Comparing direct PCR and PCR with DNA extraction kits in identifying 
TST and mecA in Staphylococcus aureus

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Abstract

Introduction: Polymerase Chain Reaction (PCR) is an accurate and sensitive test in molecular assays. DNA extraction has always been a time-consuming stage before conducting PCR and can be eliminated to significantly save time and costs. The present study was conducted to compare direct PCR and the PCR with template DNA extraction in detecting TST, femA and mecA.

Materials and Methods: In the present study, clinical isolates of Staphylococcus aureus (S. aureus) which were biochemically confirmed were assessed using direct PCR of colonies of S. aureus cultured on Muller Hinton agar and PCR with DNA extraction kits for TST and mecA. Three specific primers were used for these genes and the sequencing of all the samples was considered the gold standard.

Results: The 326-bp TST and 163-bp mecA were successfully amplified using direct PCR and PCR with DNA extraction, which was associated with toxins and resistance to methicillin. All the samples were found to be femA-positive in both methods. In direct PCR, 68 (87.17%) isolates were found to be mecA-positive and 9 (11.53%) TST-positive. In PCR with DNA extraction kits, 69 (88.46%) isolates were found to be mecA-positive and 12 (15.38%) TST-positive.

Conclusions: According to the results obtained, direct PCR is recommended to be used as a time and cost effective method of identifying S. aureus.

Key words: Staphylococcus Aurous, Methicillin-Resistant, Direct PCR, TST, mecA

Introduction

PCR is a sensitive and accurate molecular test that can be used for detecting different genes in specific regions of the DNA molecule (1 and 2). Kary Mullis developed the idea of using PCR for amplifying DNA in April 1983 and ultimately received the Noble Prize in 1993 for introducing this technique (3). PCR can be used to artificially amplify single-stranded DNA molecules (4). In this reaction, template DNA strands are first separated at 95 °C. Elongation and ultimately cloning are performed using specific fragments of primer attached to the strands at a certain temperature.
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 temperature (5). PCR encompasses three steps, including separation, attachment and elongation using different number of cycles, temperatures and durations (5). DNA polymerase is the enzyme used in cloning and thermophilus aquaticus isolated from hot springs is used to facilitate cloning and maintain the power of DNA polymerase at different temperatures (6). Themophilus aquaticus was used to make Taq polymerase in 1989 by Gelfand et al. and in 1990 by Mullis et al. in PCR (6). Bain maries were originally used in PCR for definite durations to induce the desired temperatures at different stages of separation, attachment and elongation, which was both time-consuming and error-prone (7). With the advancement of science, this conventional method was supplanted by modern, cost-effective, rapid and highly-accurate PCR equipment (8), which helped with the bacterial detection and the molecular detection of encoding genes of different factors (9). The gene causing resistance to methicillin (mecA) and TST can be easily detected in S. aureus using PCR (10). The main mechanism of resistance to methicillin in S. aureus is associated with producing a protein, namely PBP2, which has little affinity to bind to β-lactamases and causes resistance to these enzymes. mecA encodes this protein and ultimately creates methicillin-resistant S. aureus (11). mecA is located on a mobile genetic element, namely staphylococcal cassette chromosome mec (11). Given that a substantial number of hospital staff carry this bacterium in their skin and nose, they can easily transmit it to others and cause the circulation of risky strains producing toxic shock syndrome toxin-1 (TSST-1) (12). Moreover, the horizontal transfer of TST caused by mobile genetic elements along with the circulation of antibiotic resistance among S. aureus strains can cause numerous treatment problems and increase pathogenicity (13). Despite its accuracy, PCR with DNA extraction kits may impose excessive costs on researchers and retard processes such as DNA preparation and extraction, which is normally carried out using boiling, phenol chloroform and extraction kits (13). The quality of the extracted DNA is crucial in achieving the highest cloning ratio in PCR and obtaining the best fragment (14). Different DNA extraction methods have thus been proposed, including boiling, phenol chloroform, ethanol precipitation and use of detergents or extraction kits (15). All these methods are time-consuming, too costly and very problematic (13). Phenol–chloroform is too slow and unsafe, due to using a dangerous toxin that causes problems in long-term (15). Ethanol precipitation may also cause errors and even the loss of the final product owing to the transfer of ethanol in final stages (14). Boiling also causes relatively lower quality of the extracted DNA owing to being time-consuming (16). In addition, bacterial genes can be successfully amplified by inoculating newly-cultured colonies in the final mixture without extracting DNA. The speed of PCR can be increased by eliminating the stage of DNA extraction when studying a very large number of samples, and the time saved can be used for more detections (16). Multiplex PCR is recommended for simultaneously detecting several genes. This method simultaneously uses several pairs of specific primers for different targets. The advantages of this method include time saving and simultaneously studying several genes using the lowest amount of ingredients.
Direct PCR and harvesting bacterial colonies can be used to eliminate DNA extraction in some bacteria such as *S. aureus*. The present study was conducted to investigate the amplification results of *femA*, TST and *mecA* in *S. aureus* using direct multiplex PCR and PCR with DNA extraction kits, and to save time and reduce side costs in case of obtaining positive cases.

**Materials and Methods**

**Separation and isolation of bacteria**

In the present comparative study, samples of blood, urine, sputum, saliva, trachea, wound, discharge, abscess, etc. (the criterion for the samples being bacterial) were collected from inpatients in health centers of Zahedan, Iran, over six months and transferred to the Microbiology Laboratory of Zahedan University of Medical Sciences using the transport medium of BHI (Merck, Germany). These samples were kept in a fridge at -20 °C by the time of the initial culture on blood agar (Merck, Germany) using 5% of sheep blood, when gram-positive cocci were isolated using gram staining. The colonies obtained were screened using biochemical tests including coagulase, catalase, Manithol Salt Agar (Merk, Germany), DNAs (Sigmaalderich, USA) and *S. aureus* samples were isolated. The colonies confirmed were used in the following steps.

**DNA extraction**

Genomic DNA was extracted as follows: The clinical isolates stored at -20 °C were cultured on Blood Agar (Merck, Germany) and incubated at 37 °C for 24 hours. One colony of every cultured isolate was then inoculated on 5 ml of LB Broth (Merck, Germany) and incubated at 37 °C for 20 hours. The culture medium had already been poured into capped glass tubes with the same number as the isolates. These tubes were then removed from the incubator. A total of 1.5 ml of the culture medium obtained was poured into capped 1.5-ml plastic microtubes and extraction kits (CinnaGen, Cat.No. PR881614) were used as per the manufacturer’s protocol to extract genomic DNA. To confirm the quality of the extracted DNAs, they were diluted and their OD was measured. Moreover, 5 lambda of the DNA products was loaded on 1% agarose gel after being combined with loading dye (Thermo Co., US). The extracted DNAs were kept at -20 °C before conducting PCR.

**Primer preparation and PCR using the extracted DNA**

Table 1 shows the primers used to identify *femA*, *mecA* and TST. To obtain an initial dilution of 100 pM, lyophilized distilled water was added to all these primers as per recommendations of the manufacturer, i.e. Zist Fanavaran Co. Each primer was then diluted to 15 pM to be used in PCR with DNA extraction kits (Table 1).

<table>
<thead>
<tr>
<th>Gene Size (bp)</th>
<th>Primer Sequence</th>
<th>Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>132</td>
<td>AAAAAAGCACATAACAAGCG GATAAAGAAGAAACCAGCAG</td>
<td>femA</td>
<td>Manisha Mehrotra et al. (16)</td>
</tr>
<tr>
<td>163</td>
<td>ACTGCTATCCACCCTCAAAC CTGGTGAAGTTGTAATCTGG</td>
<td>mecA</td>
<td>Manisha Mehrotra et al. (16)</td>
</tr>
<tr>
<td>326</td>
<td>AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GATTGG</td>
<td>TST</td>
<td>Manisha Mehrotra et al. (16)</td>
</tr>
</tbody>
</table>
To conduct multiplex PCR, 50 μl of the final solution including 1 μl of the template DNA, 1 μl of each primer at a 10 pM concentration and 12.5 μl of 2x Master Mix and 1.5 mM MgCl2 (Ampliqon, Germany) containing 0.2% Tween20, 3 mM MgCl2, (NH4) SO4, Tris-HCl PH8.5, MmdNTP 4.4, 0.2 unit of Ampliqon polymerase, Insert red dye and stabilizer were used. Deionized distilled water was used to achieve the final volume. The PCR mix lacking the template DNA was used as the negative control (14). The Multiplex PCR performed for femA, mecA and TST using a thermocycler (BioRad, US) included the following steps: initial denaturation at 95 °C for 5 min, 35 denaturation cycles at 94 °C for 2 min, primer bonding at 57 °C for 2 min and the amplification of the target fragment at 72 °C for 1 min. The PCR products were kept in a fridge at +4 °C before using electrophoresis.

Direct PCR using colonies

Given the lack of an approved and definite protocol, efforts were made to obtain the best result by trial and error. A proper initial dilution was first prepared and the primer concentration was raised to 25 pM. To perform direct PCR and avoid the template DNA and DNA extraction, a colony of the newly-cultured bacteria was removed by foldipatin and inoculated in the mix. To minimize potential errors, Muller Hinton Agar (Merck, Germany) was used to isolate the bacteria and remove a single colony. Furthermore, the final mix was vortexed for 10 seconds to better dissolve the colony. To prepare the final mix, the final volume was set at 50 μl using the previously mentioned method and 3 μl of each primer was added rather than the formerly used 1 μl, and the same thermal cycle was used to perform the reaction.

Electrophoresis on 3% agarose gel

Electrophoresis was conducted using 5 μl of the PCR solution and 3% agarose gel. Five μl of the Gel Red solution (Biotium, US) was added to the gel. The Gel Documentation System CCD (Tab1 model, KIAGEN, Iran) was used to examine and take images of the final result. A 100-bp fermentas marker (Thermofisher, US) was used to identify the desired band. The results obtained from PCR sequencing was used as the gold standard for comparing with other results.

Data Analysis

The results obtained were analyzed in SPSS-16 using descriptive statistics including frequency, relative frequency and mean.

Results

Direct PCR was successfully carried out in S. aureus using bacterial colonies and the results obtained were compared with those of PCR with DNA extraction kits. Both methods successfully amplified 132-bp femA, 163-bp mecA and 326-bp TST (Figures 1 and 2). Of 130 S. aureus isolates separated from different specimens, 69 were found to be methicillin-resistant using PCR with DNA extraction kits. Moreover, 68 isolates were found to be methicillin-resistant using direct PCR. The two methods were different in terms of methicillin resistance of mecA and TST in S. aureus strains. The sequencing test of all S. aureus samples was also considered the gold standard for the study genes. The sensitivity and specificity of different methods were
determined based on the sequencing results. The extraction method using kits respectively suggested a sensitivity and a specificity of 92.13% and 96.96% for mecA and 98.81% and 94.34% for TST. Direct PCR also respectively suggested a sensitivity and a specificity of 98.55% and 98% for mecA and 84.2% and 96.07% for TST.

All isolates confirmed to be S. aureus using phenotypic methods were found to carry femA using direct PCR and PCR with DNA extraction kits. In addition, 12 isolates were found to be TST-positive based on PCR with DNA extraction kits and 9 using direct PCR (Table 2). All products obtained from the amplification of the study genes were sequenced to determine a gold standard for comparing the methods used. The results obtained from the NCBI web blast of these sequences showed that 78 (100%) of the isolates were femA-positive, 69 (87.17) were mecA-positive and 13 (10.53%) were TST-positive.

Table 2: Sensitivity, specificity and negative and positive predictive values of direct PCR and PCR using extraction kits

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TST (Sensitivity)</th>
<th>mecA (Sensitivity)</th>
<th>TST (Specificity)</th>
<th>mecA (Specificity)</th>
<th>Positive Predictive Value (%)</th>
<th>Negative Predictive Value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct PCR using colonies</td>
<td>84.2</td>
<td>98.55</td>
<td>96.07</td>
<td>98</td>
<td>97.58</td>
<td>98.85</td>
</tr>
<tr>
<td>PCR with DNA extraction kits</td>
<td>98.81</td>
<td>92.13</td>
<td>94.34</td>
<td>96.96</td>
<td>95.98</td>
<td>96.35</td>
</tr>
</tbody>
</table>

Figure 1: The result of the gel electrophoresis of amplified methicillin-resistant mecA and TST based on direct PCR with bacterial colonies. wells 1-9: direct PCR using bacterial colonies, well 10: positive control as direct PCR, well 11: negative control, well M: 100-bp marker
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Figure 2: The result of the gel electrophoresis of amplified methicillin-resistant mecA and TST based on PCR with DNA extraction kits. wells 1-9: PCR with DNA extraction kits, well 10: positive control as PCR with DNA extraction kits, well 11: negative control, well M: 100-bp marker

Table 3: The frequency of femA, mecA and TST obtained through PCR with DNA extraction kits and direct PCR in clinical specimens of S. aureus

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Methicillin-resistant S. aureus using phenotypic methods in frequency (%)</th>
<th>Methicillin-sensitive S. aureus using phenotypic methods in frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>femA</td>
<td>mecA</td>
</tr>
<tr>
<td>Direct PCR</td>
<td>78 (100)</td>
<td>68 (87.17)</td>
</tr>
<tr>
<td>PCR using extracted DNA</td>
<td>78 (100)</td>
<td>69 (88.46)</td>
</tr>
</tbody>
</table>

Discussion

S. aureus strains identified as Penicillin-resistant in 1950 gradually become resistant to other β-lactam antibiotics, and completely methicillin-resistant strains emerged by 1975 and named methicillin-resistant S. aureus (2). These strains carry one or both mecA and mecC and prevent β-lactam antibiotics from bonding to bacteria by encoding a special PBP (4). The presence of mecA causes resistance to methicillin in S. aureus (23). mecA is located on a mobile genetic element, namely staphylococcal cassette chromosome mec (4). This bacterium can also produce different pathogens including toxins, surface antigens, extracellular enzymes and some factors associated with Polysaccharide capsules, namely biofilm (9). S. aureus strains producing superantigens can cause many problems in inpatients, including severe clinical demonstrations in burn patients and very long-stay patients such as scaly skin, desquamation, reddening of the skin and emergence of widespread hives (16). The toxin entering the body and the subsequent severe immunologic responses heavily involve the immune system and slow down the healing process, as this bacterium mostly cause secondary diseases (16). Given the significance of an early diagnosis of superantigens and the timely detection of high-risk strains, genotypic methods are highly recommended to be...
used to trace genes responsible for toxins. These genes include TST, which is responsible for TSST-1 in patients carrying S. aureus (17). TST is a chromosomal gene responsible for encoding proteins necessary for making the toxin, whose presence indicates that the bacteria are toxin producers. Genomic methods can be used to study, classify and realize the intrinsic or acquired characteristics of microorganisms and the proteins secreted by them (12). Highly-sensitive, reliable and accurate identification of genes that encode these factors is one of these fast methods (18). Multiplex PCR can be used to simultaneously study several genes and obtain accurate information about them. This fast method is preferred to conventional methods in terms of sensitivity and specificity in identifying microorganisms’ characteristics. The high sensitivity of this method in detecting bacteria and infections associated with opportunistic bacteria such as S. aureus, which often produce severe pathogens, plays a crucial role (19). Nevertheless, given the increasing number of resistant S. aureus strains, especially methicillin-resistant S. aureus strains, and the threat they pose to treatment, rapid and reliable methods should be resorted for the early diagnosis and prevention of epidemics (19). Moreover, S. aureus-associated toxins can increase the risk of bacterial pathogenesis (20), and cause diseases such as toxic shock syndrome (TSS) (21). mecA can be identified using direct PCR and acts as one of the main detectors of resistant strains that can be used to determine the definite identity of S. aureus and help abolish tedious biochemical and phenotypic methods. Phenotypic and serological methods are not good at eradicating the pathogen and quickly detecting the factors causing infections associated with resistant S. aureus (22). Determining the intrinsic or acquired characteristics of pathogenic bacteria, including antibiotic resistance and production of dangerous toxins, can help to timely treat the condition using the most appropriate prescriptions, reduce infection-associated risks and prevent the emergence of extensive resistance caused by false diagnoses and prescriptions (23). Although PCR is a simple and available method for genome amplification, it encompasses time-consuming DNA extraction. The present study found some weaker bands obtained by direct multiplex PCR compared to those obtained through DNA-based multiplex PCR, which is consistent with numerous studies. Lichtensteiger et al. (1996) recommended direct PCR for identifying toxins produced Pasteurella multocida. They also found this technique to have a proper diagnostic value, speed, accuracy and predictive value along with methods such as ELISA and injection of a lethal dose of toxin to mice (24). Nakao et al. (1997) found direct PCR to have an acceptable sensitivity for detecting the diphtheria toxin gene and for conducting a molecular assay of clinical specimens in terms of the presence of the TOX gene as the producer of diphtheria toxin (25). Vaughan et al. (2003) used direct PCR for evaluating Escherichia coli and confirmed the match between this method and the DNA extraction-based method in terms of nearly the same bands obtained in both methods (26). Inglis et al. (2003) used direct PCR for the molecular detection of Campylobacter species (27). Van Hal et al. (2007) conducted a molecular investigation of methicillin-resistant S. aureus and reported a sensitivity and
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specificity of over 80% and a considerable cost saving for direct PCR and recommended it as a proper method of diagnosing this bacterium (28). Similar studies also suggest that the procedure speed can be increased without compromising the accuracy by eliminating the DNA extraction stage and direct use of bacterial colonies (29). Ahsani et al. (2012) used direct PCR to study genes responsible for toxins in Clostridium Perfringens and found the quality of bands to be the same in both direct PCR and PCR with DNA extraction (14).

Some researchers suggested that direct PCR can be used both in bacterial studies and non-bacterial research; Nishimura et al. (2009) reported a high specificity and sensitivity for direct RT-PCR in detecting Norwalk virus even in fecal specimens (30). This method is recommended to be applied for other bacteria with different structures and unique patterns, as it has not been addressed in the present research and literature.

Conclusion
Given the results obtained, expensiveness of some DNA extraction kits and the time-intensive nature of the method for investigating high sample sizes, direct PCR is highly recommended. Furthermore, some extraction methods result in remnants associated with consumables used in the extraction process, which man negatively affect the final-stage quality and stability of DNA and cause errors or even the failure of the process. The extraction by conventional PCR method used in the present study resulted in far more quality despite the multigene nature of the study and elimination of kits. Given the acceptable results obtained using this method compared to the DNA extraction-based PCR, direct colonies are recommended to be used in PCR experiments when their certainty has been established.

Acknowledgement
The present study was approved by the Research Council of Zahedan University of Medical Sciences in 2015 with an Ethics Committee code of 7152.

Conflicts of Interest
The authors declare no conflict of interest regarding the publication of this article.

References: