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Oxford Nanopore-Based Genomic Characterization of Macrolide Resistance in Clinical *Streptococcus pyogenes* Isolates

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Abstract

Background/Objectives: *Streptococcus pyogenes* (Group A Streptococcus, GAS) remains a major human pathogen. This study aimed to determine the phenotypic profiles of macrolide resistance in *S. pyogenes* isolates from southeastern Turkey and to investigate underlying genotypic mechanisms using Oxford Nanopore sequencing. Methods: A total of 83 clinical isolates of *S. pyogenes* were collected between January 2024 and June 2025. Antimicrobial susceptibility was assessed by disk diffusion following EUCAST guidelines (Breakpoint Tables v15.0). Macrolide resistance phenotypes [inducible MLSB (iMLSB), constitutive MLSB (cMLSB), and M phenotype] were determined by the D-zone test. Whole-genome sequencing of erythromycin-resistant isolates was performed using Oxford Nanopore technology, and resistance determinants were analyzed with ResFinder pipeline. Results: All isolates were susceptible to penicillin. Six isolates (7.2%) exhibited erythromycin resistance: three iMLSB, two cMLSB, and one M phenotype. All three adult isolates were iMLSB, whereas the pediatric isolates comprised one M and two cMLSB phenotypes; given the small number of isolates, no conclusion on age-related differences can be drawn. Genotypic analysis revealed *erm(A)* in all iMLSB isolates, *mef(A)/msr(D)* in the M phenotype, and the absence of acquired macrolide resistance genes in cMLSB isolates. Co-occurrence of tetracycline resistance genes (*tetM/O*) was observed in four of the six isolates. Conclusions: Penicillin remains the cornerstone of GAS therapy; however, macrolide resistance persists through diverse mechanisms. The coexistence of *erm(A)*-mediated iMLSB, *mef(A)/msr(D)*-associated M phenotype, and unexplained cMLSB resistance highlights the need for routine D-testing and genomic surveillance. These findings emphasize the importance of integrating phenotypic and genotypic approaches to monitor resistance evolution in GAS.

Keywords: *Streptococcus pyogenes*; macrolide resistance; iMLSB; cMLSB; Oxford Nanopore sequencing; *erm(A)*; *mef(A)*; tetracycline resistance

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1. Introduction

Streptococcus pyogenes (Group A Streptococcus, GAS) is a major human pathogen responsible for a wide clinical spectrum, ranging from superficial infections such as pharyngitis and skin/soft tissue infections to life-threatening invasive diseases including streptococcal toxic shock syndrome and necrotizing fasciitis [1]. β-lactam antibiotics, particularly penicillin, remain the cornerstone of therapy for GAS infections, as they have largely retained their efficacy. In patients with a history of penicillin allergy, macrolides and clindamycin

may serve as alternative agents [2,3]. Moreover, protein synthesis-inhibiting antibiotics are of particular clinical importance in severe invasive GAS infections, as they can suppress streptococcal toxin production. However, treatment failure has been reported in strains exhibiting inducible clindamycin resistance [4].

Recent data reported from diverse geographical regions indicate that macrolide resistance among GAS isolates may increase in association with antibiotic usage patterns and the dissemination of specific clones [3,5]. In GAS strains, macrolide resistance is genetically categorized into three principal mechanisms. The first involves modification of the ribosomal target site mediated by erm genes. The second major mechanism is active efflux, most commonly associated with mef(A/E)–*msr(D)* [6,7]. The third group encompasses rarer mechanisms, including mutations in the 23S rRNA gene or in genes encoding ribosomal proteins (e.g., L4, L22), whereby phenotypic resistance is observed despite the absence of classical resistance determinants, representing mechanisms that remain incompletely elucidated [8,9].

Erythromycin was selected as the reference agent for macrolide susceptibility testing in this study for three main reasons. First, EUCAST recommends erythromycin as the screening agent for macrolide resistance in group A, B, C, and G streptococci [10]. Second, erythromycin resistance, when assessed together with clindamycin, provides the basis for differentiating the M, inducible macrolide–lincosamide–streptogramin B (iMLSB), and constitutive macrolide–lincosamide–streptogramin B (cMLSB) phenotypes by the D-test, which is clinically relevant for guiding lincosamide therapy [4]. Third, the majority of previous studies from Türkiye have evaluated macrolide resistance in group A streptococci using erythromycin [11–16], enabling direct comparison of our results with regional data.

The distribution of macrolide resistance mechanisms, their associated phenotypic patterns, and the predominant circulating clones may vary substantially across regions [17,18]. Consequently, it is essential for each center to monitor its own local resistance data and circulating genotypic characteristics, both to guide empirical therapeutic strategies and to strengthen epidemiological surveillance.

Long-read sequencing technologies, particularly Oxford Nanopore sequencing, provide a comprehensive means of assessing the presence of resistance genes, their genetic context, and their association with mobile genetic elements, thereby contributing to a more detailed characterization of phenotype–genotype correlations [19]. The objective of this study is to determine the phenotypic profiles of macrolide resistance in *S. pyogenes* isolates obtained from clinical samples in Diyarbakır—a city in southeastern Turkey—and to investigate, in a comprehensive manner, both the established and rare genotypic mechanisms underlying this resistance using Oxford Nanopore sequencing technology.

2. Results

2.1. Demographic Data and Distribution of Clinical Samples

The study included 83 *Streptococcus pyogenes* isolates obtained from patients with a mean age of 24.5 ± 22.0 years. Of these, 48 (57.8%) were children and 35 (42.2%) were adults. The gender distribution was balanced, with 42 females (50.6%) and 41 males (49.4%).

Analysis of the clinical specimens revealed that respiratory tract samples constituted the largest group, accounting for 45 isolates (54.2%), including 42 throat swabs and 3 sputum samples. These were followed by 14 aspirates (16.9%), 9 wound specimens (10.8%), 3 blood cultures (3.6%), and 12 other clinical samples (14.5%) such as urine, cerebrospinal fluid, and ear drainage.

Erythromycin resistance was detected in 3/48 (6.3%) children and 3/35 (8.6%) adults (Fisher's exact test, $p = 0.69$). By specimen type, resistance was found in 4/45 (8.9%) respiratory tract specimens compared with 2/38 (5.3%) other specimens ($p = 0.68$) and

in 2/14 (14.3%) aspirates compared with 4/69 (5.8%) all other specimens ($p = 0.27$); no resistance was found in wound, blood or other specimens (0/24). Ward information was not available. Because of the small number of resistant isolates, formal comparisons and the assessment of risk factors for acquiring resistant strains were not feasible.

2.2. Antimicrobial Susceptibility and Resistance Phenotypes

According to disk diffusion testing, all 83 isolates (100%) were susceptible to penicillin. A total of six isolates (7.2%) exhibited resistance to erythromycin. Inhibition zone diameters of erythromycin, clindamycin and tetracycline for each resistant isolate were given in Table 1. MICs were not determined. Phenotypic characterization of these erythromycin-resistant isolates revealed three with an inducible MLSB phenotype (iMLSB, D-zone positive), two with a constitutive MLSB phenotype (cMLSB), and one with an M phenotype (Figure 1).

Table 1. Characteristics of erythromycin-resistant *Streptococcus pyogenes* isolates.

Isolate	Age/Sex	Sample Type	Clonal Profile (ST, emm)	Resistance Phenotype	E (mm)	DA (mm)	TE (mm)	Resistance Genes (Identity %)
1	75/F	Aspirate	ST46, emm22	iMLSB phenotype (D-zone positive)	16.5 mm (R)	28 mm (S)	15.2 mm (R)	*erm(A)* 99.86%, *tet(M)* 99.06%
2	30/M	Sputum	ST46, emm22	iMLSB phenotype (D-zone positive)	16 mm (R)	26 mm (S)	15.7 mm (R)	*erm(A)* 99.86%, *tet(M)* 99.06%
3	38/M	Aspirate	ST63, emm77	iMLSB phenotype (D-zone positive)	12.2 mm (R)	27.2 mm (S)	16.6 mm (R)	*erm(A)* 100%, *tet(O)* 99.79%
4	3/M	Throat swab	ST49, emm75	cMLSB phenotype (constitutive resistance)	6 mm (R)	6 mm (R)	26.2 mm (S)	No acquired macrolide resistance gene detected †
5	3/M	Throat swab	ST49, emm75	cMLSB phenotype (constitutive resistance)	6 mm (R)	6 mm (R)	28 mm (S)	No acquired macrolide resistance gene detected †
6	9/F	Throat swab	ST25, emm44	M phenotype (efflux mechanism)	15 mm (R)	24.5 mm (S)	11.2 mm (R)	*mef(A)* 100%, *msr(D)* 100%, *tet(M)* 99.53%

F: Female; M: Male; E: Erythromycin; DA: Clindamycin; TE: Tetracycline; R: Resistant; S: Susceptible; Zone diameters were interpreted according to EUCAST Breakpoint Tables v15.0 (erythromycin 15 µg, S ≥ 21/R < 21 mm; clindamycin 2 µg, S ≥ 17/R < 17 mm; tetracycline 30 µg, S ≥ 23/R < 23 mm). Gene identity values (coverage 100% for all hits) were obtained with ResFinder v4.7.2. † No acquired macrolide resistance gene detected by ResFinder. Both isolates were phenotypically susceptible to tetracycline.

Macrolide-resistant isolates were further screened for tetracycline resistance. In four isolates, the presence of corresponding resistance genes was confirmed, and phenotypic resistance to tetracycline was observed. In contrast, two isolates lacking detectable resistance genes remained phenotypically susceptible to tetracycline. The remaining 77 isolates (92.8%) did not display macrolide resistance.

Among the six erythromycin-resistant isolates, three were recovered from adult patients and three from children. All adult isolates exhibited the iMLSB phenotype, whereas the pediatric isolates comprised one M phenotype and two cMLSB phenotypes. With respect to specimen type, resistant isolates from adults were obtained from two aspirates and one sputum sample, while those from children were derived exclusively from throat swabs.

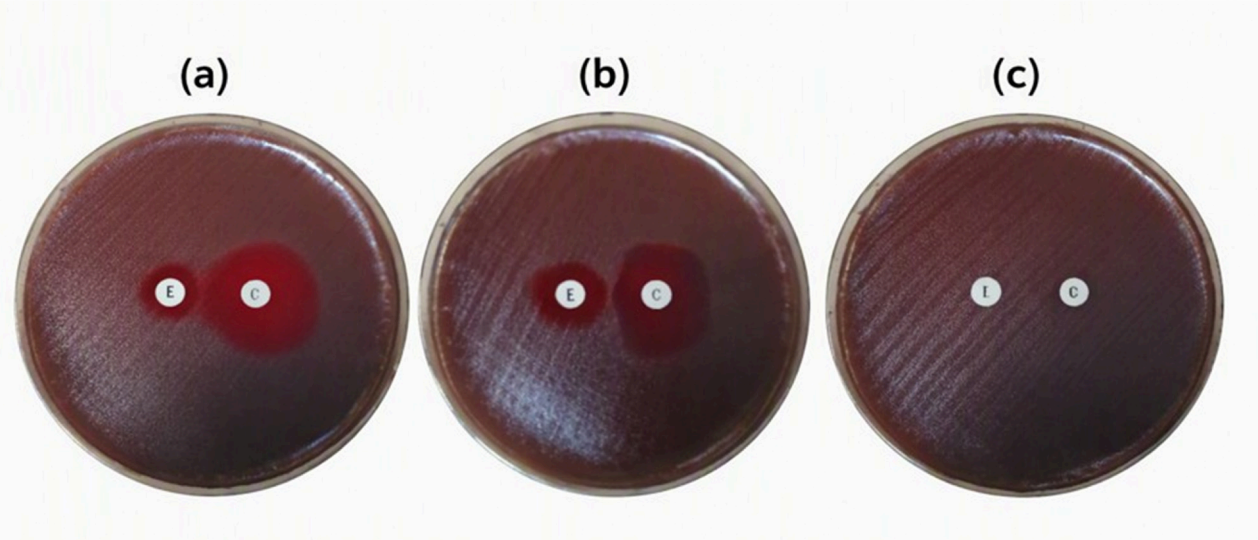


Figure 1. D-zone test results for *Streptococcus pyogenes* isolates: (**a**) M phenotype—resistant to erythromycin but susceptible to clindamycin (D-zone negative); (**b**) inducible macrolide–lincosamide–streptogramin B (iMLSB) phenotype—resistant to both antibiotics with inducible resistance (D-zone positive); (**c**) constitutive macrolide–lincosamide–streptogramin B (cMLSB) phenotype—constitutive resistance to erythromycin (E) and clindamycin (C). All plates were photographed under identical conditions. Images were cropped to the plate area, and uniform gamma and color-saturation adjustments were applied equally to each image. No features were added, removed, or relocated.

2.3. Genotypic Characteristics of Erythromycin-Resistant Isolates

Oxford Nanopore sequencing was performed on all six erythromycin-resistant *S. pyogenes* isolates. Sequencing yield ranged from 45.8 to 475.1 Mb with a median read quality of Q15.8–16.1; five genomes were assembled into one or two contigs, and the genome of isolate 6, which had the lowest yield (45.8 Mb), was assembled into five contigs, with total assembly lengths of 1.79–1.97 Mb Among the three isolates exhibiting the iMLSB phenotype, two belonged to the ST46/emm22 clonal profile and one to ST63/emm77. All three carried the *erm(A)* gene (99.86–100% identity, 100% coverage). The two ST46/emm22 isolates harbored *tet(M)* (99.06% identity), while the ST63/emm77 isolate carried *tet(O)* (99.79% identity); in each case, tetracycline resistance was phenotypically confirmed.

The isolate with the M phenotype was identified as ST25/emm44 and carried *mef(A)*, *msr(D)* (both 100% identity), and *tet(M)* (99.53% identity) genes, with tetracycline resistance also confirmed phenotypically. These three genes were located on the same contig; however, due to the fragmented nature of the assembly (five contigs, with the lowest sequencing depth among the series), their genetic context could not be analyzed further.

The two isolates displaying the cMLSB phenotype shared the ST49/emm75 clonal profile and were obtained from throat swabs of pediatric patients. The demographic, clinical, phenotypic, and genotypic characteristics of the macrolide-resistant isolates are summarized in Table 1.

Whole-genome sequencing revealed that one isolate was assembled into a single circular chromosome of 1.79 Mb, whereas the other was assembled into two circular contigs totaling 1.81 Mb. ResFinder did not identify any acquired macrolide resistance genes. However, chromosomal lmrP (99.01% identity, 100% coverage) and a low-similarity mef(E)-like sequence (80.62% identity, 97.02% coverage) were detected (Table 2). Both isolates were phenotypically susceptible to tetracycline, with inhibition zones of 26.2 and 28 mm.

Table 2. Sequencing and assembly metrics of the six erythromycin-resistant *S. pyogenes* genomes.

Isolate/Barcode	ST/emm	Yield (Mb)	Median Read Length (kb)	Median Q	Estimated Depth (×) †	Contigs	Total Length (Mb)	Circular Contigs
1/01	ST46/emm22	428.89	2.56	16.1	~229	2	1.87	2
2/02	ST46/emm22	475.07	2.58	16.1	~255	1	1.86	1
3/04	ST63/emm77	275.16	2.28	16.0	~140	1	1.96	1
4/05	ST49/emm75	151.24	1.80	15.9	~84	2	1.81	2
5/06	ST49/emm75	252.18	1.60	15.8	~141	1	1.79	1
6/07	ST25/emm44	45.80	1.61	15.9	~23	5	1.97	2

Assemblies were generated using Flye v2.9.5 in -nano-hq mode and polished using Medaka v2.2.0 within the EPI2ME wf-bacterial-genomes v2.0.1 workflow. Draft assemblies were reoriented using Dnaapler. Estimated depth was calculated as total sequencing yield divided by total assembly length. (Estimated depth (×) = Yield (Mb)/Assembly length (Mb)), † Estimated sequencing depth, expressed as average genome coverage (×).

3. Discussion

GAS is an important human pathogen responsible for diseases ranging from pharyngitis to severe invasive infections. Penicillin and other β-lactam antibiotics remain uniformly effective and continue to represent the cornerstone of current treatment strategies [1,2]. In our study, all 83 isolates were susceptible to penicillin, consistent with both national and international reports. In contrast, resistance to macrolides—key alternatives for patients with penicillin allergy—persists as a clinically relevant concern [5,9,11,12].

In this study, the erythromycin resistance rate was 7.2%. This corresponds to a low-to-moderate resistance pattern, consistent with data previously reported from Turkey. For example, Açıkgöz et al. reported a rate of 2.6% in Ankara, in Central Anatolia [11], while Akata et al. documented a rate of 2.7% in Edirne, located in northwestern Turkey [12]. In western Turkey, Telli et al. reported erythromycin resistance rates as high as 15% in Aydın [13], whereas Dündar et al. reported 9% in Kocaeli [14]. In Eastern Anatolia, Uyanık and Yazgı documented a resistance rate of 8% in Erzurum [15]. A recent study from two hospitals in Istanbul (2020–2022) likewise reported a rate of 9.5% among 92 isolates, with no penicillin resistance detected [20]. Taken together, these data indicate that macrolide resistance in GAS varies among centers in Turkey but generally remains within low-to-moderate levels. The 7.2% resistance rate observed in our study, conducted in southeastern Turkey, is consistent with this overall pattern. This variability underscores the need to examine phenotypic distributions across different regions and time periods.

When the phenotypic distributions of macrolide resistance in Turkey are considered, early studies particularly highlighted the predominance of the M phenotype. Akata et al. reported that resistant isolates predominantly exhibited the M phenotype and carried the *mef(A)* gene [12]. Similarly, Açıkgöz et al. and Çolakoğlu et al. reported that the M phenotype was the most common among resistant isolates, with *mef(A)* consistently detected in these strains [11,16]. In contrast, Telli et al. and Dündar et al. documented a notable frequency of the *erm(TR)*-mediated iMLSB phenotype [13,14]. Taken together, these findings indicate that macrolide resistance in Turkey cannot be attributed to a single phenotype or genetic mechanism but rather shows temporal and geographic variability. Consistent with this phenotypic variability, genotypic analyses from Turkey have revealed heterogeneous resistance mechanisms. Efflux determinants such as *mef(A)-msr(D)* are associated with the M phenotype, whereas ribosomal methylases including *erm(TR)* and *erm(B)* confer inducible or constitutive MLSB phenotypes. This genetic diversity underscores that macrolide resistance in GAS cannot be explained by a single mechanism but rather reflects the coexistence of multiple determinants across different regions and time periods [21].

In this study, the phenotypic distribution of six erythromycin-resistant isolates comprised three iMLSB, two cMLSB, and one M phenotype. Although the number of resistant isolates was limited, this distribution indicates that macrolide resistance in southeastern Turkey also exhibits heterogeneity. Consistent with these phenotypic findings, genotypic analysis revealed the presence of *erm(A)* (formerly designated *erm(TR)*) among iMLSB isolates, supporting the association of this phenotype with ribosomal target site modification [6–9]. Two isolates belonged to the ST46/emm22 clonal profile and one to ST63/emm77, suggesting that specific clonal lineages may co-circulate with defined resistance mechanisms. Notably, the ST63/emm77 isolate carried both *erm(A)* and *tet(O)*, resembling macrolide-resistant clones reported in Europe [3,7,17,22]; emm77 has also been reported to predominate among *erm(A)*-positive erythromycin-resistant isolates in Greece [23]. Similarly, the presence of *erm(A)* together with *tet(M)* in ST46/emm22 isolates indicates that macrolide and tetracycline resistance determinants may co-occur in the same lineage [7,14,22]; as no plasmid was identified and the genetic context was not analyzed in detail, we cannot infer whether they were co-transferred.

In the pediatric age group, one isolate belonging to the ST25/emm44 clonal profile exhibited the M phenotype and carried both *mef(A)* and *msr(D)*, indicating the presence of active efflux mechanisms of macrolide resistance in southeastern Turkey [6–9]. This finding demonstrates that classical resistance determinants can be reliably confirmed by sequencing in isolates with clear phenotype–genotype concordance. The additional detection of *tet(M)* in the same isolate is noteworthy, as it highlights the potential for co-occurrence of macrolide and tetracycline resistance genes [7,14].

One of the most notable findings of this study was the absence of acquired macrolide resistance genes in two ST49/emm75 isolates exhibiting the cMLSB phenotype. Both isolates were obtained independently from throat swabs of different three-year-old male pediatric patients and showed phenotypic resistance to erythromycin and clindamycin. The detection of the same clonal profile in two separate patients may suggest circulation of this lineage in the community; however, this observation is based on only two isolates and should therefore be interpreted with caution. In the literature, such phenotype–genotype discrepancies have been associated with mutations in the 23S rRNA gene or alterations in ribosomal protein-encoding genes such as L4 and L22 [8,9]. Therefore, similar mechanisms may also play a role in the ST49/emm75 isolates identified in this study. The absence of acquired resistance genes suggests that this lineage may differ from macrolide-resistant lineages reported elsewhere; however, this remains hypothetical. In the literature from Spain and Portugal, emm75 lineages were commonly associated with the M phenotype and *mef(A)* carriage [24,25]. Since confirmatory mutation analysis of the relevant target regions was not performed in this study, this possibility should be regarded as a working hypothesis rather than a definitive mechanism.

The distribution of resistant isolates according to age group and clinical specimen type was also noteworthy. All three erythromycin-resistant isolates obtained from adult patients exhibited the iMLSB phenotype and carried *erm(A)*, whereas among pediatric patients, one isolate showed the M phenotype and two displayed the cMLSB phenotype. Moreover, resistant isolates from adults were recovered from aspirate and sputum specimens, while those from children were exclusively obtained from throat swabs. These observations are descriptive; resistance rates did not differ significantly between age groups or specimen types, and given the limited number of resistant isolates, any inference regarding differences in circulating clones or resistance mechanisms between age groups should be considered tentative.

In the Istanbul study, emm1, emm12 and emm89 predominated, but resistance genotypes were not reported, and the authors noted that whole-genome sequencing is needed to

elucidate the genetic environment of resistance genes [20]. In Kocaeli, emm22 and emm77 were among the emm types of erythromycin-resistant isolates [14], the same types found in our iMLSB isolates. As highlighted in the review of the Middle East and North Africa region, studies describing emm types within the macrolide-resistant pool remain scarce; resistance rates in that region range from 1.1% in Cyprus to 70% in Egypt, with the M phenotype predominating in Lebanon, Saudi Arabia and Turkey, and cMLSB in Iran, Tunisia and Egypt, whereas data from neighboring Syria and Iraq are lacking [21]. Our resistance rate falls within the low-to-moderate range of the region, and the coexistence of M, iMLSB and cMLSB phenotypes within a small series distinguishes it from the single dominant patterns reported elsewhere. These comparisons, however, are limited by differences in methodology and breakpoint systems, in isolate selection, and by the fact that emm typing was performed only for resistant isolates in our study.

When international data on macrolide resistance are considered, marked geographic differences become evident. While resistance rates remain low in several European countries [3,5,17], much higher levels—particularly with *erm(B)* predominance—have been reported from regions such as China [8]. These variations are thought to be associated with differences in antibiotic usage practices, the circulation of predominant clones, and the dissemination of resistance determinants [3,5,6,9,17,18]. The diverse clonal profiles identified in this study (ST46/emm22, ST63/emm77, ST25/emm44, and ST49/emm75) further indicate that macrolide resistance in southeastern Turkey cannot be explained by a single clone or mechanism [26,27].

This study has several limitations. First, it was conducted in a single center, and the total number of resistant isolates remained limited, which precluded statistical comparison and risk-factor analysis. Second, whole-genome sequencing and emm-typing were performed only for the six erythromycin-resistant isolates rather than for all strains. Third, susceptibility testing was based solely on disk diffusion; MICs were not determined, including for the two cMLSB isolates. Fourth, ward information was not available. Fifth, the assembly of one genome was fragmented, which restricted analysis of the genetic context of resistance genes. In addition, targeted analyses to confirm potential ribosomal mutations in isolates with the cMLSB phenotype were not undertaken. Nevertheless, a major strength of the study is the integration of phenotypic resistance patterns with Oxford Nanopore–based whole-genome sequencing, enabling comprehensive genotypic evaluation of each resistant isolate [19].

4. Materials and Methods

4.1. Collection and Identification of Clinical Isolates

Between January 2024 and June 2025, 83 *Streptococcus pyogenes* isolates were obtained from diverse clinical specimens submitted to the Central Laboratory of Dicle University Hospitals. Cultures were performed on appropriate media, including 5% sheep blood agar and chocolate agar (Deva Biyoteknoloji, Kocaeli, Türkiye), under standard incubation conditions. Preliminary identification of β-hemolytic colonies was based on bacitracin and trimethoprim-sulfamethoxazole susceptibility testing using antibiotic discs and PYR testing (Bioanalyse, Ankara, Türkiye). Definitive species identification was achieved using the BD Phoenix™ M50 automated system (Becton Dickinson, Franklin Lakes, NJ, USA). Isolates were stored in Tryptic Soy Broth (TSB) supplemented with 20% glycerol (Deva Biyoteknoloji, Kocaeli, Türkiye) at −20 °C until further analysis.

4.2. Antimicrobial Susceptibility Testing and Macrolide Resistance Phenotypes

Antimicrobial susceptibility was assessed by the Kirby–Bauer disk diffusion method following EUCAST guidelines (Breakpoint Tables v15.0) [10]. Isolates were adjusted to a 0.5 McFarland standard and inoculated onto MH-F agar (Mueller–Hinton agar with 5%

defibrinated horse blood and 20 mg/L β-NAD)(RTA Laboratuvarları, Kocaeli, Türkiye). Penicillin (1 unit), erythromycin (15 µg), clindamycin (2 µg), and tetracycline (30 µg) disks (Bioanalyse, Ankara, Türkiye) were used, and zone diameters were interpreted as susceptible/resistant at ≥23/<23 mm, ≥21/<21 mm, ≥17/<17 mm and ≥23/<23 mm, respectively. MICs were not determined.

For erythromycin-resistant isolates, macrolide resistance phenotypes (cMLSB, iMLSB, M) were determined by the D-zone test, with erythromycin and clindamycin disks placed 12–16 mm apart (edge to edge). Plates were incubated at 35 ± 1 °C in 5% CO_2 for 16–20 h, and inhibition zones were interpreted according to EUCAST criteria [10]. *Streptococcus pneumoniae* ATCC 49619 served as the quality control strain.

4.3. Genomic DNA Isolation and Library Preparation

Genomic DNA was extracted using a commercial silica-membrane column kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. Due to the thick cell wall of Gram-positive cocci, the lysis step was optimized with lysozyme treatment and extended incubation. For Oxford Nanopore sequencing, libraries were prepared using the Ligation Sequencing gDNA—Native Barcoding Kit 24 V14 (SQK-NBD114.24) protocol (Oxford Nanopore Technologies, Oxford, UK) [28]. End-prep, barcode ligation, and adapter ligation were performed sequentially, with purification at each step using AMPure XP magnetic bead [28]. DNA quantity and library concentrations were measured throughout the workflow using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

4.4. Oxford Nanopore Sequencing and Bioinformatic Analysis

Reads were analyzed using the Oxford Nanopore EPI2ME wf-bacterial-genomes workflow (v2.0.1), implemented in Nextflow v25.10.2. Basecalled and demultiplexed FASTQ reads from each barcode were analyzed independently using the default workflow parameters. Read preprocessing was performed with fastcat v0.24.2, and de novo assembly was performed with Flye v2.9.5 in -nano-hq mode [29]. Draft assemblies were reoriented using Dnaapler v1.2.0 and polished with Medaka v2.2.0 [30]. Read mapping was performed using mini_align (pomoxis v0.3.16), minimap2 v2.28, and SAMtools v1.22.1, and depth of coverage was calculated using mosdepth v0.3.11 [31]. Plasmid reconstruction and typing were performed using MOB-suite v3.1.9 (mob_recon). Genome annotation was performed using Bakta v1.11.4 with the Bakta light database v6, with antimicrobial resistance gene detection by AMRFinderPlus v4.0.23 [32] and coverage estimation with mosdepth v0.3.11. emm types were assigned with emmtyper v0.2.0 [33]. Multilocus sequence typing was performed using mlst v2.23.0 with PubMLST schemes, and taxonomic identification was performed using sourmash v4.9.4 against GTDB release R226 [34]. Acquired antimicrobial resistance genes were identified using ResFinder v4.7.2 with minimum thresholds of 80% sequence identity and 60% coverage [35]. DNA extraction, library preparation, sequencing, and bioinformatic analyses were performed as outsourced services by Massive Bioinformatics (İzmir, Türkiye). All analyses were conducted according to the developers' recommendations and using default parameters unless otherwise specified.

4.5. Statistical Analysis

Resistance rates between age groups and specimen types were compared with Fisher's exact test (two-sided; $p < 0.05$ considered significant) using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA).

This study was approved by the Non-Interventional Clinical Research Ethics Committee of Dicle University Faculty of Medicine (Decision No. 35, dated 18 December 2024).

5. Conclusions

Penicillin continues to remain the first-line therapy for *S. pyogenes* infections. However, routine implementation of the D-test is critical to prevent potential treatment failures with clindamycin. The co-occurrence of tetracycline resistance genes (*tet(M)*/*tet(O)*) with macrolide resistance determinants in four of the six resistant isolates underscores the necessity of sustained molecular surveillance. In this study, all isolates retained susceptibility to penicillin, whereas macrolide resistance was mediated through diverse phenotypic and genotypic mechanisms in this small series. The coexistence of the *erm(A)*-associated iMLSB phenotype, the *mef(A)–msr(D)*-associated M phenotype, and the cMLSB phenotype not explained by major resistance genes within the same series is particularly noteworthy. These findings highlight that local surveillance efforts should not rely solely on phenotypic testing but must also incorporate genomic approaches to ensure comprehensive monitoring of resistance evolution. Future multicenter genomic surveillance studies, including MIC determination and analysis of ribosomal target mutations, will be essential to track emerging clones and resistance determinants across different regions.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Non-Interventional Clinical Research Ethics Committee of Dicle University Faculty of Medicine (Decision No. 35, 18 December 2024).

Informed Consent Statement: As the study was performed on archived bacterial isolates without involving direct patient interventions, informed consent was not applicable.

Data Availability Statement: All relevant data supporting the findings of this study are included within the article. The genomic dataset generated and analysed during this study has been deposited in the NCBI BioProject database under the accession number PRJNA1543648.

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Abbreviations

The following abbreviations are used in this manuscript:

GAS	Group A Streptococcus, *Streptococcus pyogenes*
MLSB	Macrolide–Lincosamide–Streptogramin B
iMLSB	Inducible Macrolide–Lincosamide–Streptogramin B
cMLSB	Constitutive Macrolide–Lincosamide–Streptogramin B

EUCAST	European Committee on Antimicrobial Susceptibility Testing
MH-F	MH-F (Mueller–Hinton agar with 5% defibrinated horse blood
TSB	Tryptic Soy Broth
MIC	Minimum inhibitory concentration
ST	Sequence type

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