Isolation and Characterization of Coagulase-Negative Methicillin-Resistant *Staphylococcus aureus* from Patients in an Intensive Care Unit

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Abstract

Objective: To report the isolation and characterization of four coagulase-negative methicillin-resistant *Staphylococcus aureus* isolated from patients admitted to the ICU of a Kuwait hospital by bacteriological and molecular methods. **Methods:** The isolates were characterized by cultural characteristics, Gram stain, catalase, coagulase, DNase and biochemical tests and typed by a combination of antibiogram and pulsed-field gel electrophoresis to evaluate their relatedness. **Results:** These isolates were gram-positive cocci in clusters, catalase, DNase and slide coagulase-positive but tube coagulase-negative. They gave negative results for ornithine decarboxylase and pyrrolidonyl-arylamidase tests, which indicated that these were not *Staphylococcus lugdunensis* or *Staphylococcus schleiferi*, and were identified as *S. aureus* by API Staph. They had identical antibiograms and pulsed-field gel electrophoresis patterns which suggested that they had a

common origin. **Conclusion:** The results highlight the need to complement the coagulase tests with other tests such as DNase and biochemical tests to correctly identify *S. aureus*. Coagulase-negative *S. aureus* appears to be an increasing problem that clinical laboratories should be aware of. They are as virulent as those producing coagulase and can colonize, cause infections and spread among patients.

Introduction

Staphylococcus aureus is an important cause of infections in humans including superficial skin infections, surgical wound infections, septicaemia and toxic shock syndrome [1]. They produce an array of enzymes, including coagulase, that contribute to their virulence. Coagulase is thought to contribute to the localization of the organisms at the site of

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infection by producing a fibrin barrier [1–3]. Coagulase is also used to identify *S. aureus* and differentiate it from other staphylococci that do not produce it (coagulase-negative staphylococci). Coagulase exists in two forms: a bound coagulase or clumping factor which is detected by a slide test, and free coagulase (staphylocoagulase) detected by the tube test [3]. In addition, *S. aureus* also produces DNase, an enzyme that hydrolyses DNA [1–3]. Approximately 99% of coagulase-positive strains of staphylococci also produce DNase [4, 5]. Because of the high degree of correlation between coagulase and DNase production [2–4] both tests have been recommended for use as diagnostic tests for *S. aureus*.

Besides *S. aureus*, two coagulase-negative staphylococci, *Staphylococcus lugdunensis* and *Staphylococcus schleiferi* (which are slide coagulase-positive), produce clumping factor but not free coagulase (tube coagulase-negative) and can be misidentified as *S. aureus* by the slide coagulase test [7, 8]. In addition to *S. lugdunensis* and *S. schleiferi*, rare strains of *S. aureus* have been reported to produce only clumping factor and DNase, but not free coagulase [6, 9–14]. Also *S. aureus* variants lacking clumping factor [12, 13], clumping factor and protein A [14–17] and catalase production [18, 19] have been reported. These reports suggest that although the coagulase-negative variants of *S. aureus* are considered to be rare, their numbers appear to be increasing. It is possible that a significant number of them are still missed or misidentified especially if identification is based only on the slide coagulase test. In some laboratories the tube coagulase test is performed only when the slide test is negative. Thus it is important to recognize and correctly identify these variant strains of *S. aureus* in clinical samples and distinguish them from *S. lugdunensis* and *S. schleiferi*, so that their true incidence and clinical importance can be appreciated.

Patients and Methods

Patient 1

A 43-year-old Kuwaiti lady was admitted to the Al-Amir Hospital, Kuwait on January 18, 1996 with pulmonary oedema. She has had previous admissions to the intensive care unit (ICU) for artificial ventilation with antecedent history of recurrent urinary tract and chest infections for which she was treated with several antibiotics including ampiclox, norfloxacin and cefotaxime. During her last hospital admission, she developed cardiopulmonary arrest and was admitted to the ICU. Mandatory infection control body screen for methicillin-resistant *S. aureus* (MRSA) on admission to the ICU showed no evidence of nasal carriage of MRSA. A week later, a DNase-positive and tube coagulase-negative MRSA (atypical MRSA) was isolated from the urine in mixed culture, and from the tracheotomy secretion (designated isolate 505). The growth from both sites was considered to be colonization because of the presence of a few pus cells in the direct smear and because the patient was afebrile with normal peripheral leucocyte count.

Patient 2

A 70-year-old Kuwaiti lady was admitted to the Al-Amiri Hospital on April 5, 1996. She was found to be febrile, unconscious and on a ventilator on admission. Her temperature on admission was 40°C with a WBC of $20.4 \times 10^3/\text{mm}^3$. Atypical MRSA, as described for patient 1, was isolated from her blood culture (isolate 523), the central venous pressure catheter tip and endotracheal secretion. She was treated with vancomycin for 2 weeks with the addition of rifampicin (600 mg) during the 2nd week. The central venous pressure line was changed because her temperature did not decrease within 72 h of vancomycin treatment. She continued to be febrile with high leucocyte count. The endotracheal secretion and central venous pressure tip cultures became negative for MRSA after vancomycin and rifampicin treatment. One month later, a repeat blood culture remained positive for atypical MRSA with the same antibiogram (isolate 534). The patient died on 3 June 1996.

Patient 3

A 70-year-old Kuwaiti lady was admitted to the Al-Amir Hospital on 21 May 1996 for the creation of an arteriovenous graft. She was a known case of ischaemic heart disease with recurrent attacks of left ventricular failure, hypertension, diabetes mellitus and chronic renal failure. She was also a known nasal carrier of MRSA. Nasal swab taken on admission for MRSA

screening yielded atypical MRSA (isolate 524), which was promptly cleared with the nasal mupirocin preparation. Repeat nasal swab culture after 1 week was negative for atypical MRSA.

Bacteriological Investigations

MRSA Isolates. The strains were isolated in the diagnostic microbiology laboratory of the Al-Amiri Hospital where the initial identification tests were performed. Confirmatory tests and molecular characterization were performed at the Staphylococcal Research Laboratory at the Faculty of Medicine, Kuwait University. The strains were isolated on blood agar plates (brain heart infusion agar plus 5% sheep blood) and on mannitol salt agar and identified using standard methods which included colonial morphology, Gram stain, catalase, DNase and coagulase tests. Coagulase activity was detected in both slide and tube tests using both human and rabbit plasma (Difco Laboratories, Detroit, Mich., USA) as described previously [3]. The tube coagulase test was repeated and results were read after 1-, 2-, 3- and 4-hour incubation at 35°C and after overnight incubation at room temperature. DNase production was detected by using DNase test agar (BBL) inoculated with a short streak and tested after 24-hour incubation by flooding the plate with 1 N HCl [4]. Biochemical tests were performed with the API Staph identification kit (BioMérieux, Marcy-L'Etoile, France, Ref. 20500). Protein A and the clumping factor were detected with the Staphytest latex agglutination kit (Oxoid, Basingstoke, Hampshire, England) according to the manufacturer's instructions. Ornithine decarboxylase and pyrrolidonyl-arylamidase (PYR) were detected with API 20E (ref. 20 100, BioMérieux) and API 20 Strep (ref. 20 600, BioMérieux), respectively. The tests were controlled with *S. aureus* strain ATCC-25923.

Toxin Production. Enterotoxin production and toxic shock syndrome toxin were tested by the SET-RPLA and the TST-RPLA detection kits (Oxoid), respectively. Both tests were performed by following instructions provided by the manufacturer.

Susceptibility Testing. Susceptibility to antimicrobial agents was tested by the disk diffusion method as described previously [20, 21] using the following commercial antibiotic disks (Oxoid): methicillin 5 µg), penicillin (10 U), gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg), streptomycin (30 µg), erythromycin (15 µg), clindamycin (2 µg), chloramphenicol (30 µg), tetracycline (10 µg), minocycline (30 µg), trimethoprim (2.5 µg), fusidic acid (10 µg), rifampicin (5 µg), ciprofloxacin (5 µg), mupirocin (200 µg), teicoplanin (30 µg), vancomycin (30 µg). For

testing their sensitivity to heavy metals and nucleic acid-binding compounds [21], 6-mm disks were impregnated with cadmium acetate (50 µg), propamidine isethionate (50 µg) and ethidium bromide (60 µg).

Pulsed-Field Gel Electrophoresis. Pulsed-field gel electrophoresis of *Sma*I-digested genomic DNA was performed as reported previously [22].

Results

Identification of Isolates

Six slide-positive but tube coagulase-negative MRSA were isolated from clinical samples at the microbiology laboratory of the Al-Amiri Hospital, Kuwait, between 1 January and 31 May, 1996. The 6 isolates were from 3 patients admitted at different times to the ICU. However, only 4 of them were studied further because the first 2 were not kept as they were not considered to be significant. Their significance became apparent when the other isolates were obtained.

The isolates formed creamy, non-haemolytic colonies on blood agar plates. They were gram-positive cocci in clusters and gave positive results for catalase, DNase and slide coagulase tests with both human and rabbit plasma but gave negative results for the tube coagulase test. Whereas rabbit plasma is used routinely for the coagulase test in the laboratory, human plasma was used for the slide coagulase test because it has been found to demonstrate clumping factor better than rabbit plasma [7, 8, 23]. The tube coagulase tests were consistently negative after 1-, 2- and 4-hour incubation at 35°C and after an overnight incubation at room temperature. In contrast, the positive control strain gave a positive reaction after 1 h and remained positive after on overnight incubation. The Staphytest latex agglutination test for protein A and clumping factor was also positive for all the 4 isolates.

Table 1. Biochemical reactions of coagulase-negative MRSA isolates

Tests	505	523	524	534	ATCC25923
<i>Aerobic acid from</i>					
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
Xylitol	-	-	-	-	-
D-Melibiose	-	-	-	-	-
Raffinose	-	-	-	-	-
Xylose	-	-	-	-	-
Sucrose	-	-	-	-	+
Alkaline phosphatase	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Acetoin production	+	+	+	+	-
N-Acetyl glucosamine	+	+	+	-	+
α -Methyl-D-glucoside	-	-	-	-	+
Arginine dihydrolase	+	+	+	+	+
Urease production	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-
PYR	-	-	-	-	-

When subcultured onto mannitol salt agar, they gave only a faint colour change after 48-hour incubation when compared with a mannitol fermenting control strain. However, they fermented mannitol readily in the API Staph identification kit. The biochemical profiles obtained with the API Staph identification strips are presented in table 1. Their biotypes derived from the API results were 6736153 for isolates 505, 523 and 534 and 6736113 for isolate 524. All of them fermented mannitol, maltose, produced urease and alkaline phosphatase.

Tests for ornithine decarboxylase and PYR, which were performed to distinguish these isolates from *S. lugdunensis* and *S. schleiferi*, were negative. *S. lugdunensis* strains are usually mannitol- and alkaline phosphatase-negative, ornithine decarboxyl-

ase- and PYR-positive. *S. schleiferi* are usually alkaline phosphatase- and PYR-positive, ornithine decarboxylase- and mannitol-negative [7, 8]. All 4 isolates were identified as *S. aureus* based on these biochemical reactions.

Toxin Production

Some *S. aureus* strains produce toxic shock syndrome toxin (TSST-1) which causes toxic shock syndrome, characterized by fever, skin rash, hypotension and multi-system organ failure in patients who harbour them. As the isolates were from very ill patients they were tested for the production of TSST-1 and staphylococcal enterotoxins. Only the nasal isolate 524 produced staphylococcal enterotoxin A while the blood culture isolate 534 produced TSST-1.

Fig. 1. Pulsed-field gel electrophoresis of tube coagulase-negative MRSA. Lane 1 = Lambda DNA concatemers used as molecular weight markers; lane 2 = isolate No. 505; lane 3 = isolate No. 523; lane 4 = isolate No. 524; lane 5 = isolate No. 534.

Resistance to Antimicrobial Agents

They had identical resistance profiles and were resistant to methicillin, gentamicin, kanamycin, neomycin, streptomycin, tobramycin, erythromycin, clindamycin, tetracycline, trimethoprim and ciprofloxacin. They were also resistant to the heavy metal salts, cadmium acetate, the quaternary ammonium compound propamidine isethionate and to ethidium bromide. They were susceptible to

vancomycin, teicoplanin, mupirocin, rifampicin, minocycline and novobiocin.

Molecular Characterization of the Isolates

All 4 isolates were compared by pulsed-field gel electrophoresis of *Sma*I restriction digest of their genomic DNA to assess their relatedness and establish a case for cross-contamination. They all had identical pulsed-field gel electrophoretic patterns as presented in figure 1.

Discussion

Four isolates of tube coagulase-negative, clumping factor- and DNase-positive MRSA isolated from elderly and critically ill patients have been characterized and typed by pulsed-field gel electrophoresis to establish their relatedness. Traditionally, the ability to produce coagulase has been the most widely accepted characteristic for the identification of *S. aureus* [1–3]. All 4 isolates produced catalase, DNase, clumping factor and protein A but not free coagulase. However, results of PYR and ornithine decarboxylase tests demonstrated clearly that they were neither *S. lugdunensis* nor *S. schleiferi*, the two coagulase-negative staphylococci which can be misidentified as *S. aureus* because of their ability to produce clumping factor but not free coagulase [7, 8, 23]. Therefore on the basis of their biochemical reactions presented in table 1, which were consistent with those of *S. aureus*, their biotypes and cultural characteristics, they were identified as *S. aureus*. They were also multiply resistant to antimicrobial agents, which is characteristic of the majority of MRSA [21, 22]. Their isolation for the first time in Kuwait confirms reports of other coagulase-negative variants of *S. aureus* [4, 6, 9–13] isolated from clinical samples. The results also suggest that the tube coagulase-negative

variants of *S. aureus* may not be as rare as previously thought.

The coagulase-negative MRSA was associated with bloodstream infection in 1 patient and colonization in the other 2 patients, which indicated that the loss of free coagulase production did not abolish their ability to cause an infection. This observation is in agreement with the findings by Baddour et al. [24], that laboratory mutants of *S. aureus* made deficient in coagulase production were as virulent as their parental strains in animal experiments. In addition, tube coagulase-negative *S. aureus* have been reported as a cause of infective endocarditis [4, 10] and to spread among patients [12, 15]. The observation that the bloodstream isolate produced TSST-1 which causes toxic shock syndrome and the nasal isolate produced staphylococcal enterotoxin A often association with staphylococcal food poisoning, strengthens the argument that tube coagulase-negative MRSA are important pathogens. Therefore, it is important to correctly identify these strains as *S. aureus*, as a failure to identify them as such may have both therapeutic and epidemiological implications.

The tube coagulase-negative MRSA isolates were obtained from very sick patients with histories of long-term therapy with different antibiotics. Similar observations have been made by Mackay et al. [12]. It has been observed previously that exposure of *S. aureus* in vitro to antibiotics such as oxytetracycline [2], gentamicin [25] and other chemicals can shut off coagulase production, inactivate it or delay its activity. The loss of coagulase production may also occur naturally due to an error during transcription [26]. Therefore, it is possible that these isolates were derived from a coagulase-positive MRSA which had mutated to become coagulase-deficient over time due to an exposure to different antibiotics that failed to kill them.

All 4 tube coagulase-negative MRSA isolates had identical pulsed-field patterns. This suggests that they had a common origin and that a single strain had spread among the patients. However, since the patients were admitted at different times it was difficult to establish a case of cross-contamination among them. Therefore, it would appear that each patient acquired the organisms independently. It is likely that the ICU environment served as a reservoir and the source of the organism, and the different patients acquired them from the environment when they were admitted. The environment is known to serve as a reservoir for MRSA outbreaks [27]. Unfortunately MRSA isolates from the ICU environment were not available for comparison with the patients' isolates.

This report has highlighted the dilemma faced by diagnostic microbiology laboratories attempting to identify *S. aureus* based only on results of the coagulase tests. It is constantly debated as to whether the slide or tube coagulase test alone is sufficient to accurately identify *S. aureus*. Although the tube coagulase-negative variants reported here would have been correctly identified by the slide test as *S. aureus*, they would have been missed if only the tube test was performed. Therefore both the slide and tube coagulase tests are essential for the identification of *S. aureus*. Other tests such as DNase, thermonuclease and biochemical tests can complement the coagulase tests where discrepancies occur. It is also important to distinguish *S. aureus* from the clumping factor producing coagulase-negative staphylococci.

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