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Original Article

Simple and economical method for identification and speciation of *Staphylococcus epidermidis* and other coagulase negative *Staphylococci* and its validation by molecular methods

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ABSTRACT

Coagulase-negative staphylococci (CoNS) have been increasingly recognized as a clinically important group of species that can cause several opportunistic nosocomial infections. There are at least 47 known species of Staphylococci and to differentiate all these species > 40 biochemical tests need to be performed. The present study was able to refine the CoNS identification process by using only five tests to identify *S. epidermidis* from the rest and used six other tests to identify eleven other clinically significant CoNS species. A total of 242 CoNS isolates were collected from tertiary care hospitals and included in the study. The five-biochemical test scheme devised based on mathematical probability derived from a computer algorithm included fermentation of mannitol, maltose, mannose, trehalose and novobiocin susceptibility to differentiate *S. epidermidis* from other CoNS species. The remaining CoNS isolates other than *S. epidermidis* were further characterized with the help of six additional tests, which identified another eleven species. Species-specific PCR and 16SrDNA sequencing were used to confirm and validate the identification scheme. Species-specific PCR and 16SrDNA sequencing showed 100% agreement with non-divergent phenotypic test results, indicating that the five selected assays are highly specific for identifying *S. epidermidis*. In conclusion, this study used only 11 tests to identify most of the clinically significant CoNS that can reduce cost and time. This scheme is easy to perform in any laboratory with basic resources, the results of this study were validated using more accurate molecular methods such as PCR and 16S rDNA typing to confirm the utility of the proposed scheme.

1. Introduction

Coagulase-negative staphylococci (CoNS) are increasingly recognized as being responsible for severe nosocomial infections in hospitalised patients (Lowy and Hammer 1983; Kini et al. 2010; May et al. 2014). Until the 1970s, CoNS were considered cutaneous contaminants, but it was later shown that these inhabitants have slowly developed the ability to cause nosocomial infections (Miragaia et al. 2007; Gordon et al. 2012). In the CoNS group,*Staphylococcus epidermidis* species is the most common nosocomial pathogen associated with peripheral and central venous catheters, prosthetic valves, postoperative wounds, bacteraemia, bone marrow transplants recipients, and other indwelling medical devices (Goldmann and Pier, 1993; Rupp and Archer, 1994; Weinstein and Darouiche, 2001; Mack et al. 2006; Hidron et al. 2008). In addition, emergent antibiotic resistance reported in CoNS, particularly, methicillin resistant *S. epidermidis* (MRSE) strains necessitated the need for an appropriate and accurate identification of CoNS at the species level rapidly (Bispo et al. 2014; Cidral et al. 2015). In addition, many species belonging to the CoNS group are increasingly implicated as pathogens responsible for significant nosocomial infections, several earlier researchers have also emphasized the need for species identification (Bhatt et al. 2016).

Although many species of CoNS exist in nature, very few of them have been reported as human pathogens but their importance in human pathogenesis is increasing significantly due to new human infections caused by them (von Eiff et al. 2002). This scenario may worsen due to

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the lack of an unaffordable, simplified and cost-effective method for complete speciation of various species within CoNS (van Veen et al. 2010). It is more desirable to identify individual species from clinical specimens with minimal cost, effort and time to understand the epidemiology of each of these clinically relevant species belonging to the coagulase-negative staphylococci group. Previously, Kloos and Schleifer provided a scheme of CoNS identification which is very cumbersome (Kloos and Schleifer 1975). Automated biotyping and commercial kits are available in the market, but they are not used regularly in developing countries because they are very expensive (Bannerman, 2003). Different types of manual and automated biochemical test systems are the cornerstone of identification, but in many laboratories, their performance and accuracy of identification is only about 70 to 90% (Becker et al., 2014). Moreover, the reliability of these systems for the accurate identification of all CoNS species depends on the performance of an additional range of tests recommended by the proponents and manufacturers of these systems. In addition, such automated systems are expensive and they may sometimes fail to distinguish commonly encountered CoNS species if they have unusual phenotypes (phenotypic variants) similar to phenotypes isolated from other sources such as food and livestock (Becker et al., 2014). Therefore, there is an urgent need to develop a minimal, simple, affordable and reliable phenotypic test scheme for identifying *S. epidermidis* species and other clinically important species of CoNS. The present study attempted to develop a simple scheme comprising of a few biochemical tests sufficient to identify *S. epidermidis* and other clinically important species from CoNS group in a routine clinical laboratory. Our scheme has used fermentation of sugars such as mannitol, maltose, mannose, trehalose and novobiocin susceptibility testing for complete categorization of *S. epidermidis* from other CoNS species. CoNS species other than *S. epidermidis* were identified using six additional biochemical tests. The identification of individual species within CoNS was then validated by more expensive and reliable methods such as PCR and sequencing. The proposed scheme comprising a smart panel of eleven tests accurately identified all isolates of CoNS isolated from clinical material to the species level and could be recommended as an important strategy for recognising and realizing the importance of individual species of CoNS that are increasingly responsible for nosocomial infections.

2. Materials and methods

For the present study, a total of 242 Coagulase Negative Staphylococci (CoNS) isolates were collected from two different tertiary care hospitals such as Adichunchanagiri Institute of Medical Sciences and M. S. Ramaiah Medical College located in the south of India. Phenotypic identification of CoNS isolates was performed based on colony morphology on nutrient agar, Gram stain, catalase assay and tube coagulase assay. CoNS isolates were from different clinically significant samples such as blood, urine, pus, high vaginal swab, sputum and synovial fluid collected over a one-year period from September 2013 to August 2014. The ethical clearance for the study was obtained from the respective institutional ethical committee.

Five biochemical tests were chosen from the scheme of *Bergey's Manual of Systematic Bacteriology* and were used for speciation and identification of *S. epidermidis* within the CoNS group of isolates. The present study used a computer algorithm that calculates mathematical probability to choose a minimal number of tests required that would be best, which could discriminate maximum number of species out of the total number of 44 tests used in *Bergey's* Manual (Amarnath, 1995; Beau, 1983). Our identification scheme included the fermentation of mannitol, maltose, mannose, trehalose and novobiocin susceptibility testing which are the important biochemical tests for discriminating different species within CoNS according to the *Bergey's* Manual (Schleifer & Bell, 2009). *S. epidermidis* is a non-fermenter of mannitol, trehalose, while it ferments both maltose and mannose, with sensitivity to novobiocin (Fig. 1). Different types of sugar utilization tests were performed for phenotypic identification of *S. epidermidis* and other species of CoNS. Additional biochemical tests such as lactose and xylose fermentation, ornithine decarboxylase production, urease and acetoin and finally anaerobic growth were used for the speciation of species belonging to the CoNS group. All biochemical test experiments were evaluated by including typical reference strains representative of each species belonging to CoNS as positive controls in the study. Members belonging to other Gram-postive bacteria, *S.aureus* and other MTCC reference strains were used as controls (either negative or positive) for biochemical assays. The reference strains belonging to the various species that were used in the study were given below: ATCC 35984 (*S. epidermidis*), ATCC 12228 (*S. epidermidis*), MTCC 10219 (*S. cohnii* subsp. *cohnii*), MTCC 3610 (*S. simulans*), MTCC 6151 (*S. capitis* subsp.*capitis*), MTCC 9794 (*S. cohnii* subsp. *urealyticus*), MTCC 4436^T (*S. warneri*), MTCC 3383^T (*S. haemolyticus*), MTCC 7441 (*S. xylosus*), MTCC 6150 (*S. hominis* sussp. *hominis*). There were difficulties in supplying certain standard strains, namely *S. caseolyticus* and *S. auricularis* and therefore, they were not included in the study.

The following paragraphs give details of the important biochemical tests used in our short scheme.

2.1. Tube coagulase test

Briefly, the 0.5 ml overnight culture of test bacterial samples cultured in 10 ml Luria bertani broth after inoculation of 1 to 2 overnight grown bacterial colonies ($10^9/10^{10}$ bacteria) was taken as inoculum and mixed with 0.5 ml sheep plasma taken in fresh sterile small tubes. Reference strains of *S. aureus* and *S. epidermidis* were used as positive and negative control respectively. All tubes were incubated at 37 °C and hourly monitoring of tubes until the first four hours was performed. A positive result was indicated by the solidification of the plasma.

2.2. Sugar utilization tests

Briefly, 1% sugar was added to phenol red broth containing phenol red, which is an indicator of pH. If the sugar is used by the bacteria, it will produce acid that will cause the pH of the media to drop which results in media changing its colour, red to yellow, which will read as a positive result. All sugar utilization tests were carried out for 18–24 h incubation at 37 °C. The bacteria were grown in 10 ml phenol red broth after inoculation of 1–2 overnight grown bacterial colonies ($10^9/10^{10}$ bacteria) as inoculum.

2.3. Ornithine decarboxylase test

Ornithine decarboxylase test was performed to see if the microorganism uses ornithine as a carbon source. The composition of the media is ornithine with bromo cresol purple as an indicator with 0.1% of glucose. Test bacteria ($10^9/10^{10}$ bacteria) were inoculated into the sterile medium (10 ml) and incubated for 24–48 h at 37 °C. Bacteria were able to use glucose as the primary source first and transform it into yellow colour due to the drop in pH within 24 h. The enzyme ornithine decarboxylase becomes activated when the medium is acidified. The culture was incubated again for 24 h at 37 °C to allow the bacteria to use ornithine. The final results were observed after 48 h wherein the colour change to purple from yellow, indicating a positive test for ornithine decarboxylase. The absence of yellowing after 24 h or the failure to return to purple after 48 h indicates a negative result.

2.4. Urease test

The fresh colony of test bacteria was streaked on the slant of sterile urea agar base, supplemented with 2% urea and incubated for 18–24 h at 37 °C and then observed for the colour change. The urease enzyme is produced by many bacteria in which the enzyme splits urea in the presence of water to release ammonia and carbon dioxide. Ammonium