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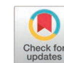

Phenotypic and Molecular Detection for Antibiotic Resistance of *Enterobacter cloacae* Isolated from Urinary Tract Infections

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Abstract

Introduction: Enterobacter cloacae has emerged as a significant pathogen in urinary tract infections, particularly in hospitals under antibiotic pressure, resulting in rapid development of resistance. The number of mechanisms, including β-lactamase production, aminoglycoside-modifying enzymes, efflux systems, and plasmid-mediated determinants used by the organism, made successful treatment difficult. Phenotypic resistance interpretation and the detection of widely used genetic markers are key components that drive therapy and contain the transmission of MDR strains.

Methods: A total of 100 urine samples were obtained from patients with UTI at three hospitals in Babylon province from November 2024 to January 2025. Isolates were confirmed as E. cloacae by routine culture and biochemical tests. Susceptibility to antimicrobial agents was determined by the CLSI disc diffusion method. All isolates were tested for AcrAB, blaTEM, strA, and tetA using molecular detection assays, whereas 16S rRNA gene sequencing was performed to validate species identification and assess genetic diversity among isolates.

Results: The isolates exhibited high-level resistance to amoxicillin-clavulanate (100%), ceftazidime (100%), and tetracycline (90%). Resistance to ampicillin, clarithromycin, erythromycin, and streptomycin was also higher. Gentamicin and ciprofloxacin demonstrated moderate activity, whereas levofloxacin and high-level streptomycin were most potent. The most common were AcrAB (80%), tetA (70%), blaTEM (40%), and strA (30%). Sequencing validated the correct assignment and identified single-nucleotide variants, indicating micro-variation.

Conclusion: The study revealed an alarming level of multidrug resistance among E. cloacae isolates in Babylon province. To prevent further dissemination of resistant strains, antimicrobial stewardship should be reinforced and routine molecular surveillance established.

Keywords: UTIs, *E. cloacae*, *16S rRNA,* Antibiotic resistance

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Introduction

Urinary tract infections (UTIs) are still one of the most prevalent bacterial infections worldwide, in people of all ages and from all regions, resulting over time in a painful burden on health services UTIs account for the high rates of cumulative incidence and recurrence, as well they are associated with serious complications such as pyelonephritis, bacteremia, or urosepsis posing an increasing public health threat. In addition, changes in global epidemiology have underscored the growing role of opportunistic and multidrug-resistant Gram-negative species in UTIs, particularly in the hospital setting under increased antibiotic pressure and compromised infection-control strategies (1, 2).

Among this broadening array of uropathogens is the Enterobacter species (specifically E. cloacae), which has proven to be a significant opportunistic urological pathogen capable of causing various forms of UTI in both the community and the hospital. E. cloacae is increasingly known to be able to induce uncomplicated cystitis, catheter-associated UTIs, upper urinary tract infections, and also life-threatening urosepsis, particularly in immunocompromised patients, diabetes mellitus (DM) subjects, as well as patients undergoing urologic invasive procedures, or those with prolonged indwelling catheters (3–4). The increasing clinical significance of this organism largely derives from its metabolic flexibility, environmental durability, and survival on medical devices and abiotic surfaces in the hospital environment, thereby contributing to persistence and spread via nosocomial transmission (5).

One of the essential factors contributing to the clinical threat of E. cloacae is its array of resistance mechanisms. This organism harbors an inducible AmpC β-lactamase that can acquire additional determinants, further reducing the activity of β-lactams (including broad-spectrum cephalosporins and carbapenems) (6). These structural changes, which increased resistance, were also

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accompanied by an increase in aminoglycoside-modifying enzymes. One of the most significant is the multidrug efflux system AcrAB–TolC, which extrudes a broad spectrum of antibiotics, including fluoroquinolones, β-lactams, chloramphenicol, and tetracycline (7–9), thereby decreasing drug accumulation within the cell and significantly contributing to multidrug resistance (MDR) phenotypes.

In addition to chromosomal resistance, there is an essential role for plasmid-borne genetic elements in facilitating the rapid spread of resistance determinants among E. cloacae and, more broadly, into other Enterobacterales. Genes blaTEM (β-lactam resistance), strA (streptomycin resistance), and tetA (tetracycline efflux) are frequently found in clinical isolates and can spread horizontally via conjugative plasmids. The fact that these determinants, such as blaCTX-M-2b and fosA3, have mobilized so quickly in the hospital setting may further facilitate the adaptability of E. cloacae and impact the global MDR uropathogen epidemic, making clinical intervention difficult to manage with higher rates of treatment failures, extended time to discharge from hospitalization, and increased healthcare costs (10–11).

In light of these hurdles, modern microbiological practice would be inconceivable without the aid of molecular diagnostic methods. PCR-based detection of resistance determinants offers a rapid and accurate identification of the genetic cause of antimicrobial resistance, whereas 16S rRNA sequence analysis remains the reference method for species-level diagnostics and for closely related members of the Enterobacter cloacae complex (12,13). These molecular tools not only enhance diagnostic accuracy but also provide valuable information on genetic diversity, phylogenetic relationships, and potential transmission routes for clinical isolates. Combined with phenotypic susceptibility profiles, such broad analyses contribute to a more complete picture of resistance dynamics and inform evidence-based antibiotic stewardship and infection control.

Owing to the increased clinical relevance of E. cloacae as a multidrug-resistant uropathogen, the study aims to characterize the phenotypic antibiotic resistance profile of E. cloacae isolates from UTI patients, detect significant resistance genes (AcrAB efflux pump, blaTEM, strA, and tetA), and confirm species identity by 16S rRNA gene sequencing. This combined phenotypic-genotypic strategy is expected to provide molecular epidemiological information on the local strains and offer insights that could enable better therapeutic management and regional surveillance prospects.

Materials and Methods

This research was conducted in November 2024-January 2025 and comprised 100 midstream urine samples from patients with suspected urinary tract infections admitted to three hospitals in Babylon province. The samples were preserved in sterilized bottles and processed without delay using standard microbiological procedures (14). Every sample was plated on blood agar and MacConkey's agar, with an incubation at 37°C for 18–24 h. Enterobacter cloacae isolates were identified by colony morphology, Gram staining, and routine biochemical tests.

Furthermore, to improve colony isolation, samples were spread onto nutrient agar (NA), tryptose soy agar (TSA), Xylose Lysine Deoxycholate (XLD) agar, and Salmonella–Shigella (SS) agar using the semi-quantitative streaking technique. Presumptive identification was verified by typical rough or smooth colony morphology and biochemical characteristics before molecular analysis (15).

Antimicrobial Susceptibility Testing

Confirmed isolates were tested for antimicrobial susceptibility by the Kirby–Bauer disc diffusion method according to CLSI 2024 guidelines (16). The antibiotics tested were ampicillin, gentamicin, clarithromycin, amoxicillin/clavulanate, tetracycline, erythromycin (ery), streptomycin (str), levofloxacin, ciprofloxacin (assay with high-level resistance to str), and ceftazidime. For all experiments, bacterial suspensions were standardized to a 0.5 McFarland standard to achieve a uniform inoculum density.

PCR Amplification and DNA Extraction

Genomic DNA was extracted using a commercial DNA purification kit according to the manufacturer's instructions. DNA quality and concentration were evaluated using a NanoDrop spectrophotometer. Specific primers were used to amplify resistance genes, including AcrAB, blaTEM, strA, and tetA, as well as the 16S rRNA gene for species confirmation. The AcrAB gene was amplified using the primers ATCAGCGGCCGGATTGGTAAA (forward) and CGGGTTCGGGAAAATAGCGCG (reverse), yielding a 312-bp product (17). The blaTEM gene was amplified using the primers GCA CGA GTG GGT TAC ATC GA (forward) and GGT CCT CCG ATC GTT GTC AG (reverse), yielding a 310-bp product (18). The strA gene was amplified with CTT GGT GAT AAC GGC AAT TC (forward) and CCA ATC GCA GAT AGA AGG C (reverse), generating a 548 bp fragment (19). The tetA gene was amplified using the primers GCT ACA TCC TGC TTG CCT TC (forward) and CAT AGA TCG CCG TGA AGA GG (reverse), yielding a 210-bp product (20). Species-level identification via 16S rRNA was performed using TCC AGA TTA CAA CTT CAC CAG G (forward) and CAA TTC ATA TCT TGT AAC G (reverse), resulting in a 1465 bp amplicon (21).

PCR reactions were performed in 50 µL volumes under standard cycling conditions: an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at gene-specific

temperatures (AcrAB: 58°C; blaTEM: 54°C; strA: 51°C; tetA: 56°C; 16S rRNA: 56°C) for 30 seconds, and extension at 72°C for 45 seconds. A final extension step was performed at 72°C for 7 minutes.

Gel Electrophoresis and Molecular Analysis

PCR products were separated on 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination. Positive bands were compared with expected product sizes for gene confirmation. Sequenced 16S rRNA products were analyzed using BLASTn for taxonomic verification. Multiple sequence alignment, SNP detection, and phylogenetic analysis were performed to evaluate genetic variability and relatedness among isolates and international reference strains.

Results

Phenotypic Antibiotic Susceptibility Patterns

The phenotypic analysis of E. cloacae isolates revealed significant differences in susceptibility to antimicrobial agents. The isolates exhibited high resistance rates to many commonly used antibiotics, as summarized in Table 1 (Phenotypic Antibiotic Susceptibility Patterns of Enterobacter cloacae Isolates). Resistance levels of 90, 100, and 100% were observed for tetracycline (TE), amoxicillin–clavulanate (AMC), and ceftazidime (CAZ), respectively. Also, AM and CLR exhibited 80% of resistance, followed by E and S with 70%.

Intermediate susceptibility was observed for AM, CLR, and TE, HLS (high-level streptomycin), S, CN, ERY(Erythromycin), LEV (levofloxacin), and CIP (ciprofloxacin) in the range of 10–30%. On the other hand, the sensitivity rates were higher for CN and CIP (50% each) and for LEV (70%) and HLS (80%), in particular. Statistically, most tested antibiotics showed that sensitive and intermediate isolates were significantly ($P<0.05$) distinguishable from resistant isolates, indicating resistance heterogeneity among all isolates; however, variation in exposure or selective pressure within the clinical context was suspected to have caused this variation.

Amplification of 16S rRNA Gene and Species Confirmation

The 16S rRNA gene was successfully amplified in all isolates. A clear, sharp band matching the anticipated 1465 bp product was observed in all lanes (Figure 1). This supported the validity of genomic DNA extraction and the selection of a gene region for molecular study.

BLASTn analysis of the sequenced amplicons indicated high sequence identity (98–100%) with reference sequences available in international databases, including those from Iraq, India, China, and Brazil. These analyses, shown in Table 2 (BLASTn identity results for the 16S rRNA gene sequences of the Enterobacter isolates), revealed that the isolates belonged to several species within the Enterobacter complex, including E. cloacae, E. hormaechei, E. asburiae, and E. aerogenes.

Multiple Sequence Alignment and SNP Characterization

The conserved and variable regions were depicted in the multiple sequence alignment of 16S rRNA gene sequences shown in Figure 2. Some single-nucleotide polymorphisms (SNPs) were observed at specific positions along the sequences. These comprised transitions (e.g., C→T and A→G) and transversions (e.g., A→T and G→C), with distinct variation at positions 22, 28, and 32. Multiple variants at these sites suggest microdiversity among the isolates.

Such a pattern of SNP distribution might indicate that this evolutionary divergence is regional, possibly driven by selective pressures or differences in host and clinical environments. The alignment reveals that, beyond a high overall similarity percentage, there are some acceptable sequence variations among strains, which may be responsible for the phenotypic or adaptive changes.

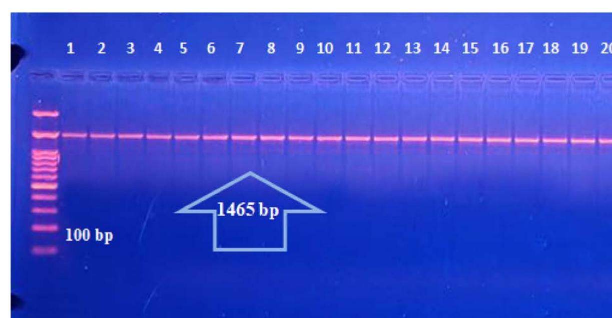

Figure 1. PCR products of the 16S rRNA gene (1465 bp) for Enterobacter cloacae isolates

Table 1. Phenotypic susceptibility to antibiotics among isolates of Enterobacter cloacae.

Antibiotic susceptibility	Antibiotic type (n=10) No. (%)											*P*-Value
	AM	CN	CLR	AMC	TE	HLS	E	LEV	S	CIP	CAZ	
R	8 (80%)	2 (20%)	8 (80%)	10(100%)	9 (90%)	0 (0%)	7 (70%)	0 (0%)	7 (70%)	2 (20%)	10(100%)	<0.0001*
I	2 (20%)	3 (30%)	2 (20%)	0 (0%)	1 (10%)	2 (20%)	3 (30%)	3 (30%)	1 (10%)	3 (30%)	0 (0%)	<0.0001*
S	0 (0%)	5 (50%)	0 (0%)	0 (0%)	0 (0%)	8 (80%)	0 (0%)	7 (70%)	2 (20%)	5 (50%)	0 (0%)	<0.0001*
P-Value	<0.0001*	0.0009*	<0.0001*	------	<0.0001*	<0.0001*	0.0001*	0.0001*	<0.0001*	0.0009*	------	

*Significant difference at the 0.05 level by chi-square test. (AM: Ampicillin; CN: Gentamicin; CLR: Clarithromycin; AMC: Amoxicillin+clavulanic acid; TE: Tetracycline; E: Erythromycin; LEV: Levofloxacin; S: Streptomycin; CIP: Ciprofloxacin, CAZ: ceftazidime)

Table 2. Identity in BLASTn of Enterobacter isolate 16S rRNA gene nucleotide sequences against reference strains.

Description	Scientific Name	Per. Ident	Accession	Country
Enterobacter sp. strain FMQEB15 16S ribosomal RNA	*Enterobacter* sp.	99.87%	PX257805	Iraq
Enterobacter sp. strain FMQEB17 16S ribosomal RNA	*Enterobacter* sp.	99.12%	PX257807	Iraq
E. aerogenes strain T2 16S ribosomal RNA gene	*E. aerogenes*	98.45%	GU265554.1	India
E. hormaechei strain JCS-3 16S ribosomal RNA gene	*E. hormaechei*	99.76%	PP440228.2	China
E. hormaechei strain FMQEB12 16S ribosomal RNA	*E. hormaechei*	100.00%	PX257802	Iraq
E. hormaechei strain FMQEB11 16S ribosomal RNA	*E. hormaechei*	99.91%	PX257801	Iraq
E. cloacae strain 344 16S ribosomal RNA gene	*E. cloacae*	99.54%	J0435862.10	Brazil
E. cloacae strain FMQEB7 16S ribosomal RNA	*E. cloacae*	99.82%	PX257797	Iraq
E. cloacae strain 341 16S ribosomal RNA gene	*E. cloacae*	99.21%	J0435861.11	Brazil
E. cloacae strain FMQEB8 16S ribosomal RNA	*E. cloacae*	100.00%	PX257798	Iraq
E. asburiae strain G3 16S ribosomal RNA gene	*E. asburiae*	98.93%	OR143363.3	China
Enterobacter asburiae strain FMQEB14 16S ribosomal RNA	*E. asburiae*	99.65%	PX257804	Iraq
E. asburiae strain FMQEB13 16S ribosomal RNA	*E. asburiae*	99.77%	PX257803	Iraq
E. cloacae strain RJ04 16S ribosomal RNA gene	*E. cloacae*	98.88%	KC990807.8	India
E. cloacae strain FMQEB5 16S ribosomal RNA	*E. cloacae*	99.43%	PX257795	Iraq
E. cloacae strain RJ20 16S ribosomal RNA gene	*E. cloacae*	99.02%	KC990811.7	India
E. cloacae strain FMQEB4 16S ribosomal RNA	*E. cloacae*	99.91%	PX257794	Iraq
E. cloacae strain 6nak4 16S ribosomal RNA gene	*E. cloacae*	98.67%	PV954740.12	India
E. cloacae strain FMQEB9 16S ribosomal RNA	*E. cloacae*	100.00%	PX257799	Iraq
E. cloacae strain RN1 16S ribosomal RNA gene	*E. cloacae*	99.25%	KC990821.5	India
E. cloacae strain FMQEB6 16S ribosomal RNA	*E. cloacae*	99.56%	PX257796	Iraq
E. cloacae strain FMQEB3 16S ribosomal RNA	*E. cloacae*	99.84%	PX257793	Iraq
E. cloacae strain FMQEB2 16S ribosomal RNA	*E. cloacae*	99.73%	PX257792	Iraq
E. cloacae strain CS8 16S ribosomal RNA gene	*E. cloacae*	98.79%	OR230271.9	India
E. cloacae strain RJ30 16S ribosomal RNA gene	*E. cloacae*	99.10%	KC990813.6	India
E. cloacae strain RN2 16S ribosomal RNA gene	*E. cloacae*	99.33%	KC990822.4	India
E. cloacae strain FMQEB1 16S ribosomal RNA	*E. cloacae*	100.00%	PX257791	Iraq
E. cloacae strain CIFRLU13 16S ribosomal RNA gene	*E. cloacae*	98.56%	PV834981.13	India
E. cloacae strain FMQEB10 16S ribosomal RNA	*E. cloacae*	99.48%	PX257800	Iraq
E. cloacae strain FMQEB20 16S ribosomal RNA	*E. cloacae*	99.62%	PX257810	Iraq

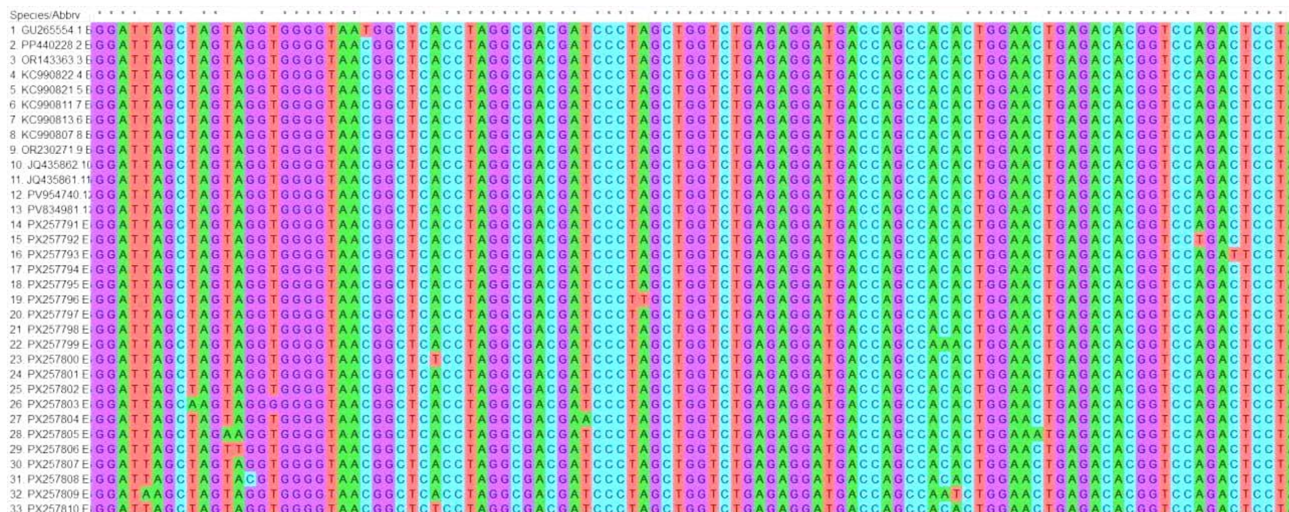

Figure 2. A multiple sequence alignment of the 16S rRNA gene of *cloacae* isolates sequences with the consensus nucleotide positions and variable regions

Phylogenetic Analysis

A phylogenetic analysis based on 16S rRNA gene sequences was used to assess the evolutionary relationships among isolates. The resulting tree (Figure 3) displayed distinct clades that agreed well with the species-level designations (Phylogenetic Tree Based on 16S rRNA Gene Sequences of Enterobacter cloacae, E. hormaechei, E. asburiae, and E. aerogenes Isolates Relative to Global Reference Strains).

In the phylogenetic analysis conducted in our study, isolates clustered with reference strains from India, China, and Brazil, indicating a cosmopolitan distribution of these species. As confessed brothers or historical slave routes between the strains. On the other hand, slight differences in branched characteristics within species clusters indicate micro-evolutionary divergence among local isolates and are consistent with SNP results inferred from sequence alignment.

Molecular Detection of Antibiotic Resistance Genes

PCR screening indicated significant diversity in the proportion of resistance genes among isolates. Amplification patterns of all four target genes are summarized in Figure 4 (Figure 4 Composite Agarose Gel Electrophoresis Showing PCR Amplification of Antibiotic Resistance Genes in Isolates of Enterobacter cloacae: (A) AcrAB efflux pump gene (312 bp), (B) blaTEM beta-lactamase gene (310 bp), and (C) strA streptomycin-resistance gene (548 bp); The arrow indicates the expected one band from all strains; panel (D) shows a higher DNA template concentration for tetA tetracycline-efflux pumps gene amplification (210 bp) than other bands.

The AcrAB gene was identified in 16 of the 20 isolates (80%). This corresponds with its established involvement in multidrug efflux and with its link to resistance to multiple drug classes. The tetA gene, associated with tetracycline efflux, was found in 14 isolates (70%), consistent with their high phenotypic resistance to tetracycline.

The blaTEM β-lactamase gene was detected in 8 isolates (40%), with a moderate distribution of this resistance determinant. In the meantime, the strA gene associated with streptomycin resistance was found in 6 isolates (30%). The corresponding summarized data are shown in Table 3 (Distribution of Antibiotic Resistance Genes in Enterobacter cloacae isolates).

The coexistence of efflux pump genes, β-lactamase genes, and aminoglycoside-resistance genes indicates that the antimicrobial resistance of Eps among these isolates is multifactorial. The association between genotypic and phenotypic results strengthens the contribution of these genes to the multidrug resistance profiles observed in this study.

Discussion

The results of the present study display a high degree of resistance to antimicrobials among clinical isolates of E. cloacae causing urinary tract infections in Babylon, governorate. The high resistance to tetracycline, amoxicillin–clavulanate, and ceftazidime is also consistent with global observations of an upward trend in resistance among Enterobacter cloacae complex isolates, particularly in areas with excessive antibiotic use and a lack of antimicrobial management policies (22,23). The high rate of resistance to β-lactam drugs can be attributed to the well-known capacity of E. cloacae strains to produce constitutive and/or acquired β-lactamases, resulting in marked loss of susceptibility to commonly prescribed antibiotics (24).

This study has demonstrated residual activity of gentamicin, ciprofloxacin, levofloxacin, and high-level streptomycin; therefore, these treatment regimens could still serve as options for mycobacterial infections but should be administered on a case-by-case basis, guided by susceptibility testing. The same resistance patterns were found in other recent clinical studies of Enterobacter species, with some preservation of aminoglycoside and fluoroquinolone activities despite high levels of resistance to most antimicrobial classes (25).

Molecular verification of the 16S rRNA gene further supported the precise identification of the isolates, with BLASTn analysis assigning them to closely related reference strains from various regions worldwide. The phylogenetic tree demonstrated a significant association between Ecuadorian isolates and strains of E. cloacae, E. hormaechei, E. asburiae, and E. aerogenes from around the world, supporting the local spread of these species. -Phylogenetic tree analysis)Similar phylogenetic structures have also been reported in other regional and international studies, providing evidence for the hypothesis that species within the E. cloacae complex share conserved evolutionary histories despite strain-level diversity (26,27).

This high structural diversity is consistent with the detection of numerous single-nucleotide polymorphisms (SNPs) in 16S rRNA sequences. The existence of transitions and transversions at multiple positions indicates ongoing evolutionary pressure on the local bacterial population. SNP-based microdiversity in Enterobacter species has previously been reported to be shaped by environmental selection, clinical exposure, and selective antibiotic pressure, potentially accounting for the heterogeneity observed here among isolates (28).

Molecular detection of resistance genes revealed a predominance of the AcrAB efflux gene, which is central to the development of resistance to various antibiotic classes. The efflux system plays an essential role in reducing intracellular accumulation of antibiotics and thereby facilitates multidrug resistance. Other studies investigating the AcrAB-TolC systems also found this gene to be among the most prevalent (31%), indicating

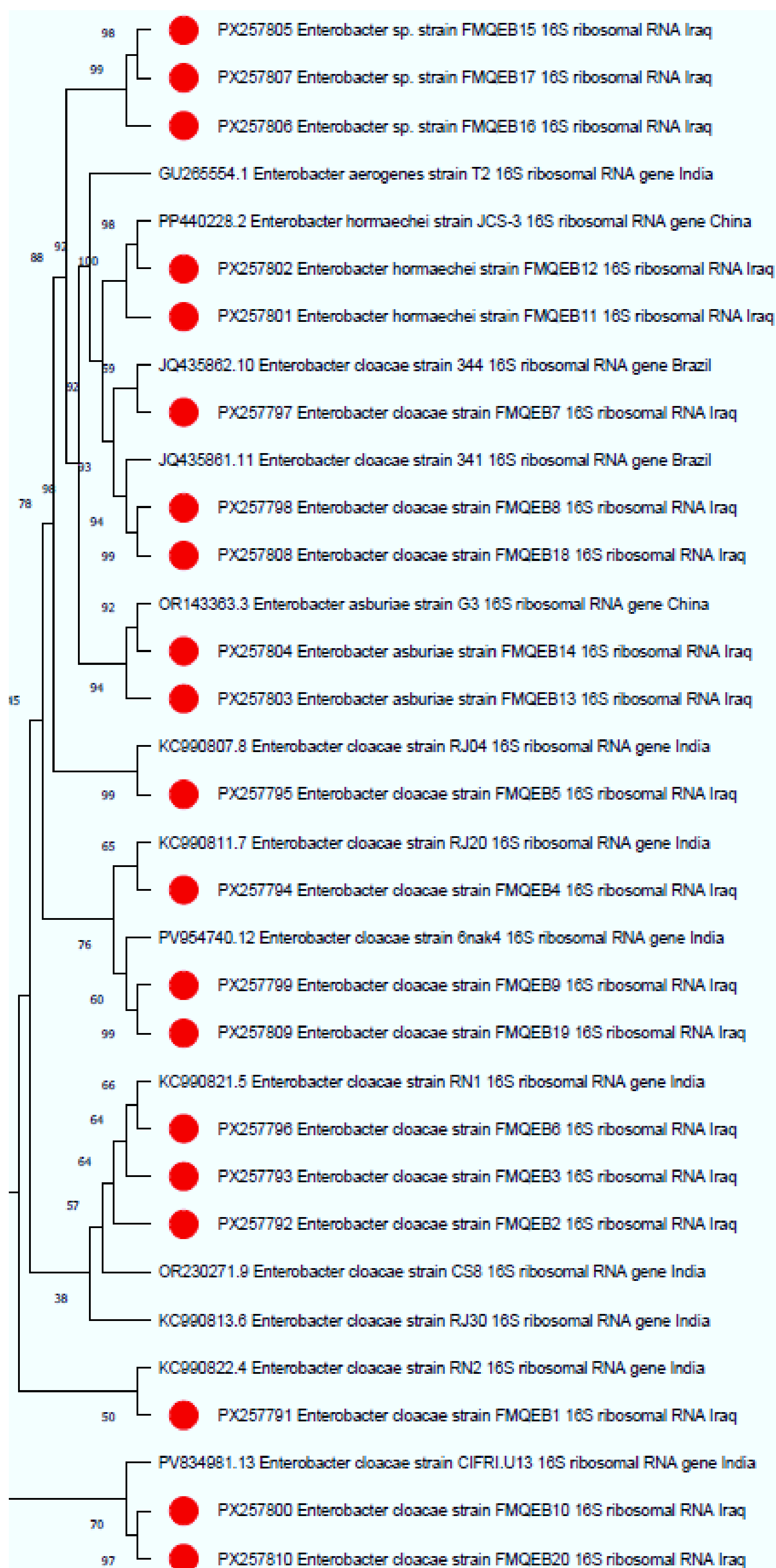

Figure 3. Phylogenetic analysis based on 16S rRNA gene sequences of clinical strains of Enterobacter cloacae, E. hormaechei, E. asburiae, and E. aerogenes, in relation to worldwide reference strains

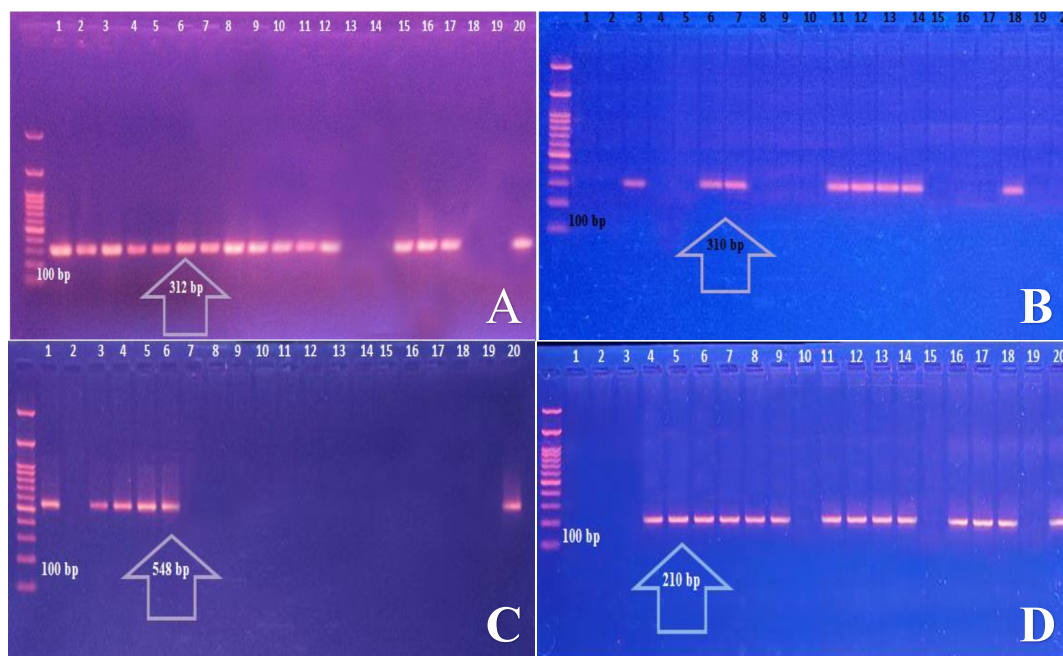

Figure 4. Agarose gels mixed with composite image of PCR amplification for antibiotic resistance genes in Enterobacter cloacae isolates: (A) AcrAB efflux pump gene (312 bp), (B) blaTEM β-lactamase gene (310 bp), (C) strA Streptomycin-resistance gene (548 bp), and (D) tetA tetracycline-efflux gene(210bp)

Table 3. Epidemiological features of isolates carrying antibiotic resistance genes in Enterobacter cloacae.

Gene name	No. of positive isolates (%)	No. of negative isolates (%)	Total No. (%)	*P* value
AcrAB	16 (80%)	4 (20%)	20 (100%)	0.0001*
blaTEM	8 (40%)	12 (60%)	20 (100%)	0.0455*
strA	6 (30%)	14(%70)	20 (100%)	0.0001*
tetA	14(%70)	6 (30%)	20 (100%)	0.0001*
P value		0.003*		

(*) Mean significant differences under a 0.05 probability level using the chi-square test

functional and clinical relevance (29).

The ubiquitous occurrence of the tetA gene (however) accords well with a quite marked phenotypic resistance to tetracycline. tetA is a plasmid-encoded determinant that can be readily acquired by strains, which may account for the high number of isolates bearing this gene. blaTEM, although not found at high frequencies, is of interest for its clinical implications of β-lactam resistance. The lower frequency of strA indicates that the streptomycin resistance might be mediated by more than one gene (other than aminoglycoside-modifying enzymes). Variability in strA carriage, frequently related to plasmid behavior and environmental characteristics, has been previously described (30-32).

Collectively, the phenotypic and genotypic profiles clearly demonstrate that the E. cloacae isolate at this region harbors a complex resistance pattern contributed by both chromosomal and plasmid-mediated mechanisms. The presence of efflux pumps, β-lactamase genes, and tetracycline resistance genes indicates the complexity of treating these infections. These results emphasized the indispensable role of strengthened AMS, regular molecular surveillance, and more rational antibiotic prescribing in response to the spread of MDR E. cloacae in clinical settings.

Conclusion

The current results indicate that E. cloacae isolates from UTI patients in Babylon province are highly resistant to multiple drugs and that such resistance is associated with efflux pump activity, β-lactamase production, and plasmid-mediated resistance determinants. The strong correlation between phenotypic resistance and genotypic identification of key elements, especially the acrAB and tetA genes, indicates that resistance mechanisms in these clinical isolates are multifaceted. Phylogenetic and SNP analyses also revealed significant genetic diversity with ongoing microevolution in the local population. These findings emphasize the importance of enhanced antimicrobial stewardship, molecular surveillance, and prudent antibiotic use to limit the dissemination of resistant E. cloacae strains in hospitals.

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