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Original article

A simple and rapid molecular detection of *Staphylococcus aureus* strain B using multiplex PCR

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Background: *Staphylococcus aureus* (*S. aureus*) is one of the most important and pathogenic species from the *Staphylococcaceae* family. *S. aureus* is capable of producing several enterotoxins (SEs) that are classified in different groups from A-R.

Objective: The present study aimed to set up simple and rapid detection of *S. aureus* strain B to develop a multiplex polymerase chain reaction (PCR) assay for the identification of genus and strain B of this bacterium.

Methods: In this work, after designing specific primers for 23S rRNA and SEB, one step multiplex PCR was carried out and the PCR product analyzed by agarose gel electrophoresis. The specificity of this method was determined using different strains of *S. aureus* A (SEA), *S. aureus* C (SEC), *Bacillus subtilis*, *Bacillus cereus*, and *Listeria monocytogenes*. Also, the sensitivity was distinguished by serial dilution of the DNA genome from *S. aureus*.

Results: The PCR product was indicated at 226 and 1,250 bp specific amplified fragments according to amplification of SEB and 23SrRNA, respectively. The sensitivity of this method determined about 2×10^3 CFU/ml and also the results showed that according to specific primers, the PCR is very specific.

Conclusion: This multiplex PCR assay can be used as a simple diagnostic test in clinical laboratories for identification of *S. aureus*.

Keywords: *Staphylococcus*, detection, multiplex PCR, sensitivity.

Staphylococcus aureus (*S. aureus*) is one of the most important and pathogenic species of the *Staphylococcaceae* family. These bacteria are found in the normal microflora in various parts of human and animal bodies.⁽¹⁾ This bacterium in special conditions, such as viral diseases, tissue injury, and any agent which causes weakness in the immune system, can cause different diseases such as dermatological infection, food poisoning, and toxic shock syndrome (TSST1).⁽²⁾ Some strains of the *Staphylococcus* genus cause food corruption. Foods are the appropriate environment for *Staphylococcal* contamination and bacterial growth.⁽³⁾ This bacterium exist in the nasal cavity of adults, therefore, people who work in the centers of supply and distribution of food, can transfer bacterium to food.⁽⁴⁾ This microorganism invades the host by secretion of various

toxins such as pyretic exotoxin, enterotoxins, catalytic toxins. Enterotoxins have more accentuate of other produced toxins by *S. aureus*.^(5, 6) *S. aureus* enterotoxins (SEs) are classified into different groups from A-R. These enterotoxins are similar in structure and biological activity but different in antigenic characteristics.⁽⁷⁾ These SEs are resistant to heat so that their thermal resistance in food is higher than their thermal resistance in culture plates. Furthermore, the biological activity of these toxins even after the heating stages of food preparation can remain.⁽⁸⁾ The existence of enterotoxin at a very low level from 20 ng to 1 μ g can cause symptoms of poisoning if there were 10^5 bacteria per gram of food, the bacteria can produce enterotoxin. Because of the thermal resistance of enterotoxin, even if bacteria were degraded by the heat, the toxin remains active and causes food poisoning that leads to an epidemic in the public life centers.⁽⁹⁾ *S. aureus* is a major problem for public health and millions of people suffer from *S. aureus* infections each year resulting in considerable healthcare costs. Food poisoning caused by this organism, leads to significant economic losses.⁽¹⁰⁾ The presence of enterotoxins in dairy products such as

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milk, cheese, and raw meat are associated with food poisoning.⁽¹¹⁾ Enterotoxin of the A-E groups cause food poisoning and A and D groups are the most important. Enterotoxin B (SEB) not only causes food poisoning, but is important due to its absorption through inhalation.⁽¹²⁾ The most common symptoms of food intake of SEs are nausea, diarrhea, vomiting and cramping of the abdominal muscles that occur within 2 - 6 hours after eating contaminated food.^(8, 12 - 14) Today, several methods are used for the identification of SEs from *S. aureus*. Although culture based and immunological methods such as enzyme-linked immunosorbent assay (ELISA), agglutination, and radioimmunoassay are very sensitive, they can detect up to 1 pico gram of SEB⁽¹⁵⁾, they are time consuming and laborious. In this study, molecular detection methods such as polymerase chain reaction (PCR) and multiplex PCR are appropriate alternative techniques for *in vitro* amplification of SEs genes. These techniques do not need any antigen and can even be detected in the presence of low levels of toxin genes.⁽¹⁶⁾ The aim of this study was to set up of a simple and rapid diagnostic method for detection of *S. aureus* at genus and strain levels according to multiplex PCR and amplification of 23s rRNA and enterotoxin B genes.

Materials and methods

DNA extraction

Extraction of DNA was carried out using a sonication technique. A 24-h culture of *S. aureus* in TSB medium (Tryptic Soy Broth, a general culture broth to grow aerobic bacteria) at 37 °C, cell biomass collected using centrifuge at 7,000 rpm for 5 minutes. The precipitate was resuspended in 2.5 ml of PBS buffer, and finally the bacterium genome was extracted by repeated sonication.

Primer design

According to the sequence of *S. aureus* strain KLT6 staphylococcal enterotoxin B variant v1 (SEB) gene, complete coding sequence (Accession No. KX168628.1), specific primers (Table 1) for SEB and 23S rRNA were designed using the Gene Runner software.

Gene amplification

After the primers were synthesized, in order to confirm any of the pairs of SEB and 23S rRNA

primers, multiplex PCR reaction performed in a volume of 5 µL as follows: 57ng (1 µl) DNA template, 1 µM (0.25 µl) from each primer, and 3.5 µl master mix of Taq DNA polymerase (amplicon PCR kit, Denmark). The PCR was carried out according to the program below: denaturation at 94°C for 4 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Also, multiplex PCR was carried similarly to multiplex PCR with F-23, R-23, F-SEB and R-SEB at one step/tube. Finally, PCR products were analyzed using 1% agarose gel electrophoresis.

Sensitivity and specificity

For comparison of the sensitivity of this method, serial dilutions of *Staphylococcal* genomic DNA from 2×10^8 CFU/ml to 2×10^{-2} CFU/ml were prepared and utilized as a template for the PCR assay. On the other hand, the specificity of method was determined according to a population of different bacteria containing *S. aureus* A (SEA), *S. aureus* C (SEC), *Bacillus subtilis*, *Bacillus cereus*, and *Listeria monocytogenes*. The results were analyzed by 1% agarose gel electrophoresis.

Results

After the primers were designed, we detected 23S rRNA and the SEB gene of *S. aureus* individually according to multiplex PCR (Figure 1A). The presence of 226 bp and 1,250 bp segments in agarose gel suggests SEB and 23S rRNA genes in the sample, respectively (Figure 1B). In order to determine the sensitivity of the method, we used the serial dilution of *S. aureus* cell cultures (2×10^8 to 2×10^{-2} CFU/ml). PCR product analysis, showed that amplification of 226 bp and 1,250 bp segments were performed until 2×10^3 CFU/ml (Figure 2A). Also, when we used different strains of bacteria such as SEA, SEB, SEC, *Bacillus subtilis*, *Bacillus cereus*, and *Listeria monocytogenes*, the results showed that the 1,250 bp segment was only detected in two types of *S. aureus* which contain A enterotoxin or C enterotoxin. Both fragments (226 and 1,250 bp) were observed in *S. aureus* that contained B enterotoxin. But in the case of other samples, there were no bands, which reveals the specificity of the designed primers (Figure 2B).

Table 1. Primer sequences used in monoplex and multiplex PCR.

Primer name	Target gene	Sequence	PCR product size (bp)
F-23	23S rRNA	ACGGAGTTACAAAGGACGAC	1,250
R-23		AGCTCAGCCTTAACGAGTAC	
F-SEB	SEB	GTTCGGGTATTTGAAGATGG	226
R-SEB		CAAATTTATCTCTCTGGTGCA	

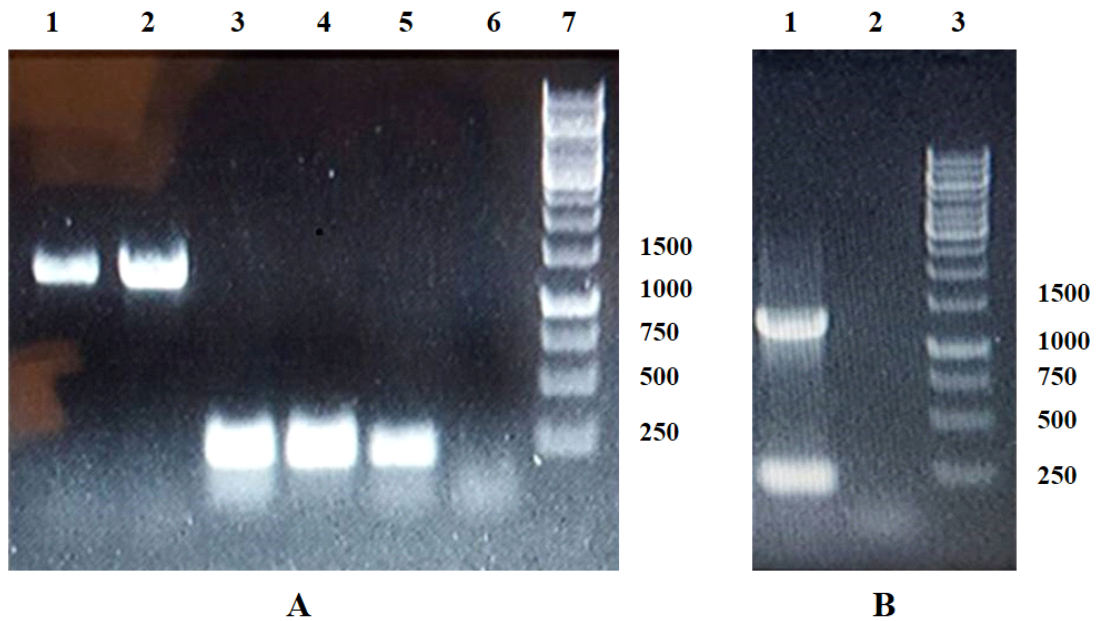


Figure 1. Agarose gel electrophoresis of monoplex (A) and multiplex (B) PCR product using 1% agarose gel electrophoresis. Lane 1A, 2A: 23S rRNA gene (1250 bp), Lane 3-5A: SEB Gene (226bp), Lane 6A: negative control, and Lane 7A: 1 Kb DNA ladder. Lane 1B: 23S rRNA (1250 bp) and SEB gene (226bp), Lane 2B: negative control, and Lane 3B: 1 Kb DNA ladder.

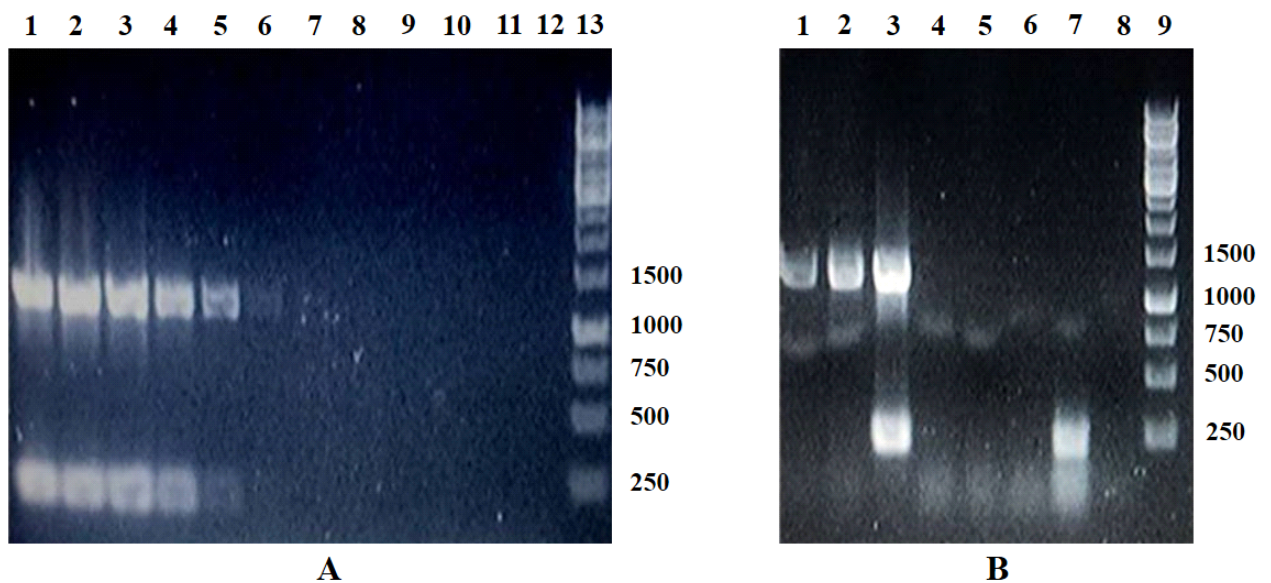


Figure 2. Gel electrophoresis of PCR products of different bacteria. (A) The sensitivity of multiplex PCR was determined by DNA serial dilutions 2×10^8 (1), 2×10^7 (2), 2×10^6 (3), 2×10^5 (4), 2×10^4 (5), 2×10^3 (6), 2×10^2 (7), 2×10^1 (8), 2 (9), 2×10^{-1} (10), 2×10^{-2} (11) CFU/ml. Lane 12 is negative control and lane 13, 1Kb DNA ladder. (B) The specificity of multiplex PCR was determined using SEA (1), SEC (2), SEB (3), *Bacillus subtilis* (4), *Bacillus cereus* (5), *Listeria monocytogenes* (6), positive control, monoplex PCR (7) negative control (8) and 1Kb DNA ladder (9).

Discussion

S. aureus is an important human pathogen that can produce various proteins and cause a wide range of diseases. Leading from the resistance of human strains to antibiotics, rapid diagnosis of the pathogen is vital. Enterotoxin is one of the proteins which causes food poisoning in human. According to reports, 14 - 40 % of foodborne diseases are attributed to this bacterium.^(4, 16) There are various immunological procedures to detect *Staphylococcal* enterotoxins, including enzyme linked immunosorbent assay and radioimmunoassay. But these methods have disadvantages and it may interfere with other toxins.⁽¹⁷⁾ In addition, ELISA commercial kits are time-consuming and expensive.⁽¹⁴⁾ Hence, molecular detection methods, such as PCR, multiplex PCR and loop-mediated isothermal amplification can be a better replacement. In PCR detection, it is possible to identify a gene that produces the toxin before producing the toxin in a bacterium which may be very effective in preventing many diseases. In multiplex PCR, in addition to the high speed and high sensitivity of the reaction, several genes can be detected in a single reaction simultaneously. In a previous investigation, Yang H, *et al.* used the PCR technique to detect *S. aureus* in foods with a sensitivity of 1.25×10^2 CFU/ml.⁽¹⁸⁾ Imani Fouladi A, *et al.* have investigated the presence of enterotoxin B and A in local dairy products by the PCR method, but did not report the numerical sensitivity, and a review of the results showed that only 1.3 % of the samples had the SEB gene.⁽¹⁹⁾ Bokaeian M, *et al.* detected SEB, and SEA with the PCR technique in clinical specimens. The specificity of the designed primers was tested, but no information for sensitivity was reported.⁽²⁰⁾ Sheikhi F, *et al.* have evaluated the SEB gene with PCR technology and a sensitivity of 200 CFU/ml has been reported.⁽²¹⁾ Sowmya N, *et al.* compared PCR and loop-mediated isothermal amplification (LAMP) methods for *S. aureus* detection and a sensitivity of the PCR was reported to be about 10^4 CFU/ml.⁽²²⁾ Ahari H, *et al.* have worked on the 23S rRNA gene. Contaminated milk samples with SEA, SEB and SEC enterotoxin were analyzed by the multiplex PCR technique. In this work, the results showed 5 of 60 samples were positive for all three toxins. Also, for the 23S rRNA gene, which is a protected gene in strains of *Staphylococcus*, a sensitivity test was performed and 103 standard *S. aureus* cells were reported.⁽³⁾ Ahani N, *et al.* examined the SEB and

SEA genes using PCR in 35 fish samples, and the results were as follows: 14.3% of the samples had the SEA gene, 8.5% had the SEB gene, and 5.7% of them had both genes.⁽²³⁾ Najera-Sanchez G, *et al.* used the multiplex PCR technique to study SEA-SEE genes in broiler and dairy products, and in all samples the lower limit of detection for the *S. aureus* cells was 10 CFU and the lower limit of detection for the isolation target was 0.5 μ g of DNA.⁽²⁴⁾ Nagaraj S, *et al.* have evaluated SEA, SEB, SEC, SEI, SEG and coa genes with multiplex PCR technology and a sensitivity of 10^6 CFU/ml has been reported.⁽²⁵⁾

In the present study, we designed two pairs of new primers for the detection of 23S rRNA and SEB genes using a multiplex PCR technique. We examined the susceptibility and specificity of this method. The results showed that the sensitivity of this method is about 2×10^3 CFU/ml and the designed primers are highly specific. Additionally, as the bacteria can produce enterotoxin in when there are 10^5 bacteria per gram in food, the sensitivity of this method is suitable for *S. aureus* identification.

Conclusion

This simple and rapid detection method could be used for the design and development of a molecular detection kits in order to identify *S. aureus*.

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Conflict of interest

The authors, hereby, declare no conflict of interest.

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