 bp DNA Ladder used as a molecular weight marker.

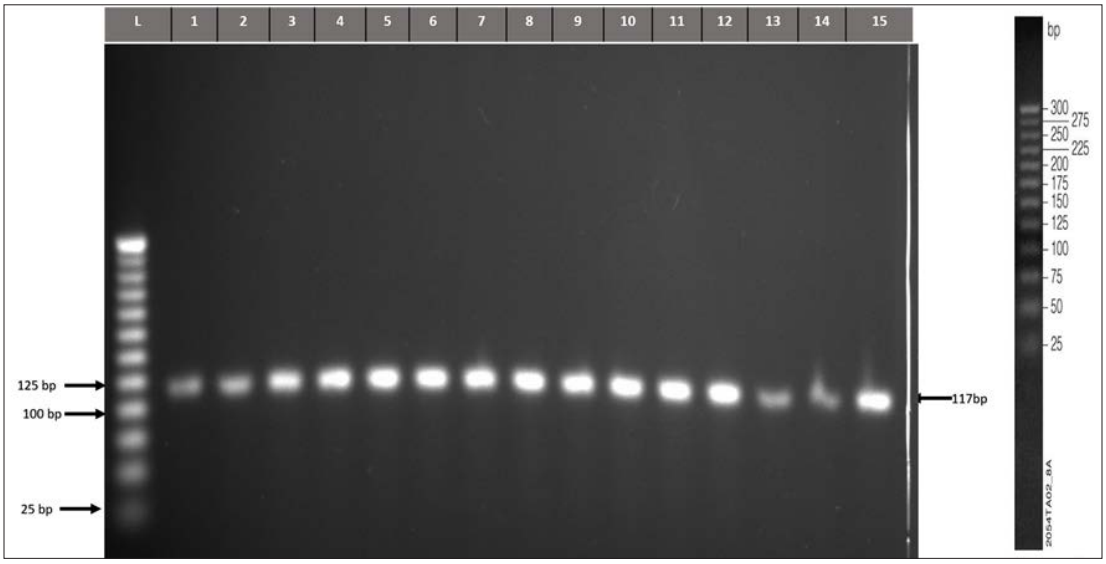


Figure 5. Gel electrophoresis on 2% agarose showing PCR amplification of the *acrA* gene (117 bp) from *E. coli* isolates. All lanes display the expected 117 bp positive band, confirming the presence of the *acrA* gene. Lane L represents the 300 bp DNA Ladder used as a molecular weight marker.

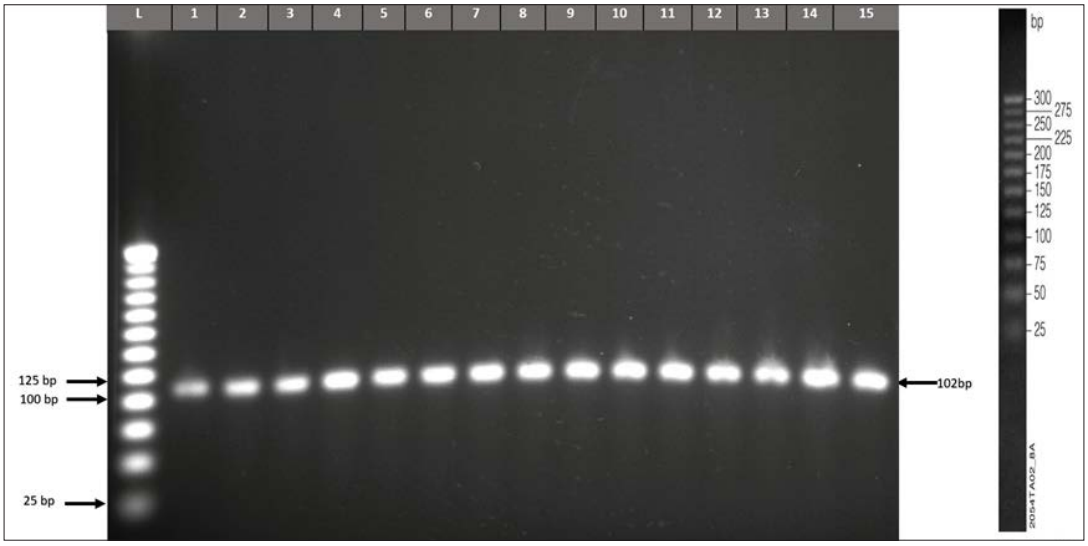


Figure 6. Gel electrophoresis on 2% agarose showing PCR amplification of the *acrB* gene (102 bp) from *E. coli* isolates. All lanes display the expected 102 bp positive band, confirming the presence of the *acrB* gene. Lane L represents the 300 bp DNA Ladder used as a molecular weight marker.

Table 4. Effect of chlorpromazine on ciprofloxacin MICs in MDR *E. coli* isolates

Isolate No.	MIC of Ciprofloxacin (µg/mL)	MIC of Ciprofloxacin + 0.5 × MIC CPZ (µg/mL)	Fold Reduction
1	16	8	2-fold ↓
2	16	4	4-fold ↓
3	32	16	2-fold ↓
4	32	16	2-fold ↓
5	2	≤1	≥2-fold ↓
6	8	2	4-fold ↓

Table 5. Effect of chlorpromazine exposure on *AcrAB-TolC* pump of efflux gene expression in *E. coli*

***AcrA* gene**							
No	**Before treatment**			**Treatment with CPZ**			**Fold change**
	gapA	**Acr A**	**ΔCt**	**gapA**	**AcrA**	**Δ Ct**	
1	11.22	17.68	6.46	15.23	23.00	7.57	0.46
2	10.57	14.67	4.10	16.48	21.90	4.17	0.40
AcrB gene							
No	Before treatment			Treatment with CPZ			Fold change
	gapA	Acr B	ΔCt	gapA	AcrB	ΔCt	
1	11.22	17.68	6.06	15.23	22.50	7.27	0.43
2	10.57	14.67	2.85	16.48	21.90	4.12	0.41
TolC gene							
No	Before treatment			Treatment with CPZ			Fold change
	gapA	Tol C	ΔCt	gapA	Tol C	ΔCt	
1	11.22	17.62	6.40	15.23	23.00	7.77	0.39
2	10.57	14.48	4.10	16.48	21.90	5.42	0.40

the baseline Ct values for the *acrA* gene in isolates 1 and 2 were 6.46 and 4.10, respectively. Following CPZ treatment, these values increased to 7.57 and 4.17, corresponding to calculated fold changes of 0.46 and 0.40. A similar trend was observed for the *acrB* gene, where pre- treatment Ct values of 6.06 and 2.85 for isolates 1 and 2 increased to 7.27 and 4.12 post-Δ treatment, resulting in fold changes of 0.43 and 0.41, respectively. Furthermore, the expression of the *tolC* gene was markedly suppressed; initial Ct values of 6.40 and 4.10 for isolates 1 and 2Δ, respectively, increased to 7.77 and 5.42 after CPZ exposure, yielding fold changes of 0.39 and 0.40. Overall, across both tested isolates, the relative expression of all three genes was reduced to approximately 0.4- fold of their baseline levels, indicating an approximate 60% reduction in transcript levels upon CPZ treatment.

Discussion

The present study established a significant association between the AcrAB-TolC efflux pump system and MDR in clinical *E. coli* isolates from UTIs. The findings demonstrated that 70% of the isolates exhibited an MDR phenotype, and 68.3% possessed active efflux mechanisms that were significantly correlated with MDR. This high prevalence is consistent with recent epidemiological data from uropathogenic *E. coli* (UPEC) isolates, where 75% of integron-positive isolates demonstrated MDR phenotypes and over 88% harbored AcrAB-TolC efflux pump genes (21). The functional activity of the AcrAB-TolC pump, rather than its mere genetic presence, is the primary driver of resistance to diverse antimicrobial agents, including β-lactams and fluoroquinolones (21,22). This distinction between genetic presence and functional activity is critical, as previous studies have noted that gene detection alone does not necessarily reflect functional efflux activity (21).

Molecular characterization confirmed the universal carriage of *acrA*, *acrB*, and *tolC* among all MDR isolates, with concurrent presence observed in 88.4% of the isolates. This finding parallels recent studies demonstrating that *tolC*, *acrB*, and *acrA* were detected in 96.1%, 92.3%, and 88.4% of UPEC isolates, respectively (21). The critical distinction between genetic presence and functional expression is demonstrated by the enhanced expression levels of these genes in resistant isolates, thereby substantiating the functional contribution of the AcrAB-TolC system to the observed resistance profiles (21). The correlation between gene expression levels and biofilm strength observed in this study aligns with the established role of efflux pump upregulation in biofilm-associated resistance (21,23).

Biofilm assays revealed that 66.6% of the isolates were strong or moderate biofilm producers, underscoring the clinical significance of biofilm-associated infections. The relationship between efflux pump activity and biofilm formation is complex and bidirectional. Recent studies have demonstrated that the AcrAB-TolC system plays a central role in biofilm biogenesis, with the deletion of *acrB* preventing biofilm formation and reducing the export of extracellular DNA (eDNA)(23). The characteristic aggregative pattern of enteropathogenic *E. coli* is strongly impaired in the absence of AcrB or in the presence of an efflux-defective AcrB transporter, whereas it is restored upon complementation with functional *acrB(24)*. These findings confirm that AcrB transporter activity is required for biofilm biogenesis and that efflux pump function is a consequence of biofilm formation.

The synergistic relationship between efflux activity and biofilm formation creates a formidable barrier to conventional antimicrobial therapy. Efflux pumps not only facilitate the extrusion of antibiotics but also actively shape cell-to-cell interactions within biofilms through the efflux of signalling molecules and the establishment of local antibiotic gradients (25). The biofilm extracellular polymeric matrix provides multiple layers of protection against antimicrobial agents, including physical barriers that limit antibiotic diffusion and the absorption of antimicrobial compounds into the matrix (26). By limiting antibiotic diffusion, the biofilm matrix reduces intracellular antibiotic concentrations, thereby allowing efflux pumps to maintain sub-inhibitory levels. This synergistic interaction between biofilm-mediated tolerance and efflux pump activity is particularly relevant to device-associated infections, where biofilms are the

predominant mode of bacterial growth(27).

The enhanced expression of *acrA*, *acrB*, and *tolC* observed in this study correlated with biofilm strength and resistance profiles, suggesting that environmental conditions within biofilms trigger the transcriptional activation of the efflux pump system. The regulation of efflux pump expression is mediated by global regulatory proteins, including MarA, SoxS, and Rob, which respond to various environmental stressors and antimicrobial exposures(28). Additionally, local repressors, such as AcrR, modulate the basal expression of the AcrAB-TolC system (28). The interplay between these regulatory elements enables bacteria to rapidly upregulate efflux pump expression in response to antibiotic pressure, facilitating the transition from susceptible to resistant phenotypes. The upregulation of efflux pump genes through the overexpression of marA, soxS, and ramA represents an efficient mechanism for the detoxification of antimicrobial agents and is a well-established mechanism of resistance in *E. coli*(28).

The present study demonstrated pronounced resistance to fluoroquinolones, with sensitivity rates ranging from 6.6% to 31.6% across the tested agents. This high prevalence of fluoroquinolone resistance reflects the multifactorial nature of resistance to this class of antibiotics. While fluoroquinolone resistance is classically associated with mutations in the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), efflux pump-mediated resistance is equally important in clinical isolates (21,29). The AcrAB-TolC system plays a central role in fluoroquinolone efflux, with increased pump expression correlating with reduced susceptibility to quinolone drugs. The synergistic effect of target-site mutations and efflux pump upregulation leads to high MICs in many clinical isolates, rendering fluoroquinolone therapy ineffective against a substantial proportion of UPEC infections. The expression of *marA*, *soxS*, *acrB*, and *ramA* is associated with nalidixic acid resistance. It is likely involved in the fluoroquinolone resistance phenotype observed in this study(29).

The evaluation of CPZ, a phenothiazine derivative, demonstrated its efficacy in modulating antimicrobial resistance. The co-administration of a sub-inhibitory concentration of CPZ with ciprofloxacin resulted in a substantial two- to fourfold reduction in the MICs across the tested MDR isolates, with some isolates exhibiting a pronounced fourfold reduction. This significant restoration of susceptibility highlights the viability of repurposing CPZ as an EPI agent. Recent investigations have demonstrated that CPZ effectively inhibits the AcrAB-TolC efflux system by interfering with substrate binding, thereby restoring the susceptibility of *E. coli* to various antimicrobial agents (13,19). Furthermore, studies indicate that CPZ not only impairs efflux-mediated resistance but also significantly attenuates quorum-sensing regulated virulence factors and prevents the formation of bacterial biofilms (13,22).

The development of AcrB-targeting inhibitors is a critical frontier in antimicrobial therapy. A comprehensive review of drug efflux pump inhibitors identified *AcrB* as a promising target for counteracting multidrug resistance in gram-negative pathogens, with multiple structural classes of compounds currently under investigation(30). The critical finding that the absence, loss-of-function, or inhibition of *AcrB* does not increase the expression of other efflux pump genes supports the discovery of AcrB inhibitors as antibiotic adjuvants(31). This observation is particularly important because it suggests that bacteria cannot readily circumvent AcrB inhibition by activating alternative efflux systems, thereby reducing the likelihood of rapid development of resistance to EPI-based therapies. The absence of compensatory mechanisms following AcrB inhibition provides a mechanistic basis for the development of AcrB-specific inhibitors as effective adjuvants to conventional antibiotics (32).

The dual action of CPZ in impairing both efflux-mediated resistance and biofilm development positions it as a highly promising candidate for combination therapies to treat recalcitrant device-associated and urinary tract infections. The strategic approach of combining antibiotics with efflux pump inhibitors represents a paradigm shift in the treatment of MDR infections, leveraging complementary mechanisms of action, whereby EPIs restore intracellular antibiotic concentrations to bactericidal levels, while conventional antibiotics exert their antimicrobial effects (30-33).

The findings of this study underscore the critical role of the AcrAB-TolC efflux pump in the pathogenesis and persistence of MDR *E. coli* infection. The high prevalence of MDR strains (70%), coupled with widespread biofilm-forming capacity (66.6% strong or moderate producers) and active efflux mechanisms (68.3%), creates a multifactorial barrier to conventional antimicrobial therapies. The universal presence of AcrAB-TolC genes among MDR isolates, combined with enhanced expression correlating with biofilm strength and resistance profiles, demonstrates the central role of this efflux system in biofilm-associated antibiotic tolerance (21,23).

The integration of targeted efflux pump inhibitors, such as CPZ, with established antibiotics holds significant promise for dismantling biofilm resilience and restoring antimicrobial efficacy against UPEC infections. The two- to four-fold reduction in ciprofloxacin MICs achieved through CPZ co-administration in this study provides direct evidence of the therapeutic potential of combination therapy. The absence of compensatory upregulation of alternative efflux systems following AcrB inhibition provides a mechanistic advantage over other resistance-modifying strategies, as bacteria cannot readily circumvent inhibition through the activation of redundant systems(31,34,35). This finding, combined with the biofilm-inhibitory properties of CPZ, suggests that combination therapies may achieve synergistic effects that exceed the additive effects of individual components(13,22,36).

Conclusion

The AcrAB-TolC efflux pump is central to the pathogenesis

and persistence of MDR *E. coli* infections. The synergistic relationship between efflux activity and biofilm formation, coupled with the regulatory integration of quorum-sensing mechanisms, creates a multifactorial barrier to conventional antimicrobial therapies. The findings of this study advocate an urgent paradigm shift towards integrating targeted efflux inhibitors, such as CPZ, into established antibiotic regimens. Such combination strategies hold significant promise for dismantling biofilm resilience, restoring antimicrobial efficacy, and ultimately improving clinical outcomes for patients with multidrug-resistant infections. The absence of compensatory upregulation of alternative efflux systems following AcrB inhibition provides a mechanistic basis for the sustainable development of targeted EPI-based therapies, offering hope for overcoming one of the most pressing challenges in contemporary infectious disease management.

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Competing Interests
The authors declare that they have no conflicts of interest.

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