Differentiation Between *Staphylococcus Aureus* and *Coagulase-Negative Staphylococcus* Species by Real-Time PCR Including Detection of Methicillin Resistant in Comparison to Conventional Microbiology Testing

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Background: *Staphylococcus aureus* has long been recognized as a major pathogen. Methicillin-resistant strains of *S. aureus* (MRSA) and methicillin-resistant strains of *S. epidermidis* (MRSE) are among the most prevalent multiresistant pathogens worldwide, frequently causing nosocomial and community-acquired infections. **Methods:** In the present pilot study, we tested a polymerase chain reaction (PCR) method to quickly differentiate *Staphylococci* and identify the *mecA* gene in a clinical setting. **Results:** Compared to the conventional microbiology testing the real-time PCR assay had a higher detection rate for both *S. au-*

reus and *coagulase-negative Staphylococci* (CoNS; 55 vs. 32 for *S. aureus* and 63 vs. 24 for CoNS). Hands-on time preparing DNA, carrying out the PCR, and evaluating results was less than 5 h. **Conclusions:** The assay is largely automated, easy to adapt, and has been shown to be rapid and reliable. Fast detection and differentiation of *S. aureus*, CoNS, and the *mecA* gene by means of this real-time PCR protocol may help expedite therapeutic decision-making and enable earlier adequate antibiotic treatment. J. Clin. Lab. Anal. 29:122–128, 2015. © 2014 Wiley Periodicals, Inc.

Key words: MRSA; bacterial DNA; PCR; microbiology; *Staphylococcus*

INTRODUCTION

Staphylococcus aureus has long been recognized as a major pathogen. Immediately upon the introduction of methicillin into clinical use, methicillin-resistant strains of staphylococci began to appear (1–3). Methicillin-resistant strains of *S. aureus* (MRSA) and *S. epidermidis* (MRSE) are among the most prevalent multiresistant pathogens worldwide, frequently causing nosocomial and community-acquired infections (4). The frequencies of both community-acquired and hospital-acquired staphylococcal infections and occurrence of MRSA have increased steadily over the last decades. Infections by these strains have now become a serious problem worldwide (5–7). Approximately 25% of nosocomial isolates of *S. aureus* are methicillin-resistant in hospitals in the United States (8).

Prompt, reliable identification of MRSA is crucial to starting appropriate antibiotic therapy and effectively eradicating infection. Early MRSA identification facilitates early contact isolation of patients and prevents unrecognized spread of MRSA within ICUs. Differentiating MRSA from *coagulase-negative staphylococci* (CoNS) is also imperative, since the latter often appears as contaminants but can also be a separate cause of infection.

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Extensive hygienic precautions that are expensive and time-consuming may be avoided in nonessential cases (9, 10). Unnecessary isolation of patients and needless use of resources could be avoided by specific, quick detection. Hence, the enormous economic burden presented by *S. aureus* infections could be directly reduced by an improved rapid testing protocol (10).

Standard identification methods and susceptibility tests are usually time-consuming. Using susceptibility tests according to the standards of the National Committee of Clinical Standards (NCCLS, now Clinical and Laboratory Standards Institute, CLSI) *S. aureus* resistance to methicillin or oxacillin can be reliably detected (11, 12). Nevertheless, false-negative or false-positive results in identification of susceptibility may be observed due to heterogeneous phenotypic expression of methicillin resistance, or to misclassifications with automated susceptibility testing systems and use of latex agglutination kits (9, 13, 14).

The presence of an additional low-affinity penicillin-binding protein 2a (PBP2a), which is encoded by the *mecA* gene, is associated with methicillin resistance in both *S. aureus* and *CoNS* (12, 15–19). Polymerase chain reaction (PCR) to detect the *mecA* gene as a rapid method for the identification of MRSA has been amply demonstrated (11, 20–27). Some even consider PCR to be the method of choice for identifying MRSA and other methicillin-resistant staphylococci (8, 28–34).

In the present pilot study, we evaluated a PCR-based method for *Staphylococci* differentiation and *mecA* gene identification in comparison to the conventional microbiology. The goal of this approach was to enable accurate and fast differentiation between *S. aureus* and *CoNS*, with rapid, specific identification of the *mecA* gene using a real-time PCR protocol.

MATERIAL AND METHODS

Study Design

A prospective sample of consecutive microbiologically confirmed cases of MRSA was used for an intraindividual comparison of experimental PCR to a standard microbiologic method. This study was approved by the local ethics committee.

Inclusion criterion was an MRSA-positive microbiological finding. To identify MRSA-positive patients, first a routine clinical screening by the conventional microbiology methods was done across all wards of the hospital. Once the microbiology department had identified an MRSA-positive patient, they informed us immediately. Since the study took place in a clinical setting, it was expected that some patients would no longer be positive using either PCR or conventional microbiology testing due

to antibiotic treatment started before the first specimens were collected for this study. After acquisition of written informed consent patients were included in the study. After inclusion patients were monitored until three consecutive microbiological cultures or PCR findings were negative for MRSA or the patient was discharged.

Polyester fiber-tipped swabs (BD Falcon SWUBE, BD, Heidelberg, Germany) were used for sample acquisition. Sites sampled included trachea, throat, nose, hair, groin, perineum, wound, and drainage. A total of 116 specimens from 12 patients were obtained. Sample bias was minimized, since specimens were acquired in parallel from each sampled location. Therefore, samples were taken from the exact same location: two swabs, one for PCR testing and one for conventional microbiology testing, were held side by side and swiped in parallel over the site. Swabs were analyzed by the conventional microbiology cultures; parallel swabs were analyzed by PCR.

Since the aim of the study was to compare the conventional microbiology testing with PCR-based technology, each patient sample is treated as an independent biological sample ($n = 116$).

Conventional Microbiology Cultures

For MRSA screening, specimens were plated on both Columbia sheep blood agar and on mannitol salt agar containing cefoxitin and read after incubation periods of 18–24 and 48 h. Species were identified by characteristic growth morphologies, Gram stain characteristics, catalase activity, and coagulase production. Susceptibility tests were performed via disk diffusion testing with cefoxitin in accordance with the National Committee for Clinical Laboratory Standards criteria described previously (35).

DNA Extraction

For analysis via PCR the swabs were processed immediately after sampling. Bacterial DNA extraction was performed with an automatic preparation device (MagNa Pure LC; Roche, Mannheim, Germany) using the LC-Microbiology Kit (Roche) according to the manufacturer's recommendations.

In brief, the swabs were incubated in bacterial lysis buffer with Proteinase K at 65°C for 10 min. After an inactivation step at 95°C for 10 min, swabs were centrifuged with the swab extraction tube system (Roche) to separate the lysis buffer from the swab. One hundred microliters of lysis buffer were placed in the MagNa Pure System for automated preparation using the DNA III protocol.

Real-Time PCR

Two different PCR runs were performed using a real-time PCR system (LightCycler, Roche).

TABLE 1. Detection Results of *S. Aureus* Obtained by the Conventional Microbiology and by Real-Time PCR

	Conventional microbiology (<i>n</i> = 116)	Real-time PCR (<i>n</i> = 116)
Positive result	32	55
Negative result	84	61

First, PCR detected and differentiated *S. aureus* from *CoNS* using the “Staphylococcus Kit M-Grade” (Roche) according to the manufacturers’ recommendations. In brief, the test consists of a primer pair specific for the ITS region, the *Staphylococcus* bacterial genome and specific hybridization probes for *S. aureus*. Differentiation of *S. aureus* and *CoNS* is based on melting curve analysis following the PCR amplification.

Second, PCR detected methicillin resistance (*mecA* gene) in *Staphylococcus spp.* using the MRSA Detection Kit (Roche) according to the manufacturers’ recommendations. The kit uses primers and hybridization probes to amplify and ascertain sequence-specific methicillin resistance associated with the *mecA* gene. Detection of the *mecA* gene is carried out by means of amplification- and melting curve analysis. Furthermore, the lyzed specimen was spiked with an internal control, then co-extracted and co-amplified with the samples of interest. This simultaneous processing of sample DNA and internal control allowed efficient extraction and inhibition control of the PCR workflow.

The reliability of this test has been established in earlier studies, showing high sensitivity of 95.7–100%, with very good specificity was 90.8–98.44% (35–37). Of note, comparison of these studies with our data is inherently biased, because the specimens in our study being collected after the initiation of antibiotic treatment (see study design above).

RESULTS

Detection of *S. aureus* by Means of Real-Time PCR and Conventional Microbiology

First, a global analysis of positive and negative *S. aureus* detection by each method was performed (Table 1). The conventional microbiology testing (*n* = 116 samples) yielded positive results for *S. aureus* in 32 cases and negative results in 84 cases, while real-time PCR (*n* = 116 samples) had positive results for *S. aureus* in 55 cases and negative results in 61 cases (Table 1).

Second, real-time PCR was cross-compared to the conventional microbiology (Table 2). Analysis showed that in 26 samples real-time PCR gave a positive signal despite a negative result in the conventional microbiology

TABLE 2. Cross-Classified Table Shows *S. Aureus* Detection Results of the Conventional Microbiology Versus Real-Time PCR

	Real-time PCR negative	Real-time PCR positive
Microbiology negative	58	26
Microbiology positive	3	29

procedure. On the other hand, real-time PCR remained negative in three samples, while the conventional microbiology testing rendered a positive result (Table 2).

Third, findings were evaluated for each patient (Table 3). Immediately after inclusion into the study, six patients were found positive for *S. aureus* by PCR testing and the conventional microbiology testing, respectively. In five of these six patients, PCR had a higher detection rate for *S. aureus* than the conventional microbiology. Only in one patient did the conventional microbiology yield more positive results for *S. aureus* than those obtained by real-time PCR (Table 3).

Detection of *CoNS* by Real-Time PCR and Conventional Microbiology

Next, global analysis for positive and negative *CoNS* detection by method was performed (Table 4). The conventional microbiology testing (*n* = 116 samples) rendered positive results for *CoNS* in 24 cases and negative results in 92 cases, with real-time PCR (*n* = 116 samples) showing positive results for *CoNS* in 63 samples and negative results in 53 (Table 4).

Second, real-time PCR was cross-compared to the conventional microbiology (Table 5). Analysis showed that in 47 samples real-time PCR yielded positive results for *CoNS*, while the conventional microbiology testing remained negative. On the other hand, in eight samples real-time PCR remained negative, while the conventional microbiology testing showed a positive result (Table 5).

Third, findings were evaluated for each patient (Table 6). Eleven patients were found positive for *CoNS* with at least one of the detection methods. In 7 of these 11 patients real-time PCR detected *CoNS* more often than the conventional microbiology. In two patients the conventional microbiology detected *CoNS* at a higher rate than real-time PCR (Table 6). Only one of 12 patients had a continuous negative result for *CoNS* with both real-time PCR and the conventional microbiology after inclusion into the study.

Detection of Methicillin Resistance by Real-Time PCR and Conventional Microbiology

After initial detection and identification of either *S. aureus* or *CoNS*, tests for methicillin resistance of the

TABLE 3. Detection of *S. Aureus* by Real-Time PCR and the Conventional Microbiology per Patient

	<i>S. aureus</i> detection by PCR		<i>S. aureus</i> detection by convent. microbiology		Total number of samples per patients
	Negative	Positive	Negative	Positive	
Patient 1	10	0	10	0	10
Patient 2	3	5	4	4	8
Patient 3	6	0	6	0	6
Patient 4	1	0	1	0	1
Patient 5	2	0	2	0	2
Patient 6	9	25	15	19	34
Patient 7	8	7	12	3	15
Patient 8	9	2	9	2	11
Patient 9	2	0	2	0	2
Patient 10	1	0	1	0	1
Patient 11	2	2	3	1	4
Patient 12	8	14	19	3	22

TABLE 4. Detection Results of *CoNS* Obtained by the Conventional Microbiology and by Real-Time PCR

	Conventional microbiology (<i>n</i> = 116)	Real-time PCR (<i>n</i> = 116)
Positive result	24	63
Negative result	92	53

TABLE 5. Cross-Classified Table Shows *CoNS* Detection Results of the Conventional Microbiology Versus Real-Time PCR

	Real-time PCR negative	Real-time PCR positive
Microbiology negative	45	47
Microbiology positive	8	16

corresponding organisms were conducted. Methicillin resistance detected by real-time PCR identified by amplification of the *mecA* gene is reported in Table 7. In all samples that were positive for monoinfection with *S. aureus* (*n* = 28) or positive for mixed infections with *S. aureus/CoNS* (*n* = 27) according to real-time PCR, the *mecA* gene was detected.

Thirty-six samples were positive for monoinfection with *CoNS* using real-time PCR. In 35 of these samples the *mecA* gene was also detected. In one case a positive result for *CoNS* was found using real-time PCR although the *mecA* gene tested negative (Table 7). None of the samples that tested negative for *S. aureus* or *CoNS* via real-time PCR showed a *mecA*-positive result.

Identified samples of *S. aureus* and *CoNS* were also tested for methicillin resistance by the conventional microbiological methods. All samples rendered positive by the conventional microbiology for *S. aureus* (*n* = 22) or

for mixed infection with *S. aureus* and *CoNS* (*n* = 10) were methicillin resistant (Table 8).

DISCUSSION

The occurrence of methicillin-resistant *S. aureus* has steadily increased since the introduction of semi-synthetic penicillins in 1961 (such as methicillin and oxacillin) and their frequent use for therapy of infections caused by *S. aureus*. Infections by these strains become a serious problem worldwide (5–7, 38, 39).

In the present study, we tested a fast and easy to use real-time PCR based method for detection of *S. aureus* and *CoNS* in the biological fluids. Moreover, the assay permitted identification of methicillin-resistant species by detecting the *mecA* gene. Two consecutive PCR runs were needed and could be performed in 90 min altogether. One vial was needed for each run and sample. It has to be pointed out, that the *mecA* PCR amplifies the *mecA* gene from *S. aureus* as well as from *CoNS*, and that either can carry *mecA*. Therefore, in mixed infections it is not possible to determine with this assay, which of the *Staphylococcus* species is carrying the *mecA* gene. To meet these concerns, we split the data for analysis into mono-infected and mixed-infected samples. Nevertheless, there are strong indications of horizontal gene transfer between *CoNS* and *S. aureus* (40–45). Therefore, *mecA*-positive mixed infections of *S. aureus* and *CoNS* may be considered to have a higher risk of becoming MRSA positive. One hundred and sixteen clinical samples were tested with this method and compared to the conventional microbiology testing (Table 1).

The real-time PCR assay identified *S. aureus* and *CoNS* in more samples than with the conventional microbiology testing (55 vs. 32 for *S. aureus*, Table 1; 63 vs. 24 for *CoNS*, Table 4). Twenty-six samples with *S. aureus* detected by

TABLE 6. Detection of *CoNS* by Real-Time PCR and the Conventional Microbiology per Patient

	<i>CoNS</i> detection by PCR		<i>CoNS</i> detection by convent. microbiology		Total number of samples per patients
	Negative	Positive	Negative	Positive	
Patient 1	3	7	8	2	10
Patient 2	0	8	4	4	8
Patient 3	0	6	5	1	6
Patient 4	0	1	0	1	1
Patient 5	2	0	2	0	2
Patient 6	10	24	28	6	34
Patient 7	13	2	13	2	15
Patient 8	4	7	9	2	11
Patient 9	1	1	2	0	2
Patient 10	1	0	0	1	1
Patient 11	0	4	4	0	4
Patient 12	19	3	17	5	22

PCR were missed by the conventional microbiology testing. The higher detection rate of the molecular testing method is due to the fact that DNA from dead bacteria can still be detected by PCR, while the conventional testing identifies only viable organisms. On the other hand, three samples were missed by PCR but detected with the conventional microbiology (Table 2). All samples that gave a positive result for monoinfection with *S. aureus* ($n = 28$) or showed a positive result for mixed infections with *S. aureus/CoNS* ($n = 27$) via real-time PCR also tested positive for the *mecA* gene (Table 7). There may have been a bias due to the fact that only routinely identified MRSA patients were included into the study. Nonetheless, this analysis could show that the number of *CoNS*-positive samples detected by real-time PCR in comparison to the conventional microbiology testing was surprisingly high. This is likely due to the high sensitivity of PCR. In this context, contamination of the samples during acquisition and during the workflow especially with *S. epidermidis* must to be considered, even though samples were processed with maximum care to avoid contamination. Nevertheless, the rate of monoinfected *mecA*-positive *CoNS* was also surprisingly high (35 out of 36 samples, Table 7), confirming that not only *S. aureus* but also *CoNS* show methicillin resistance to a high degree in patients with nosocomial infections and should be taken into clinical consideration (17, 27, 46, 47).

Moreover, we evaluated the performance of real-time PCR versus the conventional microbiology with respect

TABLE 8. MRSA Detected by the Conventional Microbiology

	Positive for <i>S. aureus</i>	Positive for <i>CoNS</i> and <i>S. aureus</i>
Methicillin-sensitive <i>S. aureus</i>	0	0
Methicillin-resistant <i>S. aureus</i>	22	10

to samples per patient. Six patients converted back to being *S. aureus*/MRSA-negative upon inclusion into the study. The number of patients initially positive for *S. aureus/CoNS* who reverted back to negative is not surprising, as these patients were already treated with antibiotics, due to the time that elapsed since initial positive MRSA screening and inclusion into the study. On the other hand, six patients were found still to be positive for *S. aureus* after inclusion into the study. The fact that some of our MRSA patients remained positive after clinical treatment might be attributed to the organism having resistance to new antibiotics (e.g., Mupirocin). PCR had a significantly higher detection rate than the conventional microbiology in detecting *S. aureus* (in five vs. one patients, Table 3) and *CoNS* (seven vs. two patients, Table 6), respectively.

The time that elapses between taking a sample and obtaining a definite result is of great importance and should be minimized. The conventional microbiology testing usually has a delay of 24 to 48 h until microbiological testing results become available (48). Earlier studies using PCR

TABLE 7. *MecA* Gene Detection in Samples ($n = 116$) by Real-Time PCR

	Negative for <i>CoNS</i> and <i>S. aureus</i>	Positive for <i>CoNS</i>	Positive for <i>S. aureus</i>	Positive for <i>CoNS</i> and <i>S. aureus</i>	Total
<i>mecA</i> gene negative	25	1	0	0	26
<i>mecA</i> gene positive	0	35	28	27	90

techniques have used rather complicated manual DNA extraction protocols, followed by single or multiplex PCR with detection of amplification products on agarose gels (11, 20–27). Most of these methods are time consuming, easily susceptible to contamination due to the manual protocols, and largely unsuitable for routine diagnostic laboratories. Previously real-time PCR for detection of *S. aureus* and *mecA* gene has been used (49–54), but with significant differences. Tan et al. (54) did not employ an internal control; Hope et al. (52) preincubated the samples; Reischl et al. (53) did not test their method in a clinical setting with clinical specimens; and Fang et al. (49) and Hagen et al. (51) used overnight cultivation.

The method we used in this study has a hands-on time of less than 5 h for preparation of DNA, carrying out PCR, and evaluating its results, speeding up the process considerably compared to the 48 h using the conventional method. Other commercial real-time PCR assays with similar specifications are available. Among the advantages of this assay are its largely automated nature, easy adaptability, high sensitivity, specificity, and reliability. Hence, detection and differentiation of *S. aureus* and *CoNS* including the *mecA* gene by means of real-time PCR may accelerate therapeutic decisions and enable even earlier adequate antibiotic treatment.

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