



A Pilot Molecular Study on The Association Between Sortase Genes (*SrtA*, *SrtB*, *SrtC*) and Antibiotic Resistance in Clinical Isolates of *Streptococcus Pyogenes*

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Abstract

Background: *Streptococcus pyogenes* is a major human pathogen causing respiratory tract infections. The occurrence of macrolide resistance has created a clinical concern to the effectiveness of treatment and control of the disease. The objectives of this study were to molecularly confirm the identity of *Streptococcus pyogenes* isolates, to identify the presence of sortase genes (*srtA*, *srtB*, and *srtC*) and to assess the statistical correlation between these genes and the phenotypic resistance to the antibiotics. **The methods** used to collect the specimens were throat and tonsil swabs from patients with symptoms of respiratory tract infections, done on a total of 300 patients. Standard microbiological testing was followed by PCR confirmation with the diagnostic *spy1258* gene, to identify and isolate bacteria. Antibiotic susceptibility test was done by the Kirby-Bauer disk diffusion method as per CLSI guidelines. The *srtA*, *srtB* and *srtC* genes were detected using PCR assays. **Results:** Out of 300 samples, 16 (5.33%) were found to be positive for *S. pyogenes*. The *srtA* gene was found in all 16 isolates (100%), the *srtB* gene was present in 43.75% of isolates and *srtC* gene was present in 81.25% of isolates. All three sortase genes were present in 7 (43.75%) of the isolates. All the isolates were sensitive to penicillin G (100%), whereas resistance was noted to erythromycin (62.5%), tetracycline (50%) and clindamycin (37.5%). Fisher's Exact Test showed that there was a significant relationship between the presence of the triple gene combination (*srtA*+*srtB*+*srtC*) and resistance to both erythromycin ($p = 0.011$) and clindamycin ($p = 0.001$). **Conclusions:** The distribution of multiple sortases, especially *srtC*, seems to be linked to higher macrolide resistance of *S. pyogenes*. This correlation indicates an indirect involvement of sortase enzymes in antibiotic tolerance, probably as an aid for pilus assembly and biofilm formation.

Keywords: *Streptococcus pyogenes*; sortase genes; antibiotic resistance; biofilm formation; virulence factors

Introduction

Streptococcus pyogenes is currently one of the most common pathogens in humans, and can lead to a range of diseases from mild mucosal diseases to highly invasive systemic infections. Recurrent infections and adaptive virulence pose increasing difficulties in clinical management, despite the availability of effective antibiotic therapies. mechanisms^{1, 2}.

Surface-associated proteins play an important role in the ability of bacteria to attach to surfaces, colonize and interact with host tissues. Sortase enzymes are cysteine transpeptidases that recognize sorting motifs that are used to anchor these essential proteins to the cell wall of bacteria^{3, 4}. Sortase A (srtA) is a “housekeeping” enzyme that is widely distributed in Gram-positive bacteria. Other sortases, on the other hand, have specific functions such as iron acquisition (srtB and srtC) and the pilus^{5, 6} assembly (srtC).

Sortase C is especially important for the polymerization of pili (filamentous surface structures important for host cell attachment and colonization)⁷. In addition, pili are directly involved in biofilm formation, which is a protective growth mode that plays an important role in the persistence of bacteria under adverse environmental conditions^{8, 9}. The dense extracellular matrix of a biofilm acts as a formidable physical barrier against antimicrobial penetration, rendering the embedded metabolically dormant bacteria highly recalcitrant to therapy^{10, 11}. This mechanism is thought to heavily influence the growing rates of macrolide resistance observed. Therefore, understanding the distribution and implications of sortase genes in *S. pyogenes* is of highest clinical relevance. Their indirect role in the development of antibiotic resistance may reveal new pathogenic processes and new targets to reduce virulence, which would not directly promote resistance¹³. Thus, the objective of this pilot study was to isolate and molecularly confirm the presence of *Streptococcus pyogenes*; to find the distribution of the srtA, srtB, and srtC genes; to study antibiotic susceptibility patterns; and to examine the relationship between the sortase gene profile and antibiotic resistance among streptococcal species¹².

Methods

Ethical approvals

Ethical approval for the collection and laboratory use of clinical samples was obtained from the Local Ethics Committee of College of Sciences/University of Anbar, approval No. 39545, dated 8/12/2025. Written informed consent was obtained from all adult participants before sampling. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Sample Collection and Processing

A total of 300 throat and tonsil swabs were collected from patients presenting with suspected respiratory tract infections. Demographic and clinical characteristics, including previous antibiotic usage, were documented for all participants. Swabs were cultured on blood agar and blood azide agar (selective medium) and incubated at 37°C for 24–48 hours. Growing colonies were subsequently subcultured on blood agar to obtain pure isolates. Identification of *S. pyogenes* was achieved based on the observation of β -hemolysis, Gram staining morphology, negative catalase test, bacitracin sensitivity, and serological grouping using a Streptex kit (Thermo Fisher Scientific / Remel Europe Ltd, UK)¹⁴.

Antibiotic Susceptibility Testing

The susceptibility of confirmed isolates was tested by the Kirby-Bauer disk diffusion method. Two pure colonies were suspended in sterile saline with a 0.5 McFarland Standard turbidity for

preparing the bacterial inoculum. This resuspension was subsequently streaked to Mueller-Hinton agar (MHA) with 5% sheep blood, where *S. pyogenes* should grow well ¹¹. It was tested with a panel of antibiotic disks such as penicillin G, erythromycin, clindamycin and tetracycline. The plates were incubated for 20–24 hours at 37 °C in a humidified, 5% CO₂-enriched atmosphere. The inhibition zones were measured, and the results (Sensitive, Resistant or Intermediate) were determined strictly following Clinical and Laboratory Standards Institute (CLSI) ¹⁵.

DNA Extraction and PCR Amplification

Genomic DNA was extracted using a commercial kit (Promega, USA) following the provided protocol, and purity was verified via spectrophotometry (Thermo Fisher Scientific, USA). Target sequences were amplified using specific primers: *spy1258* (Forward: 5'-AAAGACCGCCTTAACCACCT-3', Reverse: 5'-TGGCAAGGTAACTTCTAAAGCA-3'; 407 bp) *srtA* (Forward: 5'-TCATGAAGCTGCCTATCGAC-3', Reverse: 5'-CCTGTTTTACCTGCTTCGTG-3'; ~480 bp) *srtB* (Forward: 5'-AAGCTTGGTGGATCAACCTC-3', Reverse: 5'-GTCGACAGCACCATTTCAG-3'; 307 bp) *srtC* (Forward: 5'-GCAACTGGCACAAGTACAAG-3', Reverse: 5'-ATCCTTGCCCTGAATACCTG-3'; ~450 bp) Reactions (20 µL) contained 2 µL of each primer, 3 µL master mix, 5 µL template DNA, and 8 µL water. Cycling conditions were: 95°C for 5 min; 35 cycles of 94°C for 45s, annealing for 45s (55°C for *spy1258*, 52°C for *srtA*, 54°C for *srtB*, 56°C for *srtC*), and 72°C for 60s; with a final extension at 72°C for 10 min. Amplicons were separated on 1% agarose gels at 70V for 50–60 minutes.

Statistical Analysis

The SPSS version 26 software was used for data analysis. Sortase gene distribution and antibiotic resistance levels were summarized using descriptive statistics (frequencies and percentages). A Fisher's Exact Test was used to compare the relationship between the presence of multiple sortase genes and antibiotic resistance (erythromycin and clindamycin) because the number of confirmed isolates (N=16) was small. A p value < 0.05 was deemed to be statistically significant.

Results

Isolation and Identification

Of 300 samples processed, 16 (5.33%) were phenotypically determined to be *S. pyogenes*. All 16 isolates were then confirmed using the diagnostic gene (*spy1258*) which produces a 407 bp amplicon when amplified by PCR (Figure 1).

Detection of Sortase Genes

All 16 isolates (100%) were found to be positive for the housekeeping gene *srtA* by molecular screening. *srtC* gene was identified in 13 isolates (81.25%) while *srtB* gene was detected in 7 isolates (43.75%). Of these, 7 were found to contain the full set of three gene products (*SrtA*, *SrtB* and *SrtC*) (Table 1; Figures 2, 3, and 4).

Antibiotic Susceptibility Patterns

The resistance pattern of the isolates was varied. Penicillin G was still the most effective, with none of the items being resistant (100% susceptible). Erythromycin showed the highest level of resistance at 62.5% (10/16 isolates), while the lowest level was against tetracycline at 25% (4/16 isolates). The resistance rates of tetracycline and clindamycin were 50% (8/16 isolates) and 37.5% (6/16 isolates), respectively. A few isolates were found to have multidrug-resistant phenotypes (Table 2).

Sortase genes (*srtA*) and antibiotic resistance are interrelated. The interrelatedness between **sortase genes (*srtA*) and antibiotic resistance**.

The genotypic distribution of the isolates was analysed against the resistance phenotypes and it was found that the isolates with more than one sortase gene had higher resistance levels. Interestingly, all seven isolates with the triple genotype (srtA + srtB + srtC) were completely resistant to erythromycin and clindamycin (Table 3). The triple gene combination was found to be highly significantly associated with resistance to erythromycin ($p = 0.011$) and clindamycin ($p = 0.001$) by statistical evaluation using Fisher's Exact Test.

Discussion

In the present study the statistical significance ($p < 0.05$) was obtained when compared the antibiotic resistance pattern of clinical *S. pyogenes* isolates with the distribution of sortase genes. The complete isolates with the triple genotype (srtA + srtB + srtC) were all resistant to erythromycin and clindamycin, indicating that the three virulence factors might act synergistically or as a group to contribute to bacterial resistance to antimicrobial selective pressure. These observations are in line with recent reports describing the intricate relationship between antibiotic tolerance and virulence factors^{16, 17}.

Given that srtA has been found in all organisms (100%) and known to be a housekeeping gene essential for the covalent attachment of a wide variety of surface proteins involved in basic colonization and tissue recognition processes^{18, 19}, its widespread occurrence suggests its physiological importance rather than a specific mechanism associated with different levels of antibiotic resistance.

On the other hand, the lack of srtB and, especially, srtC suggests strain-specific virulence augmentation. The prevalence of srtC (81.25%) is interesting because it is one of the major functions of srtC is to polymerize pili on the bacterial surface^{20,21}. Pili are important contributors to host cell adhesion and for structural scaffolding during biofilm initiation and maturation^{22,23}. These biofilms have a dense extracellular matrix that hinders the diffusion of antibiotics, and they also create a low metabolic state for any resident bacteria, making them less susceptible to antibiotics that inhibit protein synthesis like macrolides^{24, 25}.

Thus, the increased macrolide resistance of srtC-positive organisms could be due to robust biofilm production which provides physical and metabolic resistance to the macrolides. Pili-dependent bacterial persistence in host tissues leads to adaptive bacterial response to sub-inhibitory antimicrobial levels. It is important to note that sortase genes per se (e.g. srtA, srtB, and srtC) do not encode ability to biochemically resist antibiotics, such as by drug inactivation, target modification, or active efflux. Rather, the increased macrolide resistance they observed is indirect, due to their increased tolerance of biofilms. Through its role in facilitating pilus assembly, surface adherence, especially via srtC, sortases help to create a dense biofilm matrix. It is a structural barrier that prevents antimicrobial molecules from entering the cell and helps to maintain a slow metabolism, which significantly decreases the effectiveness of the antibiotic and makes it easier for bacteria to remain in the body under antibiotic pressure²⁶.

Having all three of the sortase genes may have an additive effect on the pathogenic potential. The srtB gene, which is less studied, is also involved in iron acquisition, helping to ensure survival in the limiting host environment²⁷. These sortase pathways are a highly promising avenue for antivirulence approach. In principle, blocking the activity of sortase enzymes could make the bacteria harmless without preventing them from being able to live, thereby reducing the quick evolution of classic resistance^{17, 28}. The potential therapeutic applications of these may play an important role in the worldwide increasing crisis of macrolide-resistant streptococci²⁹.

This pilot study provides a solid statistical relationship, but due to its descriptive molecular nature and the limited final sample size, it is necessarily limited. The results are preliminary and do not prove functional causation. Further phenotypic validation is needed to complete elucidation of this pathway.

Conclusions

Based on the data obtained, the following conclusions can be made: 100% of the isolates with a complete gene set of sortases (srtA, srtB and srtC) were resistant to the macrolides erythromycin and clindamycin. The srtC gene is likely to play an indirect but clinically important role in antibiotic tolerance, allowing for a greater number of pili to be assembled and a protective effect on biofilm formation, thus physically preventing antibiotics from reaching the cell. It should however be stressed that this association is a proposed mechanism in the light of genotypic data. All *S. pyogenes* isolates were fully sensitive to penicillin G, reaffirming penicillin G as the drug of choice for *S. pyogenes* infections. Further research should include larger numbers and utilize quantitative phenotypic biofilm assays such as microtiter plate assays to quantify the amount of biofilm produced by the organisms and to confirm this functional causality; and should include the investigation of classical macrolide resistance genes to map the synergistic pathways involved in streptococcal persistence.

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Table 1. Distribution of Sortase Genes among *S. pyogenes* Isolates (N=16)

Gene	No. of Isolates	Percentage (%)
<i>srtA</i>	16	100.0
<i>srtB</i>	7	43.75
<i>srtC</i>	13	81.25

Table 2. Antibiotic Susceptibility Pattern of *S. pyogenes* Isolates (N=16)

Antibiotic	Resistant No.	Resistant (%)	Sensitive No.	Sensitive (%)
Penicillin G	0	0.0	16	100.0
Erythromycin	10	62.5	6	37.5
Clindamycin	6	37.5	10	62.5
Tetracycline	8	50.0	8	50.0

Table 3. Relationship Between Sortase Genotypes and Antibiotic Resistance

Genotype	No. of Isolates	Erythromycin Resistant n (%)	Clindamycin Resistant n (%)
<i>srtA</i> only	3	1 (33.3)	0 (0.0)
<i>srtA</i> + <i>srtC</i>	6	2 (33.3)	1 (16.7)
<i>srtA</i> + <i>srtB</i> + <i>srtC</i>	7	7 (100.0)	7 (100.0)

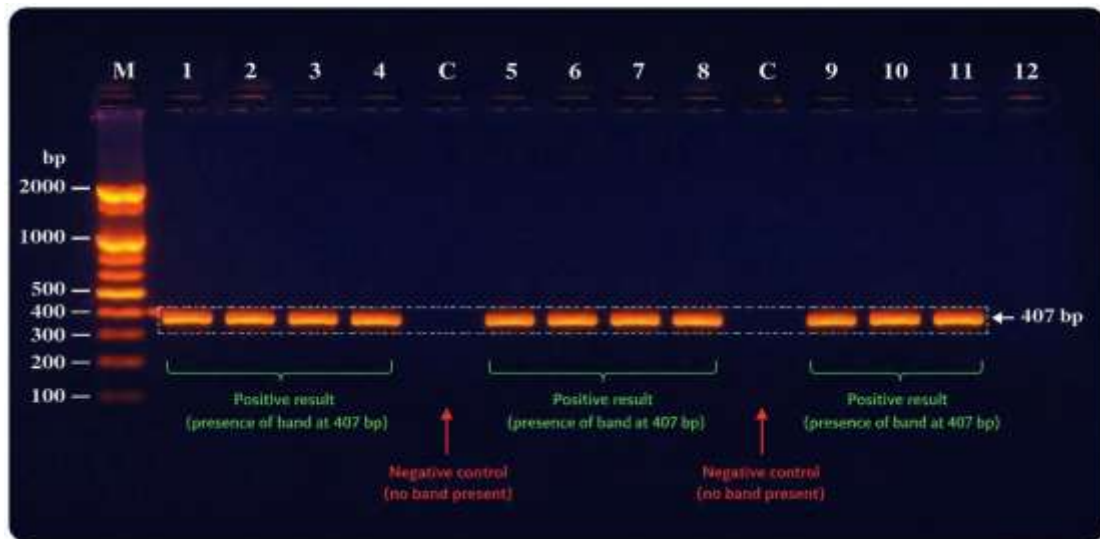


Figure 1. Representative agarose gel electrophoresis showing PCR amplification of the diagnostic *spy1258* gene (407 bp) in confirmed *S. pyogenes* isolates (Lanes 1–12 represent selected clinical isolates; C: Negative control; M: 100 bp DNA ladder).

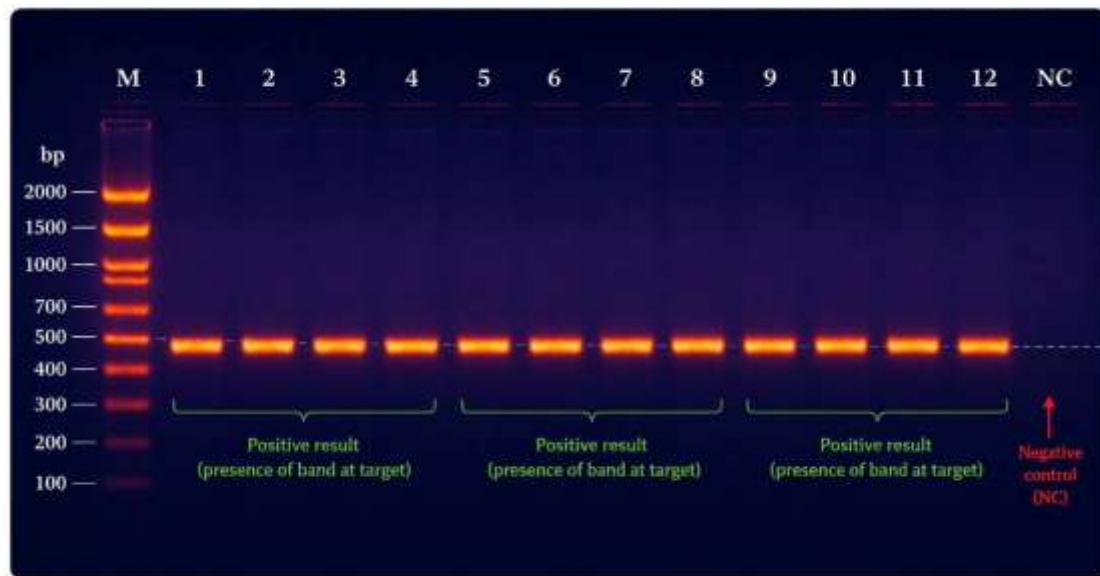


Figure 2. PCR amplification of the *srtA* gene (~480 bp) in *S. pyogenes* isolates (Lanes 1–12 represent selected positive clinical isolates out of 16; NC: Negative control; M: 100 bp DNA ladder).

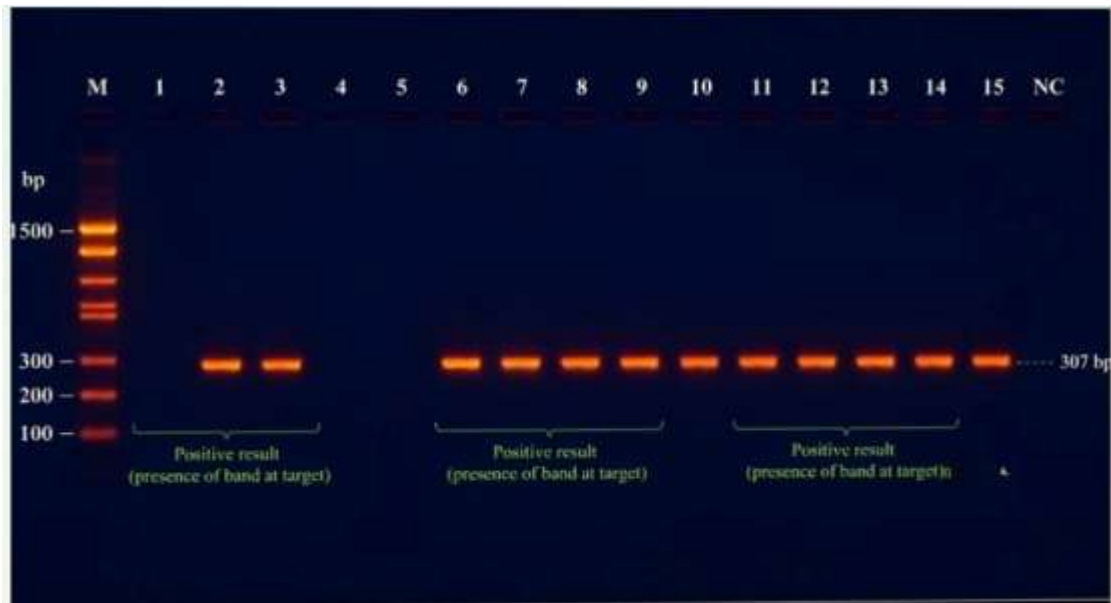


Figure 3. Agarose gel electrophoresis for PCR detection of the *srtB* gene (307 bp) in *S. pyogenes* isolates (M: DNA ladder; NC: Negative control; positive bands correspond to the 7 *srtB*-positive isolates listed in Table 1).

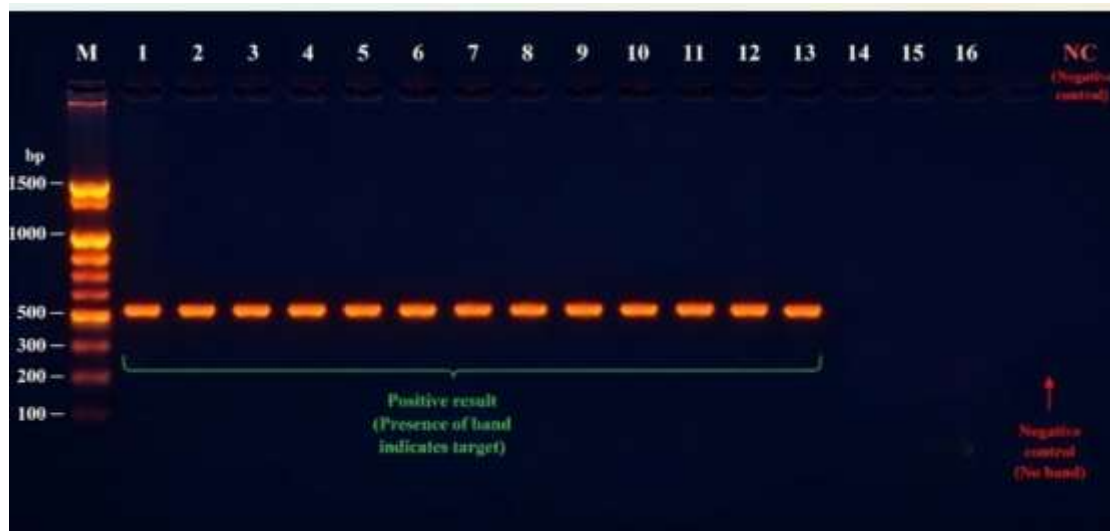


Figure 4. Agarose gel electrophoresis showing PCR amplification of the *srtC* gene (~450 bp) in representative *S. pyogenes* isolates (M: DNA ladder; NC: Negative control).