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Full Length Research Paper

Pitfalls in the routine diagnosis of *Staphylococcus aureus*

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Two hundred isolates of Presumed *Staphylococcus aureus* from routine clinical specimens, collected from two government hospitals in Abha, Saudi Arabia, had their identity verified. We used the tube coagulase test as our gold standard. Twenty (10%) of the isolates were mis-identified. Reliance by the two laboratories on DNase test, Mannitol fermentation reaction and improper performance of the coagulase test, were responsible for the mis-diagnosis.

Key words: *Staphylococcus aureus*, diagnosis, pitfalls.

INTRODUCTION

The genus Staphylococcus contains 32 species and 15 subspecies which are widespread in nature (Evangelista and Truant, 2002). Medically, only three of them are significant human pathogens: (A) *Staphylococcus aureus*, which causes various pyogenic infections like endocarditis, osteomyelitis; skin and soft tissue infections; toxin-mediated diseases such as food poisoning, toxic shock syndrome and the scalded skin syndrome; (B) *S. epidermidis*, which causes infections associated with foreign bodies, such as catheters and prosthetic devices and also a common member of the skin flora; and (C) *S. saprophyticus*, which causes urinary tract infections.(Ryan, 2004). *Staphylococci* and *Streptococci* are both Gram-positive cocci, but they are distinguished by two main criteria: (1) Microscopically, *Staphylococci* appear in grape-like clusters whereas *Streptococci* are in chains. (2) Biochemically, *Staphylococci* produce catalase, whereas *Streptococci* do not (Levinson and Jawetz, 2002).

Of the three species of *Staphylococci* that are human pathogens, *S. aureus* is by far the most important. *S. aureus* is distinguished from the others primarily by coagulase production (coagulase is an enzyme that causes plasma to clot by activating prothrombin). Furthermore, *S. aureus* usually ferments mannitol, haemolyzes red blood cells and possesses protein A on its surface, whereas the others do not.

In spite of the importance of *S. aureus*, especially the methicillin resistant *S. aureus* (MRSA), as a significant cause of nosocomial infections, enough attention is not paid to its diagnosis. The identification of *S. aureus* in most routine laboratories is, at best, treated casually, without due consideration. In other to prove this point, we decided to collect *S. aureus* strains isolated from routine clinical specimens from two government hospitals in Abha, Saudi Arabia and subject them to rigorous verification. Our findings are presented and discussed.

MATERIALS AND METHODS

Two hundred presumed *S. aureus* isolates from routine clinical specimens from Assir Central Hospital (ACH), a tertiary health-care facility and Abha General Hospital (AGH), a secondary health-care facility, both in Abha, Assir region of Saudi Arabia, were randomly collected between July 2002 and June, 2004. The procedures for

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Table 1. How to differentiate the three major *Staphylococci*.

Test	SA	SE	SS
Coagulase	+	-	-
Mannitol fermentation (MSA)	+	-	+
DNase.	+	(+) w	-
Sensitivity to Novobiocin 5µg disc.	S	S	R
Agglutination with S.aureus Latex reagent	+	-	-
Haemolysis on Blood agar	Beta*	-	-
Typical diseases caused	Abscesses, Food poisoning,TSS	Infection of prosthetic Heart valve and hips; Common skin flora	Urinary tract infection

SA = *S. aureus*; SE = *S. epidermidis*; SS = *S. saprophyticus*.
(+) w = weak positive reaction.
Beta* = Beta haemolysis not always present.
TSS = Toxic shock syndrome.

identifying *S. aureus* in both hospitals were similar. This consists of slide and/or tube coagulase test, using human plasma, fermentation of mannitol on MSA medium and DNase test. Sometimes, only one or varying combinations of these tests are used. The reason for collecting the isolates was never disclosed to the two hospitals so as not to influence their techniques. The isolates were sub-cultured on nutrient agar slopes, labeled and stored at 4-8°C. They were tested in batches every three months; but before carrying out the tests, the cultures were re-isolated on sheep blood agar, streaked for isolated colonies, and incubated in ambient air at 35°C overnight.

For the test, 2-3 identical colonies were picked, inoculated onto nutrient agar plate and 1 ml sterile nutrient broth, and incubated in ambient air at 35°C for 18-24 h. The next day, the broth culture was equivalent to 1-2 McFarland opacity tube. Table 1 shows the tests carried out on each isolate.

Coagulase test

Slide and tube coagulase tests were carried out using rabbit plasma with EDTA (Remel, TI No. 21050, Lenexa, USA). *S. aureus* ATCC 25923 and *S. epidermidis* ATCC12228 were used as positive and negative controls. 0.5 ml each of the overnight broth culture was added to 0.5 ml of rabbit plasma and incubated at 37°C for tube coagulase test. The tubes were observed gently every 30 min for clot-formation. If negative at 4 h, they were left at the same temperature overnight and a final reading taken at 24 h. Any tube that did not form a clot at 24 hours was regarded as negative.

Latex agglutination

The Pastorex Staph-plus (Code No. 56353, Bio-Rad, France), was used. One drop of this reagent was added to one drop of the overnight broth culture of the test organism (McFarland tube 1 or 2), on the test card and mixed. It was observed visually for agglutination. Each batch of tests had one positive and one negative control added.

DNase test

The DNase agar was prepared one day before it was used and stored in the fridge at 4-8°C. Each plate was divided into four segments. A sterile wire loop was dipped into the overnight broth culture of the *S. aureus* and inoculated straight down the middle of the segment. All four segments were similarly inoculated and the plates were incubated at 35°C in ambient air overnight. The next day, the plates were flooded with 1 N HCl and the excess decanted. The HCl was left to act for about ten minutes after which the plates were observed for clear zones around each streak of *S. aureus*.

The presence of a clear zone indicates a positive result, showing that DNase was produced and it digested the DNA. The absence of a clear zone indicates a negative result, that is, DNase was not produced. In order to avoid confusing the weak positive DNase reaction by *S. epidermidis*, we observed that the diameter of the clear zone around the *S. aureus* streak was always greater than 0.5 mm; whereas it was less for *S. epidermidis*.

Haemolysis

We noticed that several of the *S. aureus* isolates were non-haemolytic on sheep blood agar. Haemolysis therefore, is not a reliable index for identifying *S. aureus*.

RESULTS

Out of the 200 presumed *S. aureus* isolates collected from the two hospitals, 180 (90%) were confirmed as *S. aureus*; 13 (6.5%) were *S. saprophyticus* and 7(3.5%) were *S. epidermidis.* These aberrant results were traceable to the laboratories dependence on DNase and Mannitol fermentation as sole tests for identifying *S. aureus.* (Table1). Forty-two (21%) of the 200 isolates came from urine and high vaginal swabs. Twelve of the 180 *S. aureus* (6.7%) isolates, did not form bound coagulase. They were detected by tube coagulase test only. The tube coagulase test detects both the bound and free coagulase. Seven of the 180 *S. aureus* (3.9%), produced fibrinolysin and lysed their clot within 4 h. If they had been slide-coagulase negative and the tube coagulase test were done overnight, these strains would have been mis-diagnosed as coagulase negative *Staphylococci*.

Table 2 shows that DNase and the latex agglutination tests have comparable specificity but differ in sensitivity. Mannitol fermentation, however, has a higher sensitivity but much lower specificity. This makes it a good screen-

Table 2. Comparing the *S. Aureus*. Three diagnostic tests.

	DNase Test %	Latex agglutination (Pastorex Staph plus) %	Mannitol fermentation (MSA) %
Sensitivity	93	96	98
Specificity	96	98	85

Table 3. Practical points on coagulase test.

1	Do not use colonies from media with high-salt content such as MSA. It gives false positive result.
2	In the slide coagulase test, rule out auto-agglutination before adding plasma.
3	In slide and tube tests, the plasma must attain room temperature before use.
4	Observe the tube test every 30minutes for the first four hours or else you may miss an early clot, which may be lysed by fibrinolysin.
5	In the tube test, do not agitate or shake the tube or else the clot may dissolve and never forms again.
6	Cultures older than 18-24 hours may give false negative results.
7	Free coagulase is produced in broth culture only; therefore, use overnight broth culture.
8	No strains that produce free coagulase produce bound coagulase.
9	The tube test detects both free and bound coagulase.
10	Do not use Citrated plasma. Use only EDTA/Oxalated/Heparinized plasma. Check cultures that give positive result with citrated plasma for purity (Gram-stain and culture). Human plasma gives discordant results, so use rabbit plasma.
11	Perform the tube coagulase test only if you have a minimum of four hours to observe the clot-formation. If it is negative at 4 hours, then, incubate it overnight and take the final reading at 24 hours.
12	Positive and negative controls must be included in all test procedures.

ing test, especially where genital specimens are common. Table 3 lists key practical points that are often glossed over. The observance of these points would eliminate most, if not all the, pitfalls associated with the performance of coagulase test.

DISCUSSION

Routine laboratories worldwide face several constraints, particularly in the developing countries. Such constraints vary from inadequate resources, shortage of personnel and inundation with specimens. All these have their consequences on the quality of their results.

Medical laboratories worldwide play a key role in patient management as physicians demand and depend upon laboratory tests and investigations to support their diagnosis and as a basis for the treatment of their patients (Savellano, 2004). Conscious of this demand, most laboratories try to ensure that their products are both accurate and timely.

Some of the above constraints are present in our two hospitals. For example, most of the time they did the coagulase test with human plasma because rabbit plasma was unavailable. Some other times, however, the technicians felt that DNase test and mannitol fermentation on mannitol salt agar were good enough substitutes for identifying *S. aureus.* Forty-two (21%) of the isolates, came from urine and high vaginal swabs. This makes such dependence extremely dangerous. The regular half-hour inspection for clot-formation when doing the tube coagulase test, was often not observed, even when the test was done during the long hours of work, because of lack of time. Seven (3.9%) of the *S. aureus* produced fibrinolysin which lysed their clot within the first four hours. These strains would have been mis-diagnosed as coagulase negative *Staphylococci*, if the tubes were not so observed.

The manufacturers of the latex agglutination reagent that we used, (Bio-Rad, France), claimed a sensitivity of 99-100 percent, but we got a sensitivity of 96 percent and a specificity of 98 percent. The reason for this difference may lie in strain variations between European and Saudi Arabian isolates. Whatever is the answer, the fact remains that this test should never be used to the exclusion of tube coagulase test.

Out of the two hundred presumed *S. aureus* isolates, we confirmed one hundred and eighty (90%), as correctly diagnosed (Table 1). The reasons above are responsible for the few cases of mis-diagnosis that we found in this

study. We listed the key practical points in Table 3 that will help eliminate such errors. In addition, we recommend that there is no need for laboratories to introduce routine DNase test and Mannitol fermentation into their diagnosis of *S. aureus.* The only time such a practice may be required is when they have no access to plasma of any sort. In that case, such tests may be used as screening tests and positive isolates must be confirmed with coagulase test when plasma becomes available. When using human plasma, it is advisable to pool together samples from at least five to ten persons and then distribute them in small aliquots sufficient to last one week and freeze the rest until required. This will help eliminate errors due to insufficient fibrinogen or the presence of inhibitory substances in any particular patient's sample.

We know that *S. pyogenes, Moraxella* and *Serratia* species, may give a positive DNase test. In addition, some virulent strains of *Yersinia pestis* are coagulase positive (Cheesbrough, 2000). It is therefore essential that basic bacteriological principles must be observed, such as colony morphology, Gram reaction, and pure-culture technique, before performing any biochemical tests. These will certainly help to minimize errors.

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