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Phenotypic-Genotypic Characterization of Macrolide-Lincosamide-Streptogramin B Resistance in *Staphylococcus saprophyticus* UTI Isolates from Iraq

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Abstract: *Staphylococcus saprophyticus* is a significant uropathogen, particularly in young women. Rising macrolide-lincosamide-streptogramin B (MLSB) resistance poses therapeutic challenges. This study characterized phenotypic and genotypic MLSB resistance patterns among S. saprophyticus UTI isolates in Najaf, Iraq. Forty-two *S. saprophyticus* isolates were collected from urine samples (February-April 2025) from patients aged 1-70 years. Phenotypic resistance was assessed using the D-test methodology following CLSI guidelines. PCR amplification targeted eight resistance genes: *erm*(C) and *msr*(A). Isolates predominantly originated from females (28/42, 66.7%), with the highest frequency in age groups 21-30 (11/42, 26.2%) and 31-40 years (10/42, 23.8%). The D-test revealed that 25/42 (59.5%) isolates exhibited MLSB resistance: 9/42 (21.4%) demonstrated an inducible MLSB (iMLSB) phenotype, 16/42 (38.1%) exhibited a constitutive MLSB (cMLSB) phenotype, and 8/42 (19.0%) displayed a macrolide-streptogramin B (MS) phenotype, while 9/42 (21.4%) were MLSB-negative. Molecular analysis detected resistance genes in only 9 of 25 (36%) phenotypically MLSB-positive isolates. The erm(C) gene alone was present in 2/25 (8%) isolates, while *erm*(C)+*msr*(A) combination dominated at 7/25 (28%). High MLSB resistance prevalence (59.5%) among *S. saprophyticus* isolates, with cMLSB predominance and significant genotype-phenotype discordance (64% phenotypically positive lacking detected genes), emphasizes the necessity for routine D-testing and expanded molecular surveillance to guide antimicrobial therapy in Iraqi healthcare settings.

Keywords: *Staphylococcus saprophyticus*; UTIs; MLS resistance; cross-resistance; PCR

1. Introduction

Across the world, uncomplicated UTIs rank among the most frequently encountered bacterial infections, placing a significant burden on healthcare systems due to their high prevalence, recurrence rates, and association with the emergence of antimicrobial resistance. Among young, sexually active females, Staphylococcus saprophyticus ranks as the second leading etiological agent of community-acquired UTIs, with prevalence ranging from 10-20% in this population [1, 2]. Recent studies from Iraq and neighboring countries have documented increasing resistance rates among S. saprophyticus strains, highlighting the urgent need for robust epidemiological surveillance strategies [3, 4]. MLSB antibiotics, with erythromycin, clindamycin, and streptogramins,

remain clinically important for treating staphylococcal infections. However, resistance to these agents is increasing globally. The main mechanisms of resistance comprise ribosomal target modification mediated by erm gene–encoded methylases, along with efflux pumps driven by msr genes, and the enzyme-mediated inactivation carried out by mph or lnu genes [5-7]. These genetic determinants produce three distinct resistance phenotypes: constitutive MLSB (cMLSB), inducible MLSB (iMLSB), and macrolide-streptogramin B (MSB), each carrying significant clinical implications [8-10].

The D-test remains the gold standard for detecting inducible clindamycin resistance in staphylococci. This screening method identifies isolates that appear susceptible but may develop resistance under antibiotic pressure, potentially leading to treatment failure [11, 12]. Molecular techniques, particularly PCR, complement phenotypic testing by detecting specific resistance genes and strengthening epidemiological surveillance through genotype-phenotype correlation analysis [5,13,14]. Among MLSB-resistant *S. saprophyticus* isolates, the erm(C) and msr(A) genes are most frequently identified, although novel resistance mechanisms continue to emerge across different geographic regions [15, 16]. Global resistance trends demonstrate concerning increases. Uruguay reported elevated MLSB resistance prevalence among S. aureus isolates with strong genotype-phenotype correlation [17]. Similarly, studies from Iran and Iraq documented high erythromycin and clindamycin resistance rates alongside multidrug resistance patterns [18-20]. These findings reflect broader trends in antimicrobial resistance, which are linked to the misuse and overuse of antibiotics. In Iraq, erythromycin resistance rates range from 50% to 67%, while clindamycin resistance varies between 22% and 46% among staphylococcal isolates [21, 22]. This variability underscores the importance of combining D-test methodology with molecular techniques for accurate resistance detection and monitoring.

Therefore, this inquiry aimed to characterize the phenotypical and genetical features of MLSB resistance across *S. saprophyticus* isolates from UTI patients in Najaf, Iraq. The objectives were to generate reliable local data for antimicrobial stewardship programs and guide clinicians toward more effective therapeutic strategies.

2. Materials and Methods

2.1 Collection of Specimens and Isolation of Bacteria

Urinary specimens were gathered from patients attending Al-Sadr Hospital in Najaf, Iraq, from February to April 2025. The study protocol received institutional approval, and all participants gave their informed consent. Participants of either sex, aged 1-70 years, presenting with UTI symptoms were included. Midstream urine specimens were collected using standard sterile techniques and processed within 2 hours of collection.

2.2 Identification of bacterial isolates

MacConkey and Blood agar dishes were used for culturing urine specimens, which were incubated at 37 °C for 24–48 hours. Staphylococcus saprophyticus isolates were recognized using blood and mannitol salt agar, Gram stain, and conventional biochemical tests, including catalase and adverse coagulase reactions, novobiocin resistance (5 μg disc), and mannitol fermentation, as per established protocols [23].

2.3 Phenotypic Detection of MLSB Resistance

2.3.1 D-Test Methodology

In accordance with CLSI instructions [24], the D-test was used to screen for resistance to clindamycin. In this procedure, bacterial inocula were standardized to a 0.5 McFarland turbidity and subsequently streaked onto Mueller–Hinton agar plates. Discs of erythromycin (15 μg) and clindamycin (2 μg) were applied on the agar surface, maintaining a 15 mm spacing between their edges. Upon incubation at 37 °C for 18–24 h, interpretations were made as outlined earlier [25].

- **Inducible MLSB (iMLSB)**: A D-shaped distortion observed in the clindamycin inhibitory area in proximity to the erythromycin disc.
- **Constitutive MLSB (cMLSB)**: Resistance to the two clindamycin and erythromycin
- **MS phenotype**: Resistance to erythromycin with susceptibility to clindamycin (no D-zone)
- **MLSB-negative**: Susceptibility to both antibiotics

2.3.2 Molecular Detection of Resistance Genes

Overnight cultures were subjected to genomic DNA isolation using a commercial kit (Promega, USA) according to the supplier's guidelines. DNA yield and purity were then assessed employing spectrophotometry at an absorbance ratio of 260/280 nm. Detection of MLSB resistance genes (erm(C) and msr(A)) was performed using PCR (Table 1). Primer design and cycling conditions were adopted from earlier reports [4,5]. Reaction mixtures (25 μL) comprised of 12.5 μL of master mix (Promega), 1 μL of every primer (10 pmol), 2 μL of extracted DNA, and 8.5 μL of nuclease-free water. Thermal cycling involved an initial denaturation at 94 °C for 5 minutes, followed by 30 amplification cycles (94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute), and a final extension step at 72 °C for 10 minutes. The resulting amplicons were resolved on 1.5% agarose gels comprising ethidium bromide and visualized under UV illumination. Each run included both positive and negative controls.

Table 1. Primers employed for the identification of MLSB resistance genes

Gene	Primer Sequence (5'→3')	Amplicon Size (bp)	Reference
erm(C)	F: ATCTTTTAGCAAACCCGTATTC R: CTTGTTGATCACGATAATTTCC	190	[26]
msr(A)	F: AAGTTATATCATGAATAGATTGTCCTGTT R: GGCACAATAAGAGTGTTTAAAGG	940	[27]

2.4 Statistical Analysis

The dataset was evaluated through descriptive statistical methods. Resistance profiles of S. saprophyticus isolates were stratified by age and gender, and summarized as frequencies and proportions. The chi-square analysis was used to determine associations between resistance and demographic factors. The level of statistical significance was defined as $p < 0.05$, and all analyses were conducted using SPSS V 25.0.

3. Results and Discussion

3.1 Demographic Distribution and Clinical Epidemiology

Among the 42 *S. saprophyticus* isolates collected from UTI patients, a marked female predominance was observed, with 28 isolates (66.7%) from females and 14 (33.3%) from males, resulting in a 2:1 female-to-male ratio (Table 2). This gender distribution aligns with the well-established epidemiology of *S. saprophyticus* as the second leading causative agent of community-acquired UTIs among sexually active females of younger age groups, responsible for 10–20% of UTIs in this population [28,29]. The highest isolation rates occurred in patients aged 21-30 years (11/42, 26.2%) and 31-40 years (10/42, 23.8%), with progressively lower frequencies in older age groups: 41-50 years (7/42, 16.7%), 51-60 years (5/42, 11.9%), and 61-70 years (5/42, 11.9%). Notably, no isolates were recovered from children under 10 years old, and only 4 isolates (9.5%) were from the 11-20 year age group. The concentration of cases in the 21–40-year age range corresponds with peak sexual activity and aligns with anatomical, behavioral, and hormonal factors that predispose young women to S. saprophyticus UTIs [30, 31]. The absence of pediatric cases reinforces the rarity of this organism in childhood UTIs, where Escherichia coli predominates. These demographic patterns are consistent with regional studies from Iraq and neighboring countries, confirming *S. saprophyticus* as a significant uropathogen in reproductive-age women [30-32].

Table 2. Distribution of *S. saprophyticus* isolates by demographic characteristics (n=42)

Characteristic	Category	n (%)
Sex	Male	14 (33.3)
	Female	28 (66.7)
Age group (years)	1-10	0 (0.0)
	11-20	4 (9.5)
	21-30	11 (26.2)
	31-40	10 (23.8)
	41-50	7 (16.7)
	51-60	5 (11.9)
	61-70	5 (11.9)

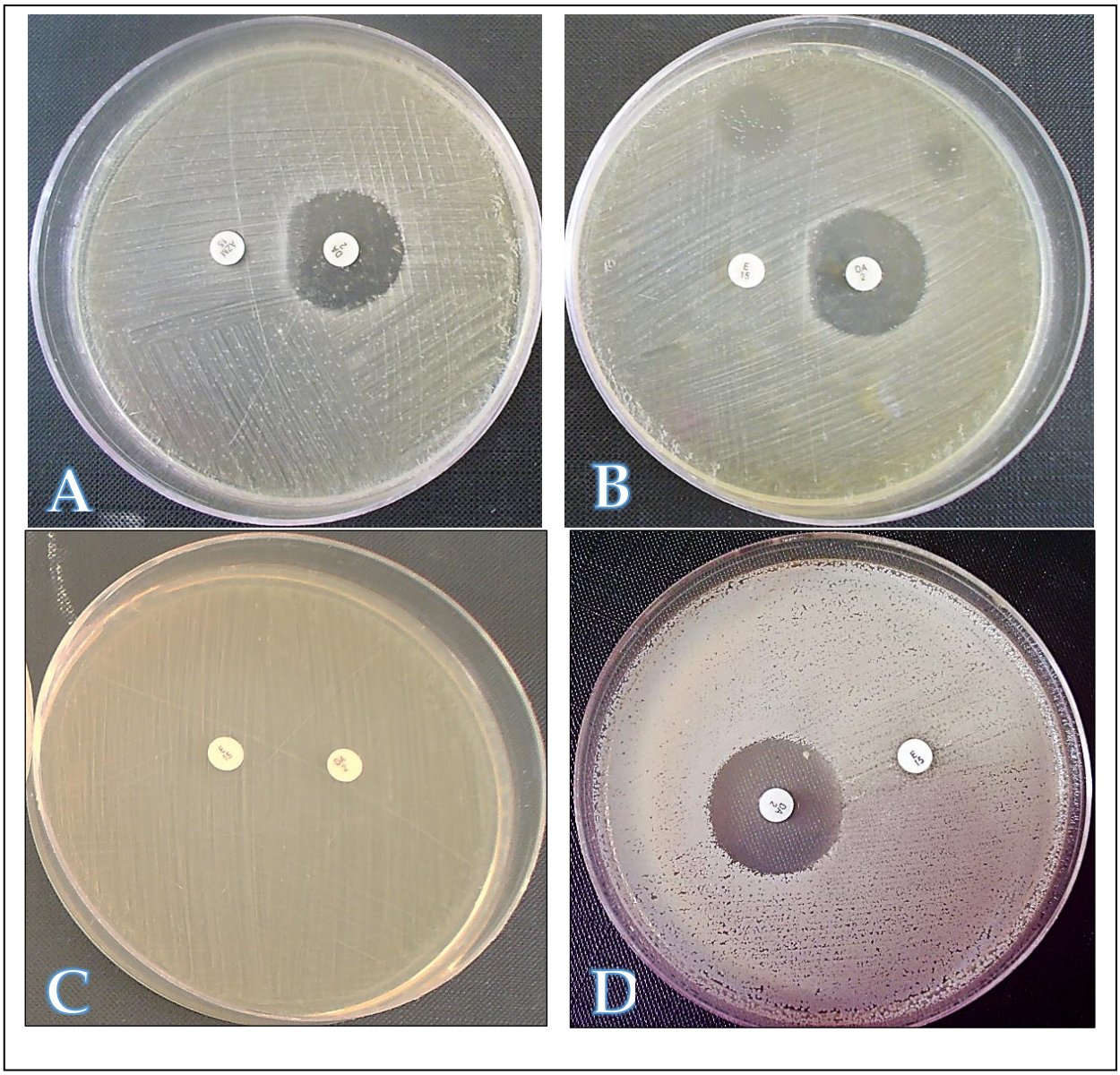

Figure 1. Representative D-test results for *S. saprophyticus*: (A) inducible resistance with azithromycin, (B) inducible resistance with erythromycin, (C) constitutive MLS resistance, (D) MS phenotype (macrolide resistance without lincosamide induction).

3.2 Phenotypic Resistance Patterns and Clinical Implications

D-test analysis revealed a concerning prevalence of MLSB resistance, with 25/42 isolates (59.5%) demonstrating MLSB-positive phenotypes (Table 3). Among these, constitutive MLSB (cMLSB) resistance predominated at 16/33 (48.4%), while inducible MLSB (iMLSB) accounted for 9/33 (27.3%) of tested isolates. Additionally, 8/33 isolates (24.3%) exhibited the MS phenotype, and 9/42 (21.5%) were MLSB-negative. The predominance of the cMLSB phenotype indicates widespread constitutive expression of ribosomal methylases that modify the drug-binding site, conferring cross-resistance to macrolides, lincosamides, and streptogramin B [33, 34]. This mechanism, typically mediated by *erm* genes, particularly *erm*(C) in staphylococci, results in high-level resistance that cannot be overcome by increasing drug concentrations [35, 36]. The detection of iMLSB in 27.3% of isolates has critical therapeutic implications, as these strains may appear susceptible to clindamycin during routine antimicrobial testing; however, they may develop resistance upon exposure to inducing agents [37, 38]. This finding strongly supports recommendations for mandatory D-testing of all erythromycin-resistant staphylococci to prevent clinical failures [39-41]. The MS phenotype observed in 24.3% of isolates represents macrolide-specific resistance, while maintaining lincosamide susceptibility, consistent with efflux pump mechanisms typically associated with *msr*(A) genes that selectively export 14- and 15-membered macrolides [42, 43]. This distinction has therapeutic relevance, as clindamycin remains effective against MS phenotype isolates despite macrolide resistance.

Table 3. Distribution of MLSB resistance phenotypes as identified through the D-test.

Phenotype	n (%)	Clindamycin (2µg)	Erythromycin (15µg)
iMLSB	9/33 (27.3)	S	R
cMLSB	16/33 (48.4)	R	R
MS	8/33 (24.3)	S	R
Total MLSB-positive	**25/42 (59.5)**	-	-
MLSB-negative	**9/42 (21.5)**	S	S

3.3 Molecular Characterization and Genotype-Phenotype Correlations

PCR analysis of the 25 phenotypically MLSB-positive isolates revealed resistance genes in only 9 samples (36%), demonstrating significant genotype-phenotype discordance (Table 4, Figures 1 and 2). The *erm*(C) gene was detected either alone (2/25, 8%) or in combination with *msr*(A) (7/25, 28%), while 16/25 isolates (64%) lacked detectable resistance genes despite expressing resistant phenotypes. The identification of *erm*(C) as the primary resistance determinant aligns with global reports establishing it as the predominant methylase gene in staphylococci [44]. The *erm*(C) gene encodes a 23S rRNA methylase that dimethylates the adenine residue at A2058, preventing antibiotic binding and conferring the MLSB resistance phenotype [45]. The co-occurrence of *erm*(C) and *msr*(A) in 28% of gene-positive isolates suggests that dual resistance mechanisms may enhance overall resistance levels through combined ribosomal modification and efflux activity [33, 46, 47]. The absence of other tested resistance genes [*erm*(A), *erm*(B), *msr*(B), *lin*(A), *mph*(C), *mef*(A)] indicates a limited genetic repertoire in this population, contrasting with the broader diversity reported in some international studies but consistent with regional patterns showing *erm*(C) predominance [48, 49]. This focused genetic profile may reflect local clonal expansion or selective pressure from specific antibiotic usage patterns in the region. The substantial genotype-phenotype discordance (64% of resistant isolates lacking detectable genes) warrants careful consideration. Several mechanisms may explain this finding: (1) presence of unexamined resistance determinants, including novel *erm* alleles or rare MLSB genes not included in our panel [35, 50]; (2) primer-template mismatches due to sequence variations in mobile genetic elements carrying resistance genes [51]; (3) regulatory mutations affecting gene expression without altering coding sequences [34]; or (4) chromosomal mutations in ribosomal proteins or RNA that confer resistance independently of classical resistance genes [52]. These findings highlight the limitations of targeted PCR approaches and suggest that whole-genome sequencing may be necessary to characterize resistance mechanisms in this population [51, 53].

Table 4. Molecular detection of resistance genes in MLSB-positive isolates

Gene Pattern	n/25 (%)	Associated Phenotype
erm(C) only	2 (8)	cMLSB/iMLSB
erm(C) + *msr*(A)	7 (28)	cMLSB + enhanced macrolide resistance
No genes detected	16 (64)	Variable MLSB phenotypes
Total genes detected	**9 (36)**	-

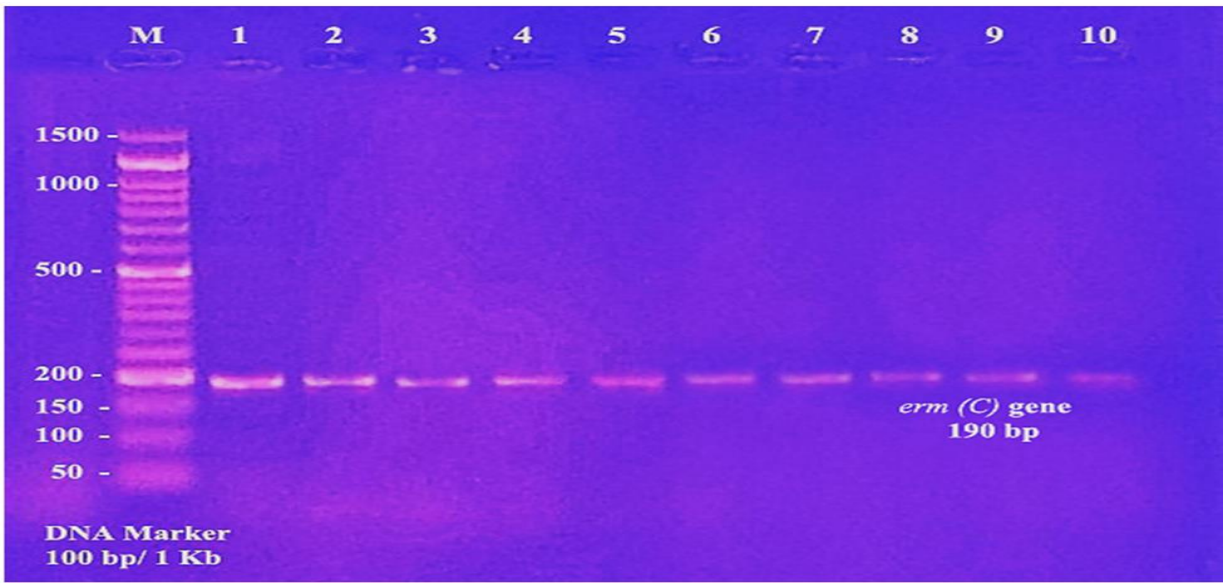

Figure 2. PCR amplification of the *erm(C)* gene (190 bp). Agarose gel electrophoresis (0.75 g agarose, 70 V, 1.5 h). Lane M: marker; lanes 1–10: positive isolates.

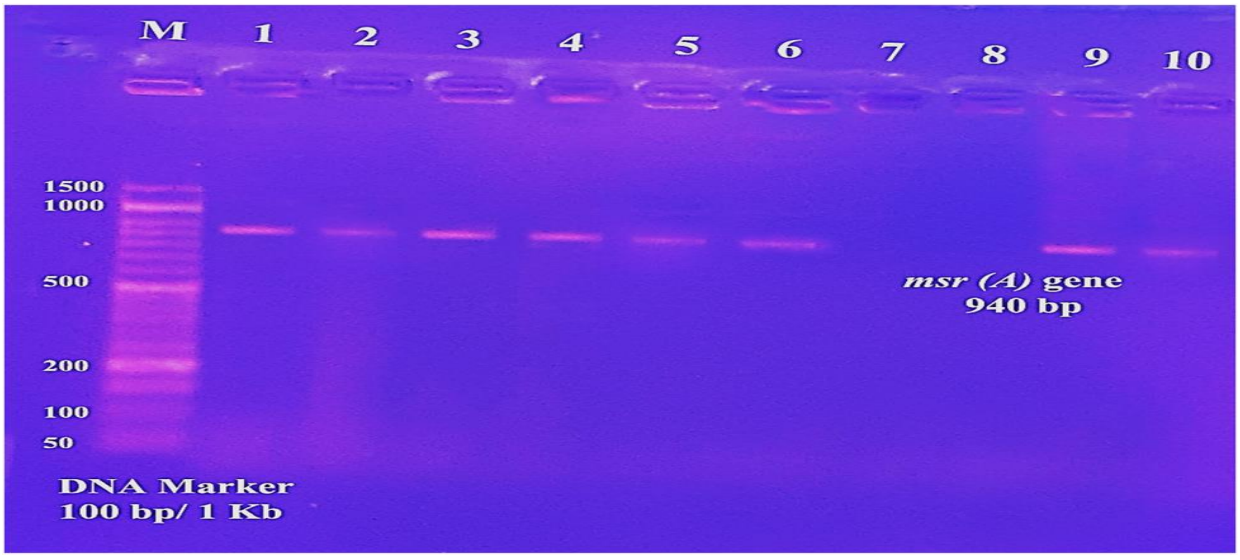

Figure 3. PCR amplification of the msr(A) gene (940 bp). Agarose gel electrophoresis (0.75 g agarose, 70 V, 1.5 h). Lane M: marker; lanes 1–6: test samples [specify positive/negative for each if clear]; lanes 7–8: negative; lanes 9–10: positive.

3.4 Clinical and Public Health Implications

The high prevalence of MLSB resistance (59.5%), particularly the significant proportion of iMLSB phenotypes (27.3%), has direct implications for clinical practice. Empirical use of macrolides or lincosamides for S. saprophyticus UTIs should be avoided in this setting, with preference given to nitrofurantoin or

trimethoprim-sulfamethoxazole as first-line agents [54, 55]. The detection of iMLSB phenotypes requires routine D-testing in clinical laboratories to prevent the inadvertent use of clindamycin in apparently susceptible isolates that harbor inducible resistance [37, 38, 56]. These resistance patterns align with broader antimicrobial resistance trends reported across Iraq, where rates of erythromycin resistance range from 50% to 67% and clindamycin resistance from 22% to 46% among staphylococcal isolates [57, 58]. The findings contribute to the growing evidence of increasing MLSB resistance in the Middle East region, likely driven by antibiotic selection pressure and horizontal gene transfer [59-61].

3.5 Study Strengths and Limitations

The combined phenotypic and molecular approach provided comprehensive resistance characterization, with D-testing offering cost-effective detection of clinically significant iMLSB phenotypes while PCR revealed underlying genetic mechanisms [37, 41, 53]. However, several limitations should be acknowledged. The single-center design may limit generalizability, the sample size of 42 isolates provides limited statistical power for subgroup analyses, and the targeted gene panel may have missed novel or rare resistance determinants. Future multicenter studies with expanded molecular panels, including whole-genome sequencing, would provide more comprehensive epidemiological data and potentially resolve the observed genotype-phenotype discordances [51, 53, 50].

4. Conclusions

This study reveals alarming levels of MLSB resistance (59.5%) among *S. saprophyticus* UTI isolates in Najaf, Iraq, with constitutive resistance predominating; however, a clinically significant proportion exhibits inducible resistance. The limited genetic repertoire dominated by *erm*(C) and *msr*(A), combined with substantial genotype-phenotype discordance, highlights the complexity of resistance mechanisms and the necessity for comprehensive surveillance approaches. These findings provide essential local epidemiological data to guide empirical therapy decisions and emphasize the critical importance of regularly implementing the D-test in clinical laboratories for the identification of inducible resistance and the prevention of treatment failures.

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