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Detection of *Staphylococcus aureus* in Sinusitis Samples by Molecular Technique

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***Staphylococcus aureus* (*S. aureus*) is among the most virulent bacterium, causing a wide spectrum of diseases, one of which is sinusitis. Sinusitis is defined as inflammation of the paranasal sinuses and can occur with an *S. aureus* infection. Since traditional diagnosis of sinusitis, such as bacterial culture, can be very time-consuming, there is an urgent need to implement a rapid and reliable method to detect *S. aureus* sinusitis. In this study, 55 sinusitis samples were provided from operating rooms in two ways: puncture and endoscopic surgery. The application of Polymerase Chain Reaction (PCR) in the detection of *S. aureus* in sinusitis samples was evaluated. The PCR test was performed with the *S. aureus nuc* gene, and produced a 279 bp DNA band, using 1.5% agarose gel electrophoresis. Finally, according to PCR analysis, 18 out of the 55 studied specimens (32.7%) from patients with sinusitis were positive for *S. aureus*. These results indicate that the use of the PCR method is a highly specific, sensitive, and reliable tool for the rapid detection of *S. aureus*-derived sinusitis. The results of this report will aid physicians in the appropriation and prescribing of antibiotics.**

Key words: *Staphylococcus aureus*, sinusitis, PCR, detection.

S. aureus is an omnipresent bacterium and the most virulent of the staphylococcal species in the micrococcaceae family. Its versatility has qualified it as the leading cause of morbidity and mortality, despite the potency of anti-staphylococcal antibiotics[1]. The primary habitat of *S. aureus* is in warm-blooded animals, including the nasal membrane and skin of humans[2], and it can cause a wide range of diseases, such as nosocomial and community-based infections, either by toxin-mediated or non-toxin-mediated mechanisms[3]. *S. aureus* is consistently found in cultures from patients with sinusitis[4]. Sinusitis is inflammation of the paranasul sinuses caused by bacterial or viral infections, allergies, and/or autoimmune diseases. There are two major types of sinusitis: acute sinusitis and chronic sinusitis. Sinusitis causes pain in different parts of head and face depending on the location of the infection, i.e., maxillary, frontal, ethmoidal, or the sphenoidal sinus cavity. Sinusitis is considered to be a

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common condition, with 24–31 million cases occurring in the United States annually[5, 6]. Given the high frequency of patients who suffer from sinusitis, a rapid and efficient diagnosis with a highly developed technique is necessary in managing the infected sinuses.

Culture-based testing is the conventional method used in the diagnosis of bacterial infections. The diagnostic results, which rely on these cultivations, are usually time-consuming. In addition, due to precarious laboratory conditions, scientists occasionally fail to cultivate the bacteria. Molecular methods based on nucleic acid amplification can be used to overcome these problems and reach more rapid and sensitive detection[5]. On the contrary, the effectiveness and sensitivity of PCR has warranted it an attractive tool in diagnostic laboratories and has subsequently led to the development of a quick diagnosis of the infection. PCR-based methods enable the direct detection of low concentrations of bacteria or bacterial products in clinical materials[7, 8].

One of the most important pathogenic factors of *S. aureus* is its thermo-stable nuclease. There are two open reading frames (ORFs) in *S. aureus* genomes that have been predicted to encode nucleases; one of these, termed *nuc*, produces an extracellular thermo-nuclease (TNase) and has a molecular weight of 17KDa[1,7]. The *nuc* gene express as an endonuclease, degrades both DNA and RNA of the host by hydrolyzing phosphodiester bonds[6]. The enzymatic activity of TNase can endure temperatures of 100ºC for up to one hour[9]. The identification of *S. aureus* isolates are conducted in different laboratories by enzymatic tests for TNase production[8]. However, streptococci, among some bacteria, can produce nucleases whose enzymatic activities are similar to the *S. aureus* TNase[7]. Conversely, earlier studies have demonstrated that *S. aureus* TNase has species-specific sequences[10] and amplifying this unique sequence of the thermo-nuclease gene can provide a rapid and sensitive method for the diagnosis of *S. aureus*. In this research, the PCR method was implemented, by which the *S. aureus nuc* gene was amplified, ensuring a rapid, specific, and sensitive method for the diagnosis of *S. aureus* in sinusitis samples; this method and its effectiveness was subsequently evaluated.

MATERIALS AND METHODS

Samples and DNA extraction

55 specimens were collected from the secretion of the maxillary, frontal, and sphenoidal sinuses at Rasuol Akram hospital (Tehran-Iran), using the endoscopic method. Genomic DNA was extracted using the DNG-plus TM DNA extraction kit (Cinagen, Iran). For DNA extraction, a 100 µl sample was mixed with 400 µl of DNG solution and was then vortexed (for 20 seconds). 350 µl isopropanol was added and mixed by gently inverting the tube (10 times). It was then centrifuged at room temperature (12000 rpm/5 min). Supernatant was subsequently and carefully removed; 1 ml 75% ethanol was added to it and mixed by inverting the tube (10 times) and centrifuged (12,000 rpm/5 min). The supernatant was removed again. The ethanol was completely poured off, and the pellet was dried (65 °C for 5 min). The DNA pellet was then dissolved in 100 µl of double distilled water (DDW), or in Tris 10 mM (pH = 8) for long time storage at -20°C.
The genomic DNA from *S. aureus* ATCC 25923, as a standard strain and control positive, was extracted using the DNG-plus DNA extraction kit (Cinagen, Iran). The lyophilized *S. aureus* ATCC 25923 was resolved in 5 ml nutrient broth and incubated at 37°C for 24 hours. The cultures of *S. aureus* ATCC 25923, present at the late-logarithmic phase, were chosen for DNA extraction.

Primers and PCR

To evaluate the presence or absence of the selected gene, *nuc*, all PCR tests were conducted in a thermal cycler using the species-specific primers set. The amplification conditions and primers for the *nuc* gene are shown in Table 1. The PCR mixture included 14 µl DDW, 2.5 µl buffer (10X), 0.75 µl MgCl2, 0.5 µl dNTP, 1 µl of each primer (forward and reverse), 0.3 µl Taq DNA polymerase enzyme, and 5 µl template DNA. Amplified DNA segments were analyzed using 1.5% agarose gel electrophoresis containing CYBER Green dye and were observed using UV light and a trans-illuminator machine.

Sensitivity and specificity test for detection

For testing the sensitivity of the PCR reaction, the extracted DNA concentration of *S. aureus* was obtained using a spectrophotometer in OD=260 nm. Genomic DNA copy numbers were determined based on the Genome Copy Number

(GCN) formula and the genome size of *S. aureus*. Serial dilution was then provided from the template (having known the genome number) by using the Kokh method and PCR analysis was performed on each dilution. In addition, the specificity of the PCR reaction was assessed by analyzing the obtained DNA from *Haemophilus influenza*, *Escherichia coli*, *Mycoplasma pneumonia*, Herpes Simplex Virus, *Pseudomonas aeruginosa*, *Brucella abortus*, *Legionella pneumophila*, and *S. aureus*.

RESULTS AND DISCUSSION

Results of PCR test on sinusitis specimens

After DNA extraction of 55 specimens, collected from pus and biopsy samples from patients with sinusitis, the PCR test was performed and PCR products were analyzed using 1.5% agarose gel electrophoresis. A total of 18 out of 55 specimens (32.7%) produced a 279 bp band related to the *S. aureus nuc* gene (as shown in Figure 1).

Result of sensitivity of the PCR test

Assessing the serial dilution of DNA extracted from *S. aureus* demonstrated that amplification of the *nuc* gene can be performed using only 10 copy numbers of DNA using PCR. However, no band was observed after the electrophoresis of PCR products obtained from dilutions with less than 10 copies of *S. aureus* DNA. These results confirm the acute sensitivity of the PCR test (as shown in Figure 2).

Result of specificity of the PCR test

Specificity of the primers directed to the *nuc* gene were evaluated on different organisms, including Escherichia coli, *Mycoplasma pneumonia*, Herpes Simplex Virus, Haemophilus influenza, *Pseudomonas aeruginosa*, *Legionella pneumophila*, and *Brucella abortus*. The results of these assessments (as shown in Figure 3) demonstrated that the S. aureus specific sequence was recognized and amplified by the utilized primer set, but none of the other microorganisms were identified and amplified by the primers. Therefore, because only the *S. aureus nuc* gene was amplified in this evaluation, primer specificity was shown to be 100%.

Table 1. The Primers and Condition of PCR Analysis

Gene	Primers Sequence(52 → 32)	Conditions			
		Denaturation	Annealing	Extension	Cycle(No)
nuc	F:5' GCGATTGATGGTGATACGGTT3' R:5' AGCCAAGCCTTGACGAACTAAAGC3'	94°C- 30s	58°C- 30s	72°C- 40s	35

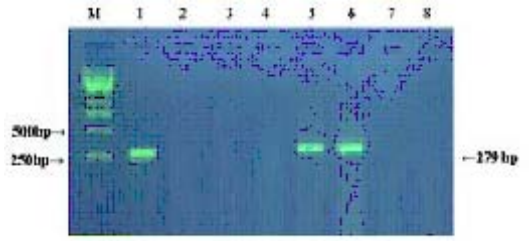

Fig. 1. Gel electrophoresis of PCR products generated by direct testing of sinus specimens. M, Marker: 1 Kb DNA ladder; lane 1: positive control (279 bp) ; lane 2 to 4 and 7, specimens which were not infected with *S. aureus*; lane 5 and 6, specimens infected with *S. aureus*; lane 8, negative control.

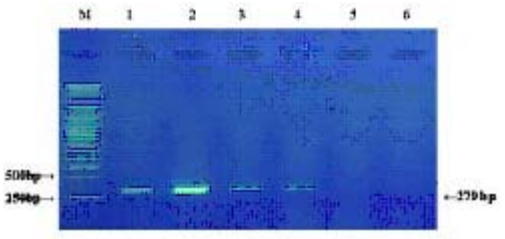

Fig. 2. Gel electrophoresis of PCR products amplified from serial dilutions of *S. aureus* indicating sensitivity of PCR test. M, Marker: 1Kb DNA ladder (Ferments, Germany); lane 1- 6, serial dilution of *S. aureus DNA:* lane1, positive control; lane 2, 10^{-1} dilution (10^{3} CFU); lane 3, 10^{-2} dilution (10^{2} CFU); lane 4: 10^{-3} dilution (10CFU); lane 5: 10^{-4} dilution (1 CFU); lane 6: negative control.

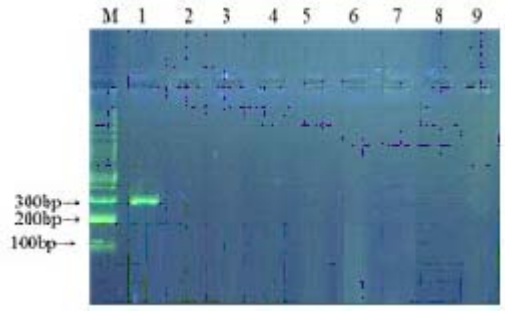

Fig. 3. Specificity of PCR test. M, Marker: 100bp DNA ladder (Ferments, Germany); lane 1, positive control; lane 2, *Haemophilus influenza* DNA; lane 3: *Herpes Simplex Virus* DNA; lane 4: *Mycoplasma pneumonia* DNA; lane 5: *Pseudomonas aeruginosa* DNA; lane 6: *Legionella pneumophila* DNA; lane 7: *Escherichia coli* DNA; lane 8*: Brucella abortus* DNA; lane 9: negative control.

DISCUSSION

Classical methods, such as bacterial culture and metabolic tests are widely used for bacterial identification in laboratories and hospitals[11]. However, these procedures are not ideal given that they can take a long time. Furthermore, despite recent advancements, some strains of bacteria experience difficulty growing in culture media[12]. As a result, several ultra-sensitive detection techniques have been developed to address these deficiencies[11]. Among these highly sensitive techniques, there are efficient methods based on nucleic acid amplification, such as the PCR[13], strand displacement amplification (SDA)[14], and ligase chain reaction (LCR)[15].

Additional studies have shown that PCR is a sensitive, rapid, and reliable test[13, 16], and consequently it has been used extensively in the detection of different pathogenic and non-pathogenic microorganisms, such as viruses, bacteria, fungi, etc[17-19]. The primary aim of this study was to implement and analyze the PCR method as a dependable technique for detecting *S. aureus* in patients with sinusitis. To achieve this aim, a suitable primer set was used to identify species-specific sequences of the *S. aureus nuc* gene encoding TNase. The *nuc* gene was amplified by primers and the amplification conditions experienced in sinus samples as well as *S. aureus* ATCC 25923, as the standard strain and control positive.

The sensitivity results of the *nuc* PCR test produced a 10 colony-forming unit (CFU) that was able to detect low levels (0.1pg) of extracted DNA. This sensitivity matches auxiliary PCR results demonstrated with tested bacteria, being between 1 and 20 CFU[20, 21] and is ten-fold more effective than that described for PCR with *S. aureus*, between 1 and 100 pg DNA in other studies[22, 23].

The specificity of the utilized primer was assessed by performing PCR on extracted DNA from different microorganisms and the designed primer set demonstrated 100% specificity for the *S. aureus nuc* gene. Current findings are supported by Brakstad and Maeland[7], who confirmed the use of the PCR test for the reliable detection of *S. aureus* infections[7]. Atanassova et al. also reported that the specificity of PCR method in detection of *S. aureus* was more accurate in comparison to traditional methods[17, 19]. In addition, ancillary evidence has proved that PCR-based tests are more specific and sensitive than conventional methods in detecting a wide range of bacteria and microorganisms[18].

The frequency of detection of *S. aureus* in sinusitis patients based on conventional methods varies and a wide range studies have reported this amount to be between 3% - 20%[24-26]. According to this study, a total of 32.7% of collected samples produced a 279 bp band related to the *S. aureus nuc* gene.

In conclusion, the results of this study clearly demonstrate that the PCR technique based on species-specific sequences in the *S. aureus nuc* gene is a valuable method with acute sensitivity, specificity, and reliability for detecting *S. aureus* in patients with sinusitis. This technique, because of its rapidity and accuracy, is a viable substitution for traditional tests, including culture, serological, and biochemical. Furthermore, *S. aureus* is an important agent of sinusitis that participates in both acute and chronic sinusitis and can be effectively detected by PCR.

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