Journal of Pharmacy & Pharmaceutics

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Research Article Open Access

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*

Received date: September 11, 2017
Accepted date: November 1, 2017
Published date: November 7, 2017

Citation: Liu, Z., et al. Molecular Identification of *Staphylococcus Aureus* Isolated from Clinical Samples by Specific PCR Assay Targeting the Signal Transduction Gene Research. (2017) J Pharm Pharmaceutics 4(2): 166- 171.

DOI: 10.15436/2377-1313.17.1682

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[1]. Apart from being a leading source of gastroenteritis via contaminated foods[2], *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[3]. 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 epidermis (S. epidermis), 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[2] 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[4-13].

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, Beiijng, 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	ATCC25923	human
	ATCC 6538	human
	ATCC29213	human
	CMCC 26001	human
	05I-043	human
	05C-054	clinic
	05C-256	clinic
	05A-176	clinic
	05A-005	clinic
	05A-075	clinic
	05C-280	clinic
	05Q-132	clinic
	05N-119	clinic
	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

	05D-261	clinic
	05N-074	clinic
	05G-064	clinic
	05D-361	clinic
	05D-154	clinic
Staphylococcus epidermidis	ATCC12228	clinic
	05A-078	clinic
	05C-088	clinic
	05C-229	clinic
	05F-028	clinic
	05G-121	clinic
	05G-157	clinic
	05K-181	clinic
	03A-103	clinic
	03L-032	clinic
	03L-029	clinic
Staphylococcus haemolyticus	05C-059	clinic
	05C-134	clinic
	05G-077	clinic
	05G-086	clinic
	05G-099	clinic
	05G-100	clinic
	05G-191	clinic
	05G-193	clinic
	05G-243	clinic
	05H-003	clinic
Enterococcus faecalis	05K-96	clinic
	05I-93	clinic
	05H-216	clinic
	05B-56	clinic
	05H-282	clinic
Enterococcus faecium	05D-54	clinic
	05G-267	clinic
	05C-384	clinic
	05D-027	clinic
	05D-034	clinic
Vibiro parahaemolyticus	AS1.1190	shrimp
	ATCC17802	shrimp
	CMCC50001	fish
	CMCC500017	fish
Salmonel choleraesuis	CMCC50004	chick
	CMCC 50035	egg
	11200	chick
	12002	chick
Escherichia coli	05A-001	clinic
	05A-008	clinic
	05A-48	clinic

DNA preparation

Purified DNA stock was prepared in our laboratory as follows. Bacteria were grown overnight in LB broth at 37°C, 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'-CTAATACTGAAAGTGAGAAACGTA-3' and 5'-TCCTGCACAATCGTACTAAA-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 × 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 × 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)

Amplicons 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 Biotechnolgy (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-M4: 5'-GTTTT CCCAG TCACG AC-3' and BcaBEST Primer RV-M 5'-GAGCG GATAA CAATT TCACA CAGG-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.

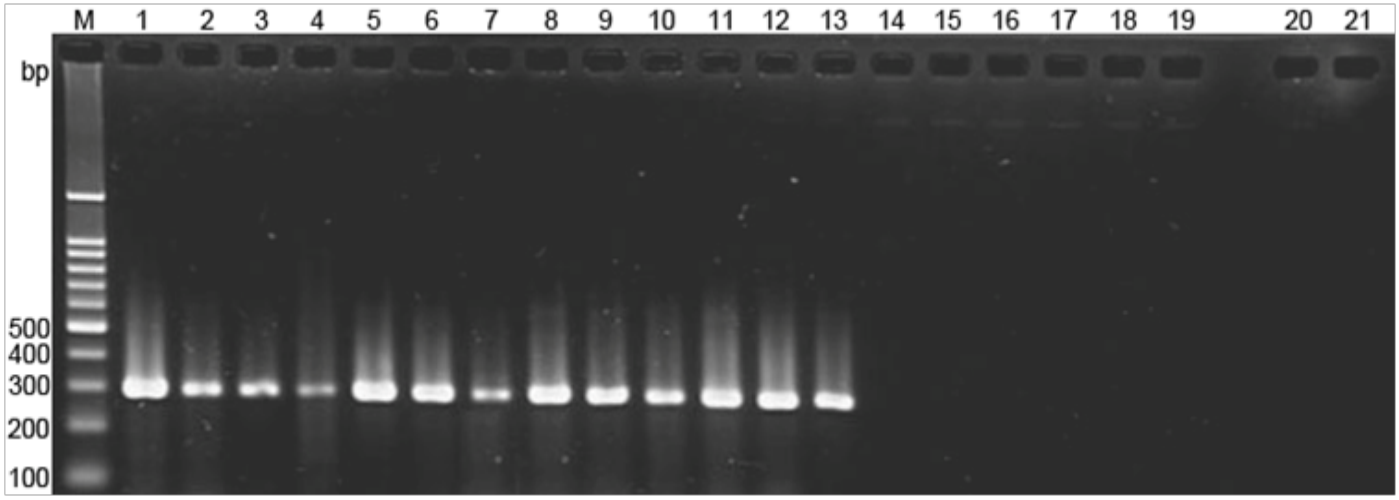

Figure 1: Electrophoretic examination of PCR products generated with *S. aureus vicK* gene primers. Lanes 1–13 contained amplified DNA products from *S. aureus* strains (ATCC12228, 25923, 6538, 29213, 26001, 05I-56, 05C-54, 05C-256, 05A-176, 05A-5, 05A-75, 05C-280 and 05Q-132 representing other 56 strains); lanes 14, *S. epidermidis* strains (05A-78 representing other 9 strains); lane 15, *S. haemolyticus* (05G-99 representing other 9 strains), lane 16, *Enterococcus faecalis* (05K-96 representing other 4 strains); lane 17, *Enterococcus faecium* (05-D54 representing other 4 strains); lane 18, *Vibiro parahaemolyticus* (ATCC 17802 representing other 3 strains); lane 19, *Salmonel choleraesuis* (CMCC50004 representing other 3 strains); lane 20, *Escherichia coli* (05A-001 representing other 2 strains) and lane 21, negative control with no template DNA,. On the left of lane 1 is a DNA molecular weight ladder (DNA ladder, Tiangen, Beijing, China).

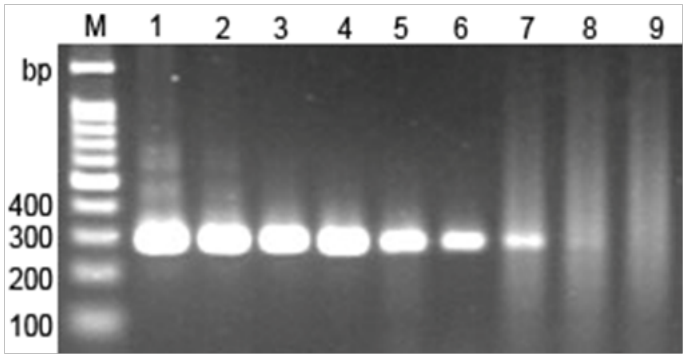

Figure 2: Sensitivity of PCR amplification targeting *vick* gene. Lane 1-9 of serially diluted plasmid containing the 289 bp fragment of *S. aureus vick* were used as template and their concentration were 5.5×10^9, 5.5×10^8, 5.5×10^7, 5.5×10^6, 5.5×10^5, 5.5×10^4, 5.5×10^3, 5.5×10^2 and 55 copies/µl, respectively. M indicates the lane containing marker DNAs, and the numbers on the left of the panel indicate the sizes of the markers

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	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 clinical 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[17]. 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*[18]. 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[22]. 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 Poisson 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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Pubmed | Crossref | Others

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