Int.J.Curr.Microbiol.App.Sci (2016) *5*(7): 778-784

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 7 (2016) pp. 778-784**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.507.089

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

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Keywords

S. aureus, coagulase test, PCR-RFLP.

Article Info

Accepted:
22 June 2016
Available Online:
10 July 2016

A B S T R A C T

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.

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 Schacchter, 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) (Flandroisand 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^{o}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^{o}C freezer) were streaked on already prepared plates on MSA and

incubated for 18-24hours. Colonies showing yellow colouration or whitish color were picked and sub-cultured on TSA and incubated at 37^{o}C between 18-24 hours. The sub-cultured organisms were used for subsequent screening.

Catalase test

Two drops of hydrogen peroxide (3%H_2O_2) 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^{o}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^{o}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^{o}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^{o}C for 10minutes. After boiling, the mixtures were then centrifuged for 5minute at 2400rpm. The supernatant were transferred into fresh sterilized Eppendorf tubes with its purity determined using Nano drop spectrophotometer (3300) and stored at -20^{o}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 $MgCl_2$, 22mM NH_4Cl, 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'- TIACCATTT 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^{o}C for 1minute, denaturation 94^{o}C for 30 seconds, annealing 58^{o}C for 30 seconds, extension 68^{o}C for 1minute and final extension 68^{o}C for 5minutes 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, 1hour) to estimate the sizes of the amplification products by comparison with a 100-bp molecular size standard ladder. Following electrophoresis at 100V for 1hour, 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 H_2O 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.

M=Molecular marker (100bp), lane 1= *S. aureus*.ATCC92535, 8, 13, 18 and 20 are *S. aureus* while the rest are CNS

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 ofprotein 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 endonulease 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 coagulanse 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.

References

Becker, K., Heilmann, C., Peters, G. 2014. Coagulase-Negative Staphylococci *Clin. Microbiol. Rev.*, 27(4): 870-926.

Bhati, T., Nathawat, P., Ahmad, I.M., Kumar, S.S., Yadav, R., Kumar, A.K. 2014. PCR-RFLP of *Staphylococcus aureus* Coagulase Gene Isolated from Bovine Subclinical Mastitis *J. Appl. Microbiol.*, 8(6): 4711-4714.

Buchanan, A.G., Booth, H., Emery, J. 1983. Coagulase negative *Staphylococcus aureus*. *Clin. Microbiol. News*, 5: 144-145.

Cooper, B.S., Medley, G.F, Stone, S.P, Kibb1er, C.C, Cookson, B.D, Roberts, J.A, Duckworth, G, Lai, R. and Ebrahim, S. 2004. Methicillin-resistant *Staphylococcus aureus* in hospitals and the community: stealth dynamics and control catastrophes, *Proc. Natl. Acad. Sci.*, 101(27): 223-228.

Davies, G.E. 1951. Factors influencing the in vitro production of staphylococcal coagulase. *J. Gen Microbiol.*, 5: 687-97.

Einsenstein, B.I., and Schaechter, M. 1994. *Biota microbiana normal. Microbiologis. Mecanismo de Las EnfermedadesInfecciosas*, 2nd edn. Barcelona, Spain: *Panamericana*, 36-46.

Flandrois, J.P., Carret, G. 1981. Study of the staphylococcal affinity to fibrinogen by passive haemagglutination: a tool for the *Staphylococcus aureus* identification. ZblBaktHyg 1 AbtOrigA 251:171-6. [PubMed].

Fung, J.C., Kaplan, M.H., Hsieh, H.C., Stephens, A., Tyburski, M. Band Tenebaum, N.I. J. 1984. Two coagulase-variant forms of *Staphylococcus aureus* isolated from blood cultures. *J. Clin. Microbiolol.*, 20: 115-117.

Ghebremedhin, B., Layer, F, Konig, W and Konig, B. 2008. Genetic classification and distinguishing of *Staphylococcus* species based on different partial gap, 16S rRNA, hsp60, rpoB, sodA, and tuf gene sequences. *J. Clin. Microbiolol.*, 46: 1019-1025.

Giambiagi-de Marval. 1999. Detection of *ileS*-2 gene encoding mupirocin resistance in methicillin-resistant *Staphylococcus aureus* by multiplex PCR. *Diagn Microbiol. Infect. Dis.*, 34: 77–81.

Gill, V.J., Zierdt, C.H., Wu, T.C., Stock, F.,

Pizzo, P.A. and NlacLowry, J.D. 1984. Comparison of lysis-centrifugation with lysis-filtration and a coniventionalunivented bottle for blood cultures. *J. Clin. Microbiolol.*, 20: 927-932.

Jarvis, W.R., and Martone, W.J. 1994. Predominant pathogens in hospitals infections. *J. Antimicrob. Chemother.*, 29 (Suppl. A), 19-24.

Kloos, W., and Bannerman, T. 1994. Update on clinical significance of coagulase negative staphylococci. *Clin. Microbiol. Res.*, 7: 117-140.

Mehndiratta, P.L., Bhalla, P, Ahmed, A. and Sharma, Y.D. 2009. Molecular typing of methicillin-resistant *Staphylococcus aureus* strains by PCR-RFLP of *spa* gene: a reference laboratory Perspective. *Indian J. Med. Microbiol.*, 27(2): 116-22.

NNIS. 2001. National Nosocomial Infections Surveillance System report data summary from January 1992 – June 2007, issued. Am J Infect Control.29, 404 – 421.

Ozen, N.S., Ogunc, D, Mutlu, D, Ongut, G, Baysan, B.O and Gunseren, F. 2011. Comparison of four methods for rapid identification of Staphylococcus aureus directly from BACTEC 9240 blood culture system. *Indian J. Med. Microbiol.*, 29(1): 42-46.

Perez-Roth, E., Claverie-Martin, F, Villar, J and Mendez- Alverz, S. 2001. Mutliplex PCR for simultaneous identification of *Staphylococcus aureus* and detection of methicillin and mupirocin resistance. *J. Clin. Microbiolol., 39:* 4037-41.

Rossney, A.S., English, L.F and Keane, C.T. 1990. Coagulase testing compared with commercial kits for routinely identifying *Staphylococcus aureus, J. Clin. Pathol.*, 43: 246-252.

Selepak, S.T., Witebsky, F.G. 1985. Inoculum size and lot-to-lot variation as significant .variables in the tube coagulase test for *Staphylococcus aureus. J. Clin. Microbiolol.*, 22(5): 835.

Tokue,, Y., Shoji, S, Satoh, K, Watanabe, A and Motomiya, M .1992. Comparison of a polymerase chain reaction assay and conventional microbiologic method for detection of methicillin-resistant Staphylococcus aureus. *Antimicrob. Agents Chemother.*, 36: 6–9.

Vannuffel, P., Gigi, J., Ezzedine, H., Vandercam, B., Delmee, M., Wauters, G., Gala. J.L. 1995. Specific detection of methicillin-resistant staphylococcus species by multiplex PCR. *J. Clin. Microbiolol.*, 33: 2864–2867.

How to cite this article:

Ezeamagu O. Cajethan, Fagade E. Obasola, Smith Stella, I. and Ogunjobi A. Adeniyi. 2016. A Two Stepwise Molecular Approach for Rapid Separation of *Staphylococcus aureus* from other Coagulase Negative Staphylococci. *Int.J.Curr.Microbiol.App.Sci.* 5(7): 778-784.
doi: http://dx.doi.org/10.20546/ijcmas.2016.507.089