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Molecular Identification of Resistance Genes in *Staphylococcus Aureus* Associated with Chronic Tonsillitis

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Annotation: Staphylococcus aureus is a pathogen responsible for many cases of tonsillitis infections (bacterial tonsillitis) which occur mostly in young people and children. In our study, we tested 40 patients suffering from recurrent tonsillitis. The samples were tested microbiologically for the presence of S. aureus. Identification of the isolates was achieved through culture on Mannitol Salt Agar, Gram staining, and biochemical testing. Determination of antimicrobial susceptibility of the isolates was performed by disk diffusion testing to a range of antibiotics. DNA was extracted from selected isolates and PCR was carried out to detect the SHV gene associated with antibiotic resistance in the isolates. The PCR products were then visualized after electrophoresis in agarose gels. PCR demonstrated the presence of the SHV gene in 4 isolates of S. aureus. Rates of infection

with S. aureus were greater in individuals resident in urban locations (60%) compared to those resident in rural areas (40%). There was a slight difference between male (21 cases) and female (18 cases) rates of infection. However, there was a strong association with reported use of antibiotics at time of infection (43 cases). Highest rates of isolation of S. aureus were observed in patients with chronic tonsillitis and in two age ranges; 10-20 years and 40-60 years.

Keywords: Resistance Genes, Staphylococcus Aureus, chronic tonsillitis, PCR, SHV gene.

Introduction

The etiology of recurrent tonsillitis is currently a subject of controversy in medicine. Recurrent tonsillitis is one of the most frequent diseases in pediatric practice (1), and much has been written on the etiology of recurrent tonsillitis, yet the exact mechanisms remain unclear. While a single microbial species can cause an acute tonsillitis, it has been suggested that recurrent tonsillitis is caused by a polymicrobial infection (2). Other than Group A Beta Hemolytic Streptococcus (GABHS), many microorganisms may be responsible for chronic tonsillitis (2). Bacterial biofilms have been identified as the major factor involved in the chronicity of infections and the resistance to antibiotic treatments. For this reason, these infections have a considerable negative impact on the quality of life of patients and represent a significant burden for public health (3).

Several researchers have claimed that the failure of the antibiotic therapy may be due to the underestimation of resistant microorganisms, which may be explained by low concentration of antibiotics in the tonsillar tissue, possibly combined with the presence of resident bacteria producing protective enzymes, or specific antibiotic resistance patterns of the involved pathogenic bacteria (4).The presence of the bacterium in the internal tissue of the tonsil may be responsible for its persistence in this location. The tonsillar surface typically presents bacteria belonging to the normal oral microbiota, whereas the internal tissue contains pathogenic microorganisms. S. aureus have been detected in both the external and internal tissues of the tonsils (5).2-1 Materials and Methods. The 40 clinical samples were collected from the tonsillar area of the subjects. Sample collection was performed using a sterile cotton swab to avoid contamination. Each swab was immediately placed on a prepared Mannitol Salt Agar plate. The inoculation was made using the streak plate method to isolate individual colonies. All inoculated plates were clearly labeled and handled under aseptic conditions. The study was conducted in a medical center where patients were undergoing routine diagnostic assessments and follow-ups. To generate relevant samples for analysis, laboratory tests and patient records were reviewed.

Colonial and Microscopically Characteristics

Colonies grown on mannitol salt agar were characterized according to their color, colony size, morphology, and colony texture, as well as their ability to ferment mannitol sugar and the change of the culture medium from pink to yellow. The colonies were stained with Gram stain on a glass

slide to observe the shapes of cells under a microscope at 1000x magnification in the presence of a drop of lens oil to observe the shape, arrangement, and color of the colonies, as they were Gram-positive.

Biochemical test

Catalase Test The catalase test was performed using 3% hydrogen peroxide (H_2O_2) to determine the presence of catalase enzyme in bacterial isolates obtained from tonsillar swabs. Oxidase Test .The oxidase test was performed to determine the presence of cytochrome c oxidase enzyme in bacterial isolates obtained from tonsillar swabs.

Genetically identification of resistant gene by Polymerase Chain Reaction (PCR)

DNA template preparation by using boiling method with lysozyme and Tris-HCl; 2 mM EDTA; 1% Triton X-100; pH 8.0; prepare fresh lysozyme buffer immediately prior to use) and resuspend the pellet by vortex or pipetting. Add 200 µl of FATG Buffer to the sample and vortex for 5 seconds.

Incubate for 10 min at 70°C or until the sample lysate is clear. During incubation, invert the tube every 3 min.

Preheat required Elution Buffer for Step DNA Elution in a 70°C water bath. (Optional Step): If RNA-free genomic DNA is required, add 5 µl of 10 mg/ml RNase A to the sample and mix by overtaxing. Incubate for 5 min at room temperature. Primers used for PCR As shown in Table 1, the genes employed in this study are related to antibiotic resistance gene necessary for Staphylococcus aureus resistance and pathogenicity. These were purchased from the Korean Alpha DNA Company.

Table -1: The primer used in the current study for PCR amplification.

Target Gene	Nucleotide sequences and direction (5\-------3\)		Product size (bp)
SHV	F	**5-GCCTTTATGGGCCTTGACTGAAG-`3**	6950.6
SHV	R	**5-TTAGCGTTGCCAGTGCTCGATCA-`3**	7030.6

Twelve selected isolates underwent a PCR-based genotypic assessment. The oligonucleotide primers for the SHV (Table 1) as per the (Alpha DNA) manufacturer's instructions were prepared by diluting the oligonucleotides in TE Buffer to obtain a final concentration of 100 µl. The reactions were performed in a PCR thermocycler; the reaction mixtures were constructed according to the manufacturer's recommendations (Maxime PCR PreMix kit (i-Taq)) and are listed in Table 2.

Table 2: component and volumes of PCR mixture

PCR Master Mix	Volume
DNA Template	5 µl
Forward primer	2 µl
Reverse primer	2 µl
Nuclease free water	3.5 µl
Total	12,5 µl

Conditions for PCR amplification which were set according to this study for the two primers was shown in table 3

Table 3 : Program of thermal cycles to amplify DNA

Gene	Temprature (C) /Time					Cycle No.
	Initial denaturation	Cycling Condition			Final Extention	
		Denaturation	Annealing	Extention		
SHV	94/2 min	94 /30 min	55/45 min	72/45min	72/45min	35
SHV	94/2 min	94/30 min	55/45 min	72/45 mi	72/45min	35

The PCR product was evaluated using agarose gel electrophoresis with 1.5% agarose that had been treated with ethidium bromide 0.5 µg/ml for 1 hr (16). A DNA ladder (bp 100–1500) was used to assess the PCR results; the gel was visualized under UV light at 336 nm, then photographed digitally.

Results and Discussion

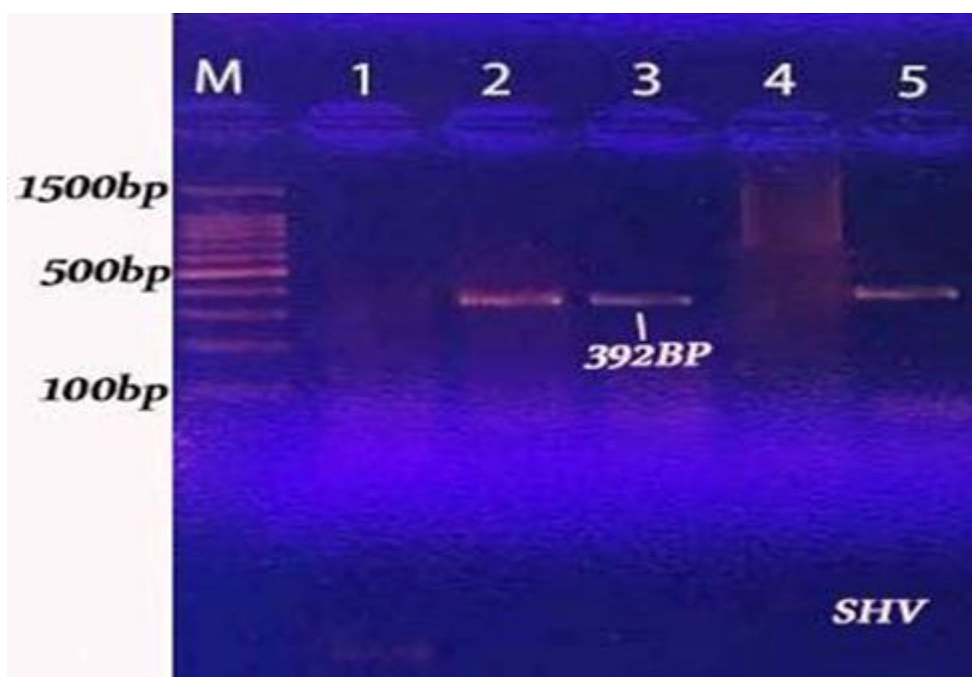
The rapid and widespread dissemination of S aureus throughout hospitals and other health care institutions constitutes a significant concern. All 40 tested samples revealed the same biochemical characteristics which identified them as S aureus and further confirmed through microscopic examination (5) to confirm the identification of these organisms. Further, an antibiotic sensitivity test was conducted as seen in Fig 1

Figure 1: Antibiotic sensitivity results

The finding supports some other research that have shown that Fluoroquinolone drugs, such as Norfloxacin are very active against Staphylococcus aureus as they inhibit DNA Gyrase and Topoisomerase IV, and therefore cause inhibition of the bacterial replication (6). Amoxicillin (30 µg) inhibited S. aureus isolates S5

It also corresponds to prior reports which indicated that the main reason why Staphylococcus aureus develop resistance to β-Lactams is due to the enzyme β-Lactamase and the presence of the SHV gene, which encodes an altered Penicillin-Binding Protein PBP2a (7).Therefore the sensitive response obtained with Ampicillin (25 µg) in the present investigation agrees with prior investigations indicating that S. aureus has been found to be resistant to Ampicillin because it produces β-Lactamase, which causes decreased susceptibility (8).

The results of the Polymerase Chain Reaction (PCR) test which were carried out to detect the SHV gene in the bacterial isolates. Lane M represents the DNA molecular weight marker (DNA ladder), with fragment sizes of 100 bp to 1500 bp, which was taken as reference for the estimation of the size of the amplified PCR products (9).



Distinct bands on the gel in lanes 2, 3, and 5 were seen at approximately 392 base pair sizes and represented the expected size of the amplified SHV gene. This indicates that the SHV gene was present in those isolates. Bands were absent in lanes 1 and 4, where it could either be that there is no target gene or that the amount of target gene was too low for the product to be detectable.A clear band representing the target gene without any nonspecific products was evidence that the primer used to amplify the target gene are very specific and that the PCR conditions were optimal and that the extracted DNA was of good quality (10). The distinct band pattern found among the different isolates also supports that the isolates have been shown to vary genetically regarding the carriage of resistance genes (11).These findings indicate that a number of Staphylococcus aureus isolates collected from tonsil swabs possess the SHV gene, which has been linked to resistance to β-lactam antibiotics (12) and may account for some therapeutic failures. Therefore, using PCR as a molecular detection tool to identify antibiotic resistant genes provides a fast and accurate way to select the best treatment options and develop effective infection control strategies (13).

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