Molecular Identification of *Staphylococcus Aureus* Isolated from Clinical Samples by Specific PCR Assay targeting the Signal Transduction gene

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**Abstract**

*Staphylococcus aureus* (*S. aureus*) is a common, Gram-positive species that is pathogenic in both human and animals. Rapid and direct identification of this bacterium specifically from clinical specimens would be useful in improving the diagnosis of *S. aureus* infections in the clinical microbiology laboratory. Although, some molecular identification assays are reported, some false-positive cases are described; it is an urgent need to develop a specific diagnostic method. Currently, the sensitivity and specificity of polymerase chain reaction targeting a signal transduction gene (*vick*) for the diagnosis of *S. aureus* isolates in clinical samples was developed. The assay is sensitive enough to detect 5500 copies of a cloned fragment of the *S. aureus* *vick* gene. This PCR assay can be used for rapid clinical diagnosis of *S. aureus* infection and detection of the pathogen in food samples.

**Keywords:** PCR; Rapid identification; Signal transduction gene; Staphylococcus Aureus; Vick

**Introduction**

*Staphylococcus aureus* is a type of bacteria that about 30% of people carry in their noses as normal flora. In healthcare settings, its infections can be serious or fatal disease entities such as toxic-shock syndrome and staphylococcal scarlet fever. Apart from being a leading source of gastroenteritis via contaminated foods, *S. aureus* has also been responsible for an increasing number of hospital-acquired infections because of its ability to acquire and develop resistance to antibiotics. In particular, the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), first noted over four decades ago, has made it one of the most important human bacterial pathogens of modern times. *S. aureus* are routinely characterized by growth properties, specific surface constituents and their ability to coagulate blood plasma from various sources (due to its ability to produce staphylocoagulase), to produce a thermostable nuclease (DNase) and to form clumps in the presence of fibrinogen (clumping factor). Several *Staphylococcus species*, such as *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus haemolyticus* (*S. haemolyticus*), and the coagulase negative species, have been isolated from ewe’s milk and were found to produce one or several *staphylococcus enterotoxins* and consequently there is a need for methods to specifically discriminate *S. aureus* from other staphylococci and non-staphylococci as quickly as possible. Conventional identification methods and novel assays based on immunofluorescence probe, DNA microarray, surface enhanced laser desorption and ionization time of flight mass spectrometry, and whole-cell SELEX methods are time-consuming and may yield false-positive or false-negative results, and misclassifications with automated susceptibility testing systems or commercially available latex agglutination kits have been reported recently.

Many laboratory diagnostic techniques, including those...
targeting femA, nuc, coa, 16S rRNA, Sa442 and other genes in *S. aureus*, and rational antibacterial therapies have played an important role in the control of this pathogen\(^6\,14-16).\) Especially, detection of *S. aureus* genes associated with antibiotic resistance including *mecA*, aacA-aphD, tetM, erm (A), erm (C), vat (A), vat (B), vat (C) by PCR facilitates the appropriate control of the pathogen\(^17,18).\) However, some false positive or negative cases by previous molecular identification assays are reported\(^16-19).\) Therefore, it is particularly important to establish species-specific detection methods, and some species- and virulence-specific genes are present and used as specific marker in many bacteria including *Listeria innocua*, *Pasteurella multocida* and *Streptococcus pyogenes* in the existing literature\(^20,21)\).

Recent description of the whole genome sequences of *S. aureus* strains provides the great opportunity to carry out detailed investigation of the molecular mechanisms, virulence and pathogenesis in the microbial genome levels. In addition, the systematic comparison of genomic sequences from different organisms represents a central focus of contemporary genome analysis. Comparative analyses of *S. aureus* sequences can identify coding and conserved non-coding regions, including regulatory elements and species-marker. Based on the previous strategy, the signal transduction gene, *vick* is described as the species-specific diagnostic marker for identification of *S. aureus*\(^17).\) In the present study, we developed a new diagnostic method for rapid identification of *S. aureus* targeting the *vick* gene.

**Materials and Methods**

**Samples**

A total of 110 strains of *S. aureus* were studied (Table 1). They comprised five *S. aureus* reference strains (ATCC29213, 6538, 25923, 12228; American Type Culture Collection, Rockville, Md. and CMCC26001; China Medical Culture Collection Center), and 64 confirmed isolates of *S. aureus* by biochemical test and other bacteria used as negative controls for the PCR experiments from patient samples isolated in the Laboratory of Microbiology (Peking University Health Science Center, Beijing, China) were used to determine the PCR *in vitro* assay specificity and sensitivity.

| Table 1: List of bacteria strains examined in the study. |
| Species | Strain code |
|---------|-------------|
| *Staphylococcus aureus* | 05L-189 clinic |
| | 05B-205 clinic |
| | 05B-272 clinic |
| | 05C-212 clinic |
| | 05N-071 clinic |
| | 05B-033 clinic |
| | 05A-095 clinic |
| | 05L-085 clinic |
| | 05C-077 clinic |
| | 05L-254 clinic |
| | 05L-103 clinic |
| | 05L-201 clinic |
| | 05N-075 clinic |
| | 05O-114 clinic |
| | 05Q-236 clinic |
| | 05I-073 clinic |
| | 05E-186 clinic |
| | 05C-250 clinic |
| | 05B-206 clinic |
| | 05B-008 clinic |
| | 05B-012 clinic |
| | 05B-038 clinic |
| | 05B-041 clinic |
| | 05B-043 clinic |
| | 05B-255 clinic |
| | 05B-289 clinic |
| | 05C-052 clinic |
| | 05C-136 clinic |
| | 05C-155 food |
| | 05C-215 clinic |
| | 05C-288 clinic |
| | 05I-056 clinic |
| | 05C-299 clinic |
| | 05C-173 clinic |
| | 05I-052 clinic |
| | 05A-100 clinic |
| | 05H-150 clinic |
| | 05L-224 clinic |
| | 05F-148 clinic |
| | 05N-138 clinic |
| | 05B-085 clinic |
| | 05G-226 clinic |
| | SJTU-4 clinic |
| | SJTI-5 food |
| | 05E-024 food |
| | 05G-021 clinic |
| | 05H-150 clinic |
| | 05L-228 clinic |
| | 05M-146 clinic |
| | 05D-005 clinic |

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and 1.5 ml cultures of the organism were centrifuged at 3000 rpm/min for 5 min, then washed twice with TE (Tris 10 mM, EDTA 1 mM, pH 8.0). The purified organism was resuspended in 456 μl of TE, and lysed by adding 24 μl of 50 mg/ml lysozyme and incubated at 37°C for one hour. Then 53 μl of 10% sodium dodecyl sulfate was added and continued incubation for 15 min at 68°C. Finally, 87 μl of 5 mol/L NaCl and 69 μl of 1% cetyl trimethyl ammonium bromide were added and incubated at 68°C for an additional 15 min to release DNA. Subsequently, the samples were extracted with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) followed by phenol and chloroform (1:1). The DNA was then precipitated by the addition of 1/10 volumes of 3 mol/L sodium acetate and 2 volumes of 100% ethanol and incubating at -20°C for 15 min. The DNA pellets were washed twice with ice chilled 70% ethanol, air dried and the DNA pellets was dissolved in distillation water, and the DNA concentrations were determined at UV 260/280 nm in a DU800 UV spectrophotometer (Beckman Coulter, Fullerton, CA). A small amount of DNA from each bacterial strain was diluted in distilled water to 10 ng/μl for PCR analysis.

Oligonucleotide primers and PCR procedure

Oligonucleotide primers were designed on the basis of the signal transduction gene vicK that is S. aureus species-specific diagnostic marker in our previous research. The sequences of the primers were 5'-CTAATACTGAAAATGAGAAACGTA-3' and 5'-TCCTGCAACATCGTACTAAA-3') and facilitated the amplification of a 289-bp DNA fragment from only S. aureus.

Each DNA amplification was performed in 200 μl microtubes using a 50 μl reaction mixture containing the 10 ng genomic DNA template, 1.0 U Taq DNA polymerase (Tiangen Biotechnology, Corporation, Beijing, China), 5 μl of 10 x PCR buffer, 50 μM dNTPs, 25 pM each primers and double-distilled water to the final volume of 50 μl. The reaction mixture with no template DNA was used as a negative control. All the amplifications were carried out in a PCR system PTC-200 (Biorad, Foster city, CA, USA) with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation 94°C for 40 s, primer annealing at 50°C for 40 s and extension at 72°C for 1 min, and followed final extension at 72°C for 10 min. After completion of all cycles, 6 μl of 10 x DNA loading buffer was added to each tube, and the amplified products were examined in 1.5% agarose gel electrophoresis in the presence of ethidium bromide (0.5 μg/ml). The stained gels were visualized under UV light and photographed using a Las300 Fuji Film (Fuji, Japan).

Amplifics identification

In order to confirm the specificity of the PCR amplifications, the PCR products were purified from agarose gels and cloned into a plasmid vector (pMD18-T vector, Takara Biotechnology (Dalian) Corporation, Dalian, China) following the manufacturer’s protocol. Escherichia coli (DH5α, Tiangen Biotechnology, Beijing, China) were transformed and colonies containing inserts were selected using blue/white screening. Plasmid DNA was isolated using Tiangen plasmid mini prep kit (Tiangen Biotechnology, Beijing, China) following the manufacturer’s protocol. Recombinant plasmid DNA was sequenced bi-directionally using the primers, M13-4: 5'-GTTTATCCAGTCACGAC-3' and BcaBEST Primer RV-M 5'-GAGGCGGATAACAATTCAACAAG-3' bi-directionally using an automat-
ed DNA sequence (ABI 3730 DNA analyzer, Foster city, CA, USA).

Sensitivity test
In order to determine the limit detection of the assay, the sensitivity test was explored with the copies of S. aureus vicK gene. Plasmid DNA containing the 289 bp fragment of the S. aureus vicK gene was quantified using spectrophotometry and was serially diluted in water. These serially diluted samples were used as template for the PCR assay for 10 reactions for each dilution. After analysis the PCR product, the sensitivity of the assay was gained.

Results

Specificity
The PCR assay specifically amplified a 289 base pair product from S. aureus, and did not produce amplicons when other organisms DNA from Staphylococcus epidermidis, Staphylococcus haemolyticus, Enterococcus faecalis, Enterococcus faecium, Vibrio parahaemolyticus, Salmonel choleraesuis and E. coli (Figure. 1; data not shown for all organisms) were used as a template for the PCR reaction (Figure.1). To prove their identity, amplicons were extracted from the gel, purified, inserted into pMD18-T Vector and sequenced, and the DNA sequence of the amplicons from the S. aureus isolates in this study was 100% identical to the S. aureus sequences in Genbank, which indicated the discriminating power of the PCR described here.

Sensitivity
The PCR was able to detect S. aureus DNA spiked with as little as 5500 gene copies/μl in DNA isolated from the plasmids containing the target sequence (Figure. 2). The frequency of amplicon detection in the replicate reactions is presented in Table 2, and consistent detection (100%) required 5500 gene copies of the target molecule per reaction.

Table 2: Sensitivity of S. aureus-specific PCR assay for the detection of vicK gene copies diluted in S. aureus samples.

| Gene copies per microliter | Success rate (%) (positive PCR reactions/ attempts) |
|---------------------------|---------------------------------------------------|
| 55000000                  | 100(10/10)                                        |
| 5500000                   | 100(10/10)                                        |
| 550000                    | 100(10/10)                                        |
| 55000                     | 100(10/10)                                        |
| 5500                      | 100(10/10)                                        |
| 550                       | 100(10/10)                                        |
| 550                       | 30(3/10)                                          |
Discussion and Conclusion

Although *S. aureus* is not difficult to grow and is easy to identify, there is a need for the development of rapid and sensitive DNA-based assay that is suitable for the detection of *S. aureus* to improve the rapidity and the accuracy of the diagnosis of *S. aureus* infections. Since introduction of PCR over thirty years ago, PCR methods for the detection of infectious organisms have been recognized as increasingly valuable diagnostic tools. The development of highly sensitive and specific PCR assays has alleviated problems typically associated with microorganisms which are found in low densities in tissue (or tissue fluids), difficult to culture, or serologically similar.

There are PCR amplification assays targeting various genes, including mecA, nuc, SEs encoding enterotoxins. However, all of these targets are not ubiquitously found in the species *S. aureus*, and consequently, these PCR assays are not suitable for the detection and identification of *S. aureus*. The coa gene coding for the coagulase protein has also been considered a candidate for the development of DNA-based diagnostic assays for *S. aureus*, however, this gene is highly polymorphic and does not allow for the ubiquitous identification of all *S. aureus* strains. Hence, there is still an urgent need for development of species-specific and rapid diagnostic method for rapid identification of *S. aureus* from clinical samples.

In our work, 110 strains isolated from clinical samples in various hospitals were identified in biochemical test, and used to identify the validity of our novel PCR method. The PCR assay results revealed a good correlation with those identified by biochemical test. The *S. aureus*-specific PCR described in this study was determined to be very sensitive and was able to detect *S. aureus* DNA in samples containing less than 5500 gene copies per reaction. The number of target molecules, in conjunction with the detection limit of the specific PCR assay, is a critically important determinant of the clinical diagnostic sensitivity of tests used to detect the presence of pathogenic organisms. This is especially applicable to PCR assays in which a negative test may not be useful in ruling out infection with a particular pathogen that can induce persistent low-level infection. The lower percentage of PCR positive test results obtained from samples with 5500 or less gene copies/μl highlights the effect that sampling has on detection in patient samples containing low copy numbers. Since it is impossible to detect less than 5500 gene per PCR reaction, the number of target molecules that are actually placed in the reaction dictates whether or not this particular sample will result in a positive result. The probability of a target molecule being placed into the reaction is determined by Poissonian statistics since the molecules in the solution should have a Poissonian distribution (Pfaff). For this assay 5500 copies *vick* gene per reaction are necessary for detection of *S. aureus* every time.

Conflict of Interest: The authors have no conflict of interest.

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