Identification of biochemically atypical *Staphylococcus aureus* clinical isolates with three automated identification systems

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Between January and April 2002, a total of 271 strains of *Staphylococcus aureus* were isolated from clinical specimens at Toho University Omori Hospital, Japan, including 201 (74.2 %) which were identified as methicillin-resistant *S. aureus* (MRSA). However, 34 (12.5 %) were biochemically atypical, because they did not produce acid on mannitol salt agar or did not agglutinate in Staphaurex testing but were categorized as MRSA by PCR analysis and by antibiotic susceptibility. Three automatic identification systems, AutoScan-4[®] (Dade Behring), BD Phoenix[™] (Becton Dickinson) and Vitek[®] 2 (bioMérieux), were evaluated by testing these atypical *S. aureus* isolates. The AutoScan-4[®] and Phoenix[™] systems identified all 34 isolates as *S. aureus*. Without additional tests such as Staphaurex, observation of colony pigment and haemolysins on sheep blood agar, Vitek[®] 2 identified only 16 isolates (47.1 %) as *S. aureus* with good or better confidence levels and misidentified one of the remaining isolates as *Staphylococcus chromogenes*. This study shows that it is possible to identify these physiologically atypical *S. aureus* isolates correctly by using the Phoenix[™] and AutoScan-4[®] fully automatic identification systems.

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INTRODUCTION

Staphylococci are widespread in nature, although they are mainly found living on the skin and mucous membranes. The coagulase-positive species *Staphylococcus aureus* is well known as a human pathogen. Serious infections produced by *S. aureus* include bacteraemia, pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, encephalitis, meningitis, choriomnionitis, mastitis, scalded skin syndrome and abscesses of the muscle, urinogenital tract and various intra-abdominal organs (Murray *et al.*, 2003). The species is identified on the basis of a variety of conventional physiological or biochemical characters. The key characters for *S. aureus* are colony pigment, free coagulase, clumping factor, protein A, heat-stable nuclease and acid production from mannitol (Murray *et al.*, 2003). In addition, *S. aureus* can be identified by PCR methods. Sequences targeted by PCR include *tst* (encoding the toxic shock syndrome protein), *eta* and *etb* (encoding exfoliative toxins A and B, respectively), staphylococcal enterotoxin genes such as *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej* and *sel*, *nuc* (encoding thermostable nuclease) and the Sa442 DNA fragment (Becker *et al.*, 2003; Martineau *et al.*, 1998; Pinto *et al.*, 2005). Since the 1980s,

methicillin-resistant *S. aureus* (MRSA) has spread widely to become a major clinical and epidemiological problem in many medical centres (Maple *et al.*, 1989; Matsushashi *et al.*, 1986). Some MRSA isolates may be biochemically atypical compared to methicillin-susceptible *S. aureus*, particularly in coagulase production or acid production from carbon sources (Berke & Tilton 1986; Smole *et al.*, 1998; Wilkerson *et al.*, 1997). In clinical microbiology laboratories, it is very important to distinguish *S. aureus* from other staphylococci, because *S. aureus* is an important nosocomial pathogen (Murray *et al.*, 2003).

A total of 271 *S. aureus* isolates were isolated by the Clinical Laboratory Department in Toho University Omori Hospital, Japan, from January to April 2002. These isolates, including 34 biochemically atypical isolates, were analysed using Vitek[®] 2 and its optional tests. Thirty-four (12.5 %) were found to be biochemically atypical, because they did not produce acid from mannitol salt agar or did not agglutinate in Staphaurex testing but were categorized as MRSA by PCR analysis and antibiotic susceptibility. Because of an increased frequency of isolation of physiologically atypical *S. aureus* and the need to identify them accurately, this collection of isolates was used to evaluate three automatic identification systems, AutoScan-4[®], BD Phoenix[™] and Vitek[®] 2.

Abbreviation: MRSA, methicillin-resistant *Staphylococcus aureus*.

METHODS

Bacterial strains. The date of isolation and origin of the clinical isolates used in this study are given in Table 1. All atypical isolates were isolated at Toho University Omori Hospital between February and April 2002. Data from pulsed-field gel electrophoresis provided by the hospital infection control team indicated that the 34 *S. aureus* isolates used did not have a common origin (data not shown).

Identification of *S. aureus*. All isolates were initially evaluated by Vitek® 2 with additional tests and then stored at -80°C using 30 % glycerol. Additionally, Staphaurex (Murex Biotech) was used to

detect clumping factor, as membrane-bound coagulase, and protein A. Free coagulase production was detected by aggregation of rabbit plasma (Eiken Chemical Co.) by the tube method, according to the supplier's instruction manual. Mannitol fermentation was tested on mannitol salt agar (Murray *et al.*, 2003). Haemolysis was detected on sheep blood agar after 20 h incubation at 35°C ; yellow pigment production was defined as the visual detection of carotenoid pigments by two or more people after 24 h incubation at 35°C (Murray *et al.*, 2003).

Confirmation of *S. aureus* by species-specific PCR and detection of the *mecA* gene. DNA amplification of the 34

Table 1. Atypical *S. aureus* isolates used in the evaluation of three automatic identification systems and their biochemical characteristics

All isolates produced free coagulase, detected by aggregation of rabbit plasma. Acid production from mannitol was observed by a change in colour of the mannitol-salt agar plate. Haemolysis of colonies and yellow pigment were observed on trypticase soy agar II with 5 % sheep blood. BAL, Bronchoalveolar lavage.

Isolate	Isolation date	Specimen	Aggregated by Staphaurex	Acid production from mannitol	Haemolysin activity	Yellow pigment
1 ^{a*}	10 April 2002	Pharyngeal swab	+	—	+	+
2	29 March 2002	Umbilical swab	+	—	+	+
3 ^b	29 March 2002	Skin	+	—	+	+
4 ^b	1 April 2002	Pharyngeal swab	+	—	+	+
5 ^b	1 April 2002	Pharyngeal swab	+	—	+	+
6 ^b	1 April 2002	Pharyngeal swab	+	—	+	+
7	22 February 2002	Faeces	—	+	+	+
8†	1 April 2002	Intravenous catheter	+	—	—	+
9	1 February 2002	Faeces	—	+	+	+
10 ^a	18 March 2002	Pharyngeal swab	+	—	+	+
11 ^a	23 March 2002	Nasal swab	+	—	—	—
12	26 March 2002	BAL fluid	+	—	—	+
13	26 March 2002	BAL fluid	+	—	—	+
14	25 March 2002	Urinary catheter	—	+	+	+
15 ^a	5 March 2002	BAL fluid	+	—	—	—
16 ^a	7 March 2002	Pharyngeal swab	+	—	+	+
17 ^a	8 March 2002	Pharyngeal swab	+	—	+	+
18 ^a	8 March 2002	Pharyngeal swab	+	—	+	+
19	8 March 2002	Vaginal swab	+	—	—	+
20 ^a	8 March 2002	Vaginal swab	+	—	+	+
21	4 February 2002	Sputum	—	+	+	+
22	8 March 2002	Pharyngeal swab	+	—	+	+
23 ^a	8 March 2002	Vaginal swab	+	—	+	+
24	8 March 2002	Pharyngeal swab	+	—	—	—
25 ^a	8 March 2002	Pharyngeal swab	+	—	+	+
26	15 March 2002	Blood	—	+	+	+
27	26 February 2002	Intravenous catheter	—	+	+	+
28	4 March 2002	Vaginal swab	+	—	+	+
29 ^a	4 March 2002	Vaginal swab	+	—	+	+
30	5 March 2002	Skin	—	+	+	+
31 ^a	5 March 2002	BAL fluid	+	—	+	+
32 ^a	4 March 2002	Intravenous catheter	+	—	—	—
33	8 February 2002	Vaginal swab	—	+	+	+
34	20 February 2002	Faeces	—	+	+	+

*Resolved by additional tests: *a*, coagulase production; *b*, coagulase production, yellow pigment and haemolysis.

†Identified as *S. chromogenes* by the Expert system of Vitek® 2.

isolates was performed with colony direct PCR (Tsuchizaki *et al.*, 2000). A small portion of a colony was picked up by a toothpick, transferred directly to 50 µl of PCR mixture containing 50 pmol of each oligonucleotide primer from the Sa442 set (Martineau *et al.*, 1998), 25 µl SYBR® Green Master Mix (Applied Biosystems) and autoclaved MilliQ water. *S. aureus* FDA 209P and *Staphylococcus epidermidis* ATCC 14990 were used as positive and negative controls, respectively, for the PCR. The thermal cycling protocol was as follows: 5 min at 95 °C for hot start of DNA polymerase and initial denaturation followed by 40 cycles of two steps consisting of 1 s at 95 °C for denaturation and 55 °C for the annealing and extension steps. Real-time detection of the PCR product was performed on an ABI PRISM 7000 Sequencing Detection System (Applied Biosystems) by measuring the fluorescence signal. Specificity of the fluorescence signal was estimated by a denaturation protocol to compare with a theoretical T_m value of the PCR product after 40 cycles.

The *mecA* gene was used as the gold standard for detection of methicillin resistance by PCR assay. The specific primer set for *mecA* reported by Reischl *et al.* (2000) was used. PCR conditions used were as previously described (Martineau *et al.*, 1998). *S. aureus* N315 (Hiramatsu *et al.*, 1992) and *S. aureus* FDA 209P were used as positive and negative controls for the *mecA* gene, respectively.

Biochemical identification. Inocula for the following studies were prepared using a nephelometric device to adjust the turbidity to McFarland standard 0.5. *S. aureus* FDA 209P and *S. epidermidis* 14990 were used as positive and negative controls, respectively. Analysis of the results was based on the computerized reports from each identification system.

Identification with AutoScan-4® system. Preparation of the AutoScan Pos ID panel (Dade Behring), inoculum preparation, panel rehydration and inoculation, biochemical overlays (Pos ID only), incubation, reading of the panels and quality control were performed according to the manufacturer's instructions. Pos ID panels were read visually after 24 h incubation at 35 °C. The test reactions were read by the AutoScan-4® (Dade Behring) and the results were converted to compare with the AutoScan updated database.

Identification with Vitek® 2. The test panels (ID-GPC; bio-Mérieux) were automatically filled by a vacuum device, sealed and inserted into the Vitek® 2 reader-incubator module (bioMérieux) and subjected to a kinetic fluorescence measurement every 15 min. The results were interpreted by the ID-GPC database and final results were obtained automatically.

Identification with the Phoenix™ system. The Phoenix™ system (Becton Dickinson) was used according to the manufacturer's instructions with PMIC/ID14 panels (Becton Dickinson) for strain identification and oxacillin-susceptibility testing. Test suspensions were prepared from pure bacterial cultures grown on trypticase soy agar II with 5% defibrinated sheep blood (Becton Dickinson). The ID suspension was inoculated within 30 min into the panel, which was then loaded into the instrument for incubation at 35 °C and continuous reading. The results were interpreted by the ID-GPC database and final results were obtained automatically.

RESULTS AND DISCUSSION

One of the most important strategies to prevent and control the spread of MRSA is early and correct identification of positive strains, including those coming from diseased or colonized areas. Samples used in this study came from both. Several samples were taken as a precaution due to a high incidence of MRSA infections in the neonatal intensive care

unit (e.g. vaginal samples taken from pregnant women prior to delivery, pharyngeal samples or umbilical samples taken from newborns) (Table 1). Once an isolate has been positively identified, cases of disease can be treated appropriately, and other procedures such as patient isolation, decontamination of exposed areas and increased hygiene measures can take place. Thus, identification is crucial.

All 34 isolates in this study were confirmed as *S. aureus* by PCR using the Sa442 primer set. Free coagulase production was confirmed for all isolates by the tube method with rabbit plasma. Nine of 34 isolates did not aggregate in the Staphaurex test, 25 of 34 isolates did not produce acid from mannitol, 26 of 34 had haemolysin activity and only 30 of these clinical isolates produced yellow pigment (Table 1). In addition, these 34 strains were confirmed as MRSA (*mecA* gene positive) by PCR analysis. Antibiotic susceptibility testing also confirmed all isolates as MRSA by the Phoenix™ system.

Out of 34 isolates tested, a concordant identification to the species level was obtained by the Phoenix™ system, AutoScan-4® (Table 2) and genetic determination by PCR for all the isolates tested. On the other hand, only 16 (47.1%) isolates were identified as *S. aureus* by Vitek® 2, with good or better confidence levels, without the use of supplementary tests such as Staphaurex and/or haemolysin activity and pigment of colony on sheep blood agar (Table 2). One strain was identified incorrectly as *Staphylococcus chromogenes* by the Vitek® 2 instrument (Table 1).

The 34 isolates used formed 24 clusters by pulsed-field gel electrophoresis when the data were analysed by the criteria of Tenover *et al.* (1995) (data not shown). Therefore, these atypical MRSA isolates have 24 or more origins.

Toho University Omori Hospital uses the Vitek® 2 system for identification of clinical isolates. This system had previously proved to provide accurate and acceptable identification and antibiotic susceptibility for Gram-positive cocci (Ligozzi *et al.*, 2002). Recently, the frequency of isolation of *S. aureus* with atypical physiological characteristics has increased to approximately 12.5% (data not shown) in Toho University Omori Hospital, and it is very difficult to identify atypical *S. aureus* by the Vitek® 2 system unless extra tests are used. Furthermore, there have been several reports on the limitations of this identification system in distinguishing staphylococcal species (Becker *et al.*, 2004; Ben-Ami *et al.*, 2005).

Several genes have been targeted for PCR analysis of *S. aureus* but among these genes, *tst* genes, *eta* and *etb* and staphylococcal enterotoxin genes are not always detected in *S. aureus* (Becker *et al.*, 2003; Pinto *et al.*, 2005). The *nuc* gene has been widely used for species-specific detection, although it has also been reported in *Staphylococcus intermedius* strains (Becker *et al.*, 2005). Thus, the Sa442 DNA fragment was used to confirm *S. aureus* by PCR in the present study. Recently, Klaassen *et al.* (2003) reported that

Table 2. *S. aureus* isolates with dissenting or ambiguous results in identification by Vitek® 2, AutoScan-4® or Phoenix™

Isolate	Identification by Vitek® 2 (<i>T</i> index, confidence level)	Confidence value (%)	
		AutoScan-4®	Phoenix™
1	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.46, low)	99.9	99
2	<i>S. aureus</i> (0.31, good)	99.9	99
3	<i>S. chromogenes</i> (0.58, low); <i>S. hyicus</i> (0.50, low); <i>S. aureus</i> (0.49, low)	99.8	99
4	<i>S. aureus</i> (0.59, low); <i>S. chromogenes</i> (0.58, low); <i>S. hyicus</i> (0.50, low)	90.5	99
5	<i>S. aureus</i> (0.59, low); <i>S. chromogenes</i> (0.58, low); <i>S. hyicus</i> (0.50, low)	99.9	99
6	<i>S. chromogenes</i> (0.58, low); <i>S. hyicus</i> (0.50, low); <i>S. aureus</i> (0.49, low)	90.5	99
7	<i>S. aureus</i> (1.00, excellent)	99.9	98
8	<i>S. chromogenes</i> (0.50, good)	92.6	99
9	<i>S. aureus</i> (0.88, excellent)	99.9	99
10	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.56, low)	92.6	99
11	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.56, low)	99.9	99
12	<i>S. aureus</i> (0.56, very good)	99.9	99
13	<i>S. aureus</i> (0.56, very good)	99.9	99
14	<i>S. aureus</i> (0.90, excellent)	99.9	99
15	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.56, low)	99.9	99
16	<i>S. chromogenes</i> (0.58, low); <i>S. hyicus</i> (0.50, low); <i>S. aureus</i> (0.49, low)	99.8	99
17	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.56, low)	99.9	99
18	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.46, low)	99.9	99
19	<i>S. aureus</i> (0.56, very good)	99.8	99
20	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.46, low)	99.9	99
21	<i>S. aureus</i> (0.90, excellent)	99.9	99
22	<i>S. aureus</i> (0.84, excellent)	90.5	99
23	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.46, low)	99.8	99
24	<i>S. aureus</i> (0.56, very good)	99.9	99
25	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.56, low)	99.9	99
26	<i>S. aureus</i> (0.90, excellent)	99.9	99
27	<i>S. aureus</i> (0.65, very good)	99.9	99
28	<i>S. aureus</i> (0.56, very good)	99.8	99
29	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.46, low)	99.9	99
30	<i>S. aureus</i> (0.65, very good)	99.9	99
31	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.46, low)	99.8	99
32	<i>S. chromogenes</i> (0.58, low); <i>S. aureus</i> (0.46, low)	99.9	99
33	<i>S. aureus</i> (1.00, excellent)	99.9	99
34	<i>S. aureus</i> (1.00, excellent)	99.9	99

the Sa442 primer set did not work against the clinical isolate *S. aureus* 550226. They concluded that a number of *S. aureus* strains may have been misidentified in the past or the presence of *S. aureus* in clinical isolates may have been overlooked when identification was based solely on the Sa442 PCR assay. However, in our study, all 34 atypical *S. aureus* isolates were identified by PCR using this primer set.

Extra tests were required to confirm the identity of the strains when using the Vitek® 2 system: Staphaurex for membrane-bound coagulase and protein A, haemolysin activity and production of pigment on sheep blood agar plate. Staphaurex is a method commonly used for *S. aureus* identification, even though it only detects clumping factor and protein A. Staphaurex has also been reported as too insensitive for reliable detection of MRSA (Rappaport *et al.*, 1988). We obtained negative results with Staphaurex for

several isolates. Fortunately, isolates negative for clumping factor and/or protein A produced acid from mannitol, and all 34 isolates produced free coagulase, were identified by PCR and as oxacillin resistant by antibiotic susceptibility testing.

The semi-automatic identification system of AutoScan-4® conforms to the requirements of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) and requires a longer incubation period than Vitek® 2 or Phoenix™. However, it permits technologists to check biochemical reactions visually by observing the panels. We believe that this point is very important for clinical technologists, because some like to reconfirm the results of biochemical reactions by eye. The AutoScan-4® correctly identified the 34 *S. aureus* strains in this study without any extra tests.

The Phoenix[™] system also correctly identified all *S. aureus* strains without extra testing within 6 h. This was faster than the 18 h needed by the AutoScan-4[®] system and the more than 24 h needed by the Vitek[®] 2 system when additional tests were necessary. The confidence levels of identification were above 90 % for AutoScan-4[®] and Phoenix[™]. On the other hand, Vitek[®] 2 had low discrimination levels for 17 strains (Table 2).

It should be noted that molecular biological techniques, such as DNA sequencing (Becker *et al.*, 2004), hybridization (Sogaard *et al.*, 2005; Trindade *et al.*, 2003) or the use of DNA microarray technology (Charbonnier *et al.*, 2005), could provide a more accurate identification and classification tool, but such techniques are difficult to apply in a routine clinical laboratory. A rapid, conventional and automated identification method, based on phenotypic characters, is a more practical approach for daily clinical laboratory procedures.

In conclusion, the Phoenix[™] system and AutoScan-4[®] could provide accurate information for the identification of *S. aureus* with atypical physiological characteristics without any extra tests. The merit of AutoScan-4[®] is that technologists can check biochemical reactions by observing the panels. This study shows that biochemically atypical *S. aureus* strains were not identified as *S. aureus* by Vitek[®] 2 unless extra tests were used. The Phoenix[™] system identified all strains correctly within 6 h. Accordingly, this report suggests that Phoenix[™], a fully automatic system, can be used for rapid identification in the clinical laboratory.

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