Original Research Article

A Two Stepwise Molecular Approach for Rapid Separation of Staphylococcus aureus from other Coagulase Negative Staphylococci

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ABSTRACT

Staphylococcus aureus is the most important cause of nosocomial and community associated infections as well as the most pronounced species in drug resistance amongst Staphylococcus species. There have been reported cases of inconsistency in presumptive coagulase tests using commercial kits and conventional methods. This study therefore reports a simple, rapid, accurate and cost-effective PCR-RFLP-based molecular technique that separates and identifies to species level S. aureus from other coagulase negative staphylococci. Fifty clinical isolates of staphylococci previously isolated from various specimens were investigated for their ability to clot blood plasma. Of the 50 clinical isolates, 6 (12%) and 44 (88%) isolates yielded positive and negative results, respectively, for conventional tube coagulase test. The PCR-RFLP supplemented with species-specific primers classified these isolates into three distinct species: S. epidermidis (86%), S. aureus (12%) and S. xylosus (2%). Furthermore, the technique applied in this study classified the isolates into two RFLP patterns representing S. aureus and other coagulase negative staphylococci which correlated with coagulase test results. Since coagulase test could be sometimes misleading and inconclusive, this method could be substituted in routine laboratory program.

Keywords
S. aureus, coagulase test, PCR-RFLP.

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Introduction

The genus Staphylococcus is the pathogen of man and animals. Basically, they are classified into two based on their plasma clotting ability; coagulase positive staphylococci and coagulase negative staphylococci (CNS). Staphylococcus aureus is the most important cause of nosocomial and community associated infections as well as most prominent in drug resistance especially with emergence of methicillin-resistant strains (NNIS, 2001; Cooper et al., 2004). It is responsible for many of the human diseases which include folliculitis, impetigo, wound infection, toxin-mediated infections that includes toxic 2 shock syndrome, food poisoning, scalded skin syndrome, pneumonia, bacteremia,
endocarditis, osteomyelitis, septic arthritis are caused by this group of bacteria. Coagulase negative staphylococci belong to the group of opportunistic pathogens found as normal flora of the skin and mucus membranes in different parts of the body (Einsenstein and Schachcter, 1994; Becker et al., 2014). They were originally assumed to be contaminating clinical samples but were not involved in the primary infection; there is evidence that these bacteria may be responsible for primary infections as a result of increased use of medical in dwelling plastic devices and compromised or immunodepressed patients (Jarvis and Martone, 1994; Kloos and Bannerman, 1994, Becker et al., 2014). It is therefore imperative to identify and separate S. aureus from CNS owing to its clinical significance in infectious diseases.

In routine laboratory practice, coagulase production has been the conventional screening test to distinguish S. aureus from other staphylococci; since other coagulase positive staphylococci (CPS) such as S. hyicus, S. schleiferi subspecies coagulase or intermedius have rarely been found in human infections. However, several reports of inconsistency and inaccuracy in the conventional coagulase test have earlier been documented (Buchanan et al., 1983; Fung et al., 1984; Gill et al., 1984). These reports somehow might have contributed to the improvement of conventional coagulase detection by the development of test kits; Staphaurex (Wellcome), Staphylase (Oxoid), Staphyslide (bioMerieux), Biostaph (Medlabs) and Bacto Latex (Difco) (Flandrois and Carret, 1981; Rossney et al., 1990), but these kits are still not accurate and are not within reach of the both health institutions and local clinics in developing countries as they are expensive. In the light of this, the serological separation of CPS and CNS are largely based on slide and tube coagulation tests which are not reliable due to variability in inoculum size, incubation period and inoculation medium (Davies, 1951; Rossney et al., 1990). Beside, this technique when scarcely done has little relevance as it does not conclusively confirm S. aureus as non-staphylococcal species for example; Pseudomonas aeruginosa and Serratiamarcescens can produce coagulase (Rossney et al., 1990). This study was therefore designed to involve two stepwise molecular approaches that will separate S. aureus and CNS as well as identify S. aureus to species level using PCR-RFLP double digestion format that can be integrated into routine laboratory program.

Materials and methods

Isolates

Clinical isolates from various samples (eyes swab, ear swab, wound swab, tissue aspirates high and vagina swab) that were isolated between 2008 and 2011 under normal routine laboratory practice in University College Hospital Ibadan, Nigeria were used for the study. In all, 50 isolates were used for this study.

Re-isolation procedure MSA

Media used include Mannitol Salt Agar (MSA) and Tryptone Soy Agar (TSA), Oxoid. All the media were prepared according to the manufacturer’s instructions by weighing the specified amount of each medium into 100ml of distilled water. The media were then autoclaved. The sterilized media were allowed to cool to 45°C before dispensing into petri dishes to solidify. The petri plates were kept in refrigerator until when needed. All isolates (stored in 40% glycerol under -80°C freezer) were streaked on already prepared plates on MSA and
incubated for 18-24 hours. Colonies showing yellow colouration or whitish color were picked and sub-cultured on TSA and incubated at 37°C between 18-24 hours. The sub-cultured organisms were used for subsequent screening.

**Catalase test**

Two drops of hydrogen peroxide (3%H₂O₂) were made on a slide and a sterile non-nichrome wire loop was used to pick a colony from overnight growth culture of the test organism and then mixed. An immediate bubbling indicates positive result.

**Haemolytic test**

Mueller-Hinton Agar plates were prepared according to the manufacturer’s instructions and cooled to 45°C. Thereafter, 10% of human blood was added, mixed and were allowed to solidify. After drying, the petri plates were streaked with each organism using a sterile wire loop. The streaked plates were incubated at 37°C for between 18-24hrs. Haemolytic and non-haemolytic activities were observed after overnight incubation.

**Coagulase test**

Three test tubes were taken and labeled ‘test’, ‘negative control’ and ‘positive control’. A serial dilution (1 in 10) of human plasma with physiological saline was made. In each tube 0.5 ml of the diluted human plasma was dispensed. Subsequently, the tube labeled test was added with 0.1 ml of overnight broth culture of test bacteria. The tube labeled positive control was also treated with 0.1 ml of overnight broth culture of known *S. aureus*. However, the tube labeled negative control was added with only 0.1 ml of sterile broth. All the tubes were incubated at 37°C and observed up to four hours.

Positive result was indicated by gelling of the plasma.

**DNA extraction**

The boiling method as described by Perez-roth *et al.*, (2001) was applied as follow: Colonies from an overnight culture of the organisms on Tryptone Soya Agar (TSA) were emulsified in 500µl of sterile double distilled water contained in 1.5ml Eppendorf tubes to give turbidity equivalent to 0.5 McFarland standards. These were transferred into a heating block set at 100°C for 10 minutes. After boiling, the mixtures were then centrifuged for 5 minute at 2400 rpm. The supernatant were transferred into fresh sterilized Eppendorf tubes with its purity determined using Nano drop spectrophotometer (3300) and stored at -20°C in a refrigerator until when required.

**Confirmation of the genus staphylococci**

An aliquot of 2.0µl of DNA suspension was added to 25µl of PCR mixture consisting of 5µl standard reaction buffer (Biolabs, England) [20mM Tris-HCl, 1.8mM MgCl₂, 22mM NH₄Cl, 22mM KCl, 0.06% IGEPL CA-630, 0.05% Tween 20, pH 8.9]), a 0.2 mM concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.25µl of each primers (TstaG422 5'-GGCCGTGTT GAACGTGGTCAAATCA-3' as forward and Tstag765 5'- TIAACCATTT CAGTACC TTCTGGTAA-3' as reverse), and 0.125 U of *Taq* DNA polymerase. DNA amplification was carried out in a system 9700 thermocycler with the following thermal cycling profiles: Initial denaturation at 94°C for 1 minute, denaturation 94°C for 30 seconds, annealing 58°C for 30 seconds, extension 68°C for 1 minute and final extension 68°C for 5 minutes with a programmable number of 30 cycles. After
PCR amplification, 10μl of PCR product was removed and subjected to agarose gel electrophoresis (1.5% agarose, 1x Tris-borate-EDTA, 100 V, 1 hour) to estimate the sizes of the amplification products by comparison with a 100-bp molecular size standard ladder. Following electrophoresis at 100V for 1 hour, gels was examined for bands using a photo documentation system (TranUV, illuminator Biorad).

**PCR-RFLP by double digestion**

Ten (10) μl of each amplified product was mixed 2.5 μl of 10X buffer, 10.5μl of H2O and digested with 1.0 U of Hind III and EcoRI restriction enzymes simultaneously. The mixture was incubated at 37°C for 1-2 h. After endonuclease digestion, 10μl of product was removed and subjected to agarose gel electrophoresis (2% agarose, 1x TBE, 100 V, 1 h) to estimate the sizes of the restriction digest by comparison with a 100-bp molecular size standard ladder (Biolabs, England). The interpretation criterion for identifying different species was a single band difference.

**Results and Discussion**

**DNA extraction protocol**

To facilitate quick identification in clinical Microbiology laboratories, a simple DNA extraction procedure was given almost priority. Several DNA protocols exist in literatures where DNA can be extracted from overnight liquid cultures (Tokue et al., 1992; Vannuffel et al., 1995; Giambiagi-de Marval, 1999). However, we adopted the DNA extraction protocol described by Perez-roth et al (2001) with little time modification because it is simple, fast and cheap. This protocol yielded good quality DNA for any PCR amplification procedure.

**Correlation of coagulase test with PCR-RFLP**

All the isolates from different clinical sources were confirmed to belong to the genus staphylococci by using genus-specific primers. The genus PCR products after double digestion with restriction endonucleases classified the isolates into two RFLP patterns: A (220-50bp) and B (370bp) representing *S. aureus* and other coagulase negative staphylococci respectively (Fig 1). Of the 50 clinical isolates, 6 (12%) were identified as *S. aureus* and the rest was confirmed to be CNS using species-specific primers (Table 1). All the six *S. aureus* (100%) identified using the PCR-RFLP gave a positive coagulase test and 88% of the CNS were negative for coagulase test. This tube coagulase test correlated well with this molecular technique.

Experimental evidence of staphylococcal coagulase production in vitro has been shown to be affected by many factors and tube coagulase test results vary depending on the methods used (Buchanan et al., 1983; Fung et al., 1984; Gill et al., 1984; Rossney et al., 1990).

The conventional tube coagulase test correlated with this new technique in this study, probably due to the small sample size of *S. aureus* or as a result of the fact that atypical strains were not encountered. The inconsistency in results of various coagulase tests (conventional and commercial kits) for separating *S. aureus* from CNS have necessitated some investigators to advocate for an alternative test in addition to coagulase test (Selepak and Witebsky, 1985; Ozen et al., 2011).
**Table 1** Distribution of sample sources, organisms and confirmation of *Staphylococci* using genus-specific primers

| Sample source | No of isolates | Confirmed with TstaG442/Tstag765 primers | Organisms identified |
|---------------|----------------|------------------------------------------|----------------------|
| Semen         | 8              | 8                                        | *S. aureus* (1), *S. epidermidis* (7) |
| HVS           | 1              | 1                                        | *S. epidermidis* (1)  |
| Wound         | 16             | 16                                       | *S. aureus* (3), *S. epidermidis* (13) |
| Eye           | 13             | 13                                       | *S. aureus* (1), *S. epidermidis* (12) |
| Ear           | 7              | 7                                        | *S. epidermidis* (7)  |
| Urethra       | 1              | 1                                        | *S. epidermidis* (1)  |
| skin          | 1              | 1                                        | *S. aureus* (1)       |
| Sputum        | 1              | 1                                        | *S. epidermidis* (1)  |
| Throat        | 1              | 1                                        | *S. xylosus* (1)      |
| Soft tissue   | 1              | 1                                        | *S. epidermidis* (1)  |
| **Total**     | **50**         | **50**                                   |                      |

**Fig.1** RFLP patterns after digestion with restriction endonucleases.

Several publications have reported somehow similar biochemical characteristics of coagulase negative staphylococci to *S. aureus* (Buchanan et al., 1983; Fung et al., 1984) and this as well as other factors might have necessitated PCR-based procedures for discriminating staphylococci using different restriction enzymes and gene targets such as PCR-RFLP of protein A coding gene, coagulase gene (Mehndiratta et al., 2009, Bhati et al., 2014), PCR-RFLP of gap gene (Ghebremedhin et al., 2008). Each of these techniques selects either CPS or CNS. The disadvantages of aforementioned techniques above lie with the fact that when CPS or CNS is fingered as the cause of a particular infection(s), they require more than a pair of primers and one restriction enzyme for complete fingerprinting. Also, more resources and time are required to achieve
this purpose. However, this current procedure selects both CPS and CNS with different restriction patterns. The technique seems to take advantage of genetic differences between the coagulase and non-coagulase gene sequences rather than the individual species diversity. It is also possible that the CNS either has no restriction sites for endonucleases used or probably that the parts of restriction products were too small to be detected by conventional agarose gel electrophoresis. It is worthy to note that when Hind III restriction endonuclease was used alone, no digestion was observed, and it seemed therefore that EcoR1 was responsible for the restriction pattern observed. However, we only reported double digestion that we did. Although, other coagulase positive staphylococci such as S. hyicus, S. schleiferi subspecies coagulase or intermedius were not available for inclusion to affirm this assumption. Further work is required to clarify this assumption (i.e. genetic differences). Since coagulase test could be sometimes misleading and inconclusive, this method could be substituted for routine laboratory program especially when isolating S. aureus amidst CNS from clinical or other based samples.

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