ORIGINAL ARTICLE 10.1111/j.1469-0691.2007.01880.x

Screening for methicillin-resistant *Staphylococcus aureus* in clinical swabs using a high-throughput real-time PCR-based method

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ABSTRACT

The presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and the community is a serious problem. Accordingly, a comprehensive plan has been implemented in the County of Vejle, Denmark, to identify colonised and/or infected individuals and to control the spread of MRSA. Since 2005, all patients and healthcare personnel have been screened for MRSA colonisation, involving analysis of 300–400 samples daily. To deal with this number of samples, a PCR-based method customised for high-throughput analysis and a system for fast reporting of MRSA carrier status were developed. Swab samples were incubated overnight in a selective tryptone soya broth and were analysed by PCR the following day. Using this strategy, non-colonised individuals were identified within 24 h, while MRSA-positive samples were analysed further by traditional microbiological methods to determine the resistance pattern. This is a cost-effective approach, as the greatest expense in hospitals involves the isolation of patients of unknown MRSA status. The method was evaluated by testing 2194 clinical samples, with a sensitivity and specificity of 100% and 94%, respectively. The analytical sensitivity was 97%, with 161 of 166 different MRSA strains and isolates generating positive results according to PCR analysis. Using four control strains, the inter-assay variation was revealed to be a maximum of 2.6%, indicating good reproducibility.

Keywords Colonisation, high-throughput screening, MRSA, real-time PCR, screening, surveillance

Original Submission: 30 January 2007; **Revised Submission:** 12 July 2007; **Accepted:** 14 August 2007

Clin Microbiol Infect 2008; **14**: 22–28

INTRODUCTION

Since the first strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were observed during the early 1960s [1], the incidence of MRSA has increased dramatically worldwide, so that MRSA now constitutes a serious problem in the healthcare sector in many countries [2]. The consequences of an MRSA infection are an increased length of hospitalisation and increased healthcare costs [3], and it has also been suggested that MRSA infections are associated with an increased mortality rate [4–6]. In contrast to many other countries in Europe, Denmark had an MRSA prevalence of <2% throughout the 1980s and 1990s [2]. However, in 2002 the County of Vejle experienced a large outbreak of MRSA infections in six hospitals. To prevent the type of endemic situation observed in, e.g., the UK and Germany [7,8], a comprehensive control strategy was initiated, involving screening of all healthcare personnel and all patients upon initial hospitalisation and at discharge. In addition, the resources of the infection control department were increased in order to track and reduce the spread of MRSA, as contamination of the hospital environment constitutes a major source of infection [9]. Accordingly, following identification of an MRSA-positive patient, the patient is isolated and treated, and a follow-up procedure is started by the infection control department to ensure that the patient is cured of MRSA infection. All healthcare personnel and patients who have had contact with an MRSA-positive patient are tested for MRSA. If a known MRSA-positive patient is

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hospitalised, specific precautions are taken. This intensive infection control strategy required a fast high-throughput MRSA screening method, and was therefore not compatible with traditional microbiological testing using blood agar.

Methicillin resistance in *S. aureus* is encoded by the mobile staphylococcal cassette chromosome *mec* (SCC*mec*) element. The SCC*mec* element contains the *mecA* gene, which encodes penicillin-binding protein 2′ and is responsible for methicillin resistance [10]. Six different SCC*mec* variants have now been identified, each of which possesses a different sequence and encodes varying resistance patterns [11–14]. The SCC*mec* element is always integrated specifically in the *S. aureus* chromosome by site-specific recombinases at the 3′ end of an open reading frame (*orfX*) located near the replication origin [15,16]. Based on this knowledge, a PCR detection method was described in 2004 that targets the *orfX* of *S. aureus* and the right extremity junction of the SCC*mec* element, thereby making the method capable of distinguishing between MRSA and coagulase-negative staphylococci [17]. Using this strategy as a starting point, the aim of the present study was to develop a method for screening a large number of samples within a short time as part of the daily workflow of the hospital.

MATERIALS AND METHODS

Reference strains

The reference strains comprised 109 different MRSA strains obtained from the Statens Serum Institut (SSI; Copenhagen, Denmark). In addition, 57 clinical MRSA isolates obtained in the County of Vejle, Denmark were typed by the SSI and included in the study. The reference strains included most well-known international strains and the most prevalent strains in Denmark.

Four strains were used as positive controls: three MRSA strains typed by the SSI, i.e., 41195 (CC22), 40431 (CC5) and 40126 (CC8-t024), and one clinical isolate (8190) obtained in the County of Vejle and typed as CC30 by the SSI. The methicillin-susceptible *S. aureus* (MSSA) strain ATCC 25923 was used as a negative control.

Clinical samples

The study included samples collected from 2194 patients during March/April 2006 at the six hospitals in the County of Vejle (Kolding, Fredericia, Horsens, Braedstrup, Give and Vejle Hospitals). The samples were swabs taken from nostril, throat, perineum, wounds or skin, inserted in Stuart's transport medium (SSI) and delivered to the Department of Clinical Microbiology, Vejle Hospital.

Culture conditions and media

The swabs from the nose and throat of each patient were pooled in a 2.5-mL tryptone soya broth (TSB) containing NaCl 2.5% w/v, aztreonam 10 mg/L and cefoxitin 3 mg/L (SSI) and incubated overnight at 37°C. Swabs from wounds, skin, etc. were treated individually, but using the same procedure as for the nose/throat swabs. Following overnight incubation, 10 μL of the broth was plated on agar plates containing horse blood 5% v/v (SSI) and incubated at 37°C. The remaining broth was used for DNA extraction and subsequent PCR analysis. The agar plates were examined after incubation for 1 and 2 days for typical staphylococcal colonies. On day 2 or 3, potential *S. aureus* colonies were identified using the tube coagulase test and sensitivity to oxacillin (1-μg disk; Rosco, Taastrup, Denmark) by the agar disk-diffusion method. On day 4 or 5, coagulase-positive colonies that were resistant to oxacillin were tested phenotypically for the presence of the *mecA* gene using the Slidex MRSA Detection kit (bioMérieux, Marcy l′ Etoile, France) and for sensitivity towards 12 selected antibiotics. For some randomly chosen false-positive and MRSA-negative samples (see Results), a sample from the blood agar plate was stored in meat extract 0.7% v/v agar (SSI) at 4°C, and this was followed by subsequent identification of the bacteria present in each sample using the Vitek II system (bioMérieux).

DNA extraction

DNA extraction was performed according to the procedure described by Hagen *et al.* [18], with adjustments made to accommodate a high-throughput system using the Biomek FX pipetting robot (Beckmann, Fullerton, CA, USA). In brief, 950 μL of overnight broth culture was centrifuged at 5100 *g* for 5 min and the pellet was resuspended in 180 μL of lysis buffer (Triton X-100 1% v/v, Tween-20 0.5% v/v, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The suspension was heated to 95°C for 10 min and then centrifuged at 5100 *g* (Multifuge 3L; Heraeus, Osterode, Germany) for 10 min. The supernatant was used for real-time PCR assays.

Real-time PCR

Multiplex real-time PCR was performed using the primers and probes described previously [17], except for primer 85/1940, which is a modification of a primer described by Ito *et al.* [19]. The sequences and concentrations of all primers and probes used are shown in Table 1. For each PCR, 3.3 μL of DNA extract was transferred to 22 μL of PCR mix containing primers and probes, 2 × *Taq*Man Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.625 U of Ampli*Ta*q Gold (Applied Biosystems) and *Taq*Man Exogenous Internal Positive Control (Applied Biosystems) to monitor any PCR inhibition. PCR conditions comprised 2 min at 50°C (UNG carryover prevention), followed by 10 min at 95°C and 45 cycles of 15 s at 95°C and 75 s at 60°C, performed on an ABI 7900 instrument (Applied Biosystems). A sample was considered to be MRSA-positive when the PCR cycle threshold (Ct) value was <41, based on receiver operator characteristic curve calculations generated from the data obtained when the results of the MRSA PCR were compared with those of the reference standard of a conventional microbiological culture. The sensitivity in the receiver operator characteristic curve

Table 1. Primers and probes used in the multiplex PCR for the detection of methicillin-resistant *Staphylococcus aureus*

Primer/probe	Sequence (5′–3′)	Concentration in PCR
S. aureus orfX-specific primer	GGATCAAACGGCCTGCACA	400 nM
SCC*mec*-specific primers:		
Mecii 574	GTCAAAAATCATGAACCTCATTACTTATG	400 nM
Meciii 519	AATATTTCATATATGTAATTCCTCCACATCTC	400 nM
Meciv 511	CTTCAAATATTATCTCGTAATTTACCTTGTTC	400 nM
85/1940	TCATGAACCTCATTACTTATGATAAGCTTC	600 nM
S. aureus orfX-specific probes:		
XsauB5	6-FAM-ACTGCGTTGTAAGACGT-MGB	200 nM
XsauB9	6-FAM-ACTACGTTGTAAGACGTCC-MGB	200 nM

FAM, carboyfluorescein; MGB, minor groove binder.

analysis was set at 100% to avoid false-negative samples, and calculations were performed using the NCSS program (Number Cruncher Statistical Systems, Kaysville, UT, USA).

Assay verification

The reproducibility of the assay was evaluated by measuring inter- and intra-assay variation. The coefficient of variation was assessed in both cases by measuring the Ct values obtained for independent overnight cultures of four MRSA control strains, which were processed in the same way as normal samples. The inter-assay variation was calculated from 22 independent samples analysed on different days. The intra-assay variation was based on the average of two different runs performed with eight duplicate samples. The amplification efficiency was investigated by measuring the slope of a standard curve based on a serial dilution of the four control strains (each dilution was analysed using three duplicate samples). The PCR amplification efficiency was calculated using the equation $E = 10^{-(1/\text{slope})} \times 100\%$, with an average of two independent experiments being taken as the final volume. The standard curves were also used to determine the detection limit of the method, which denotes the number of CFUs that could be detected reproducibly by each PCR.

RESULTS

Examination of clinical samples

During this study, 2194 patient samples were tested for the presence of MRSA by multiplex PCR and conventional microbiological culture. In total, 88 samples were MRSA-positive and 1987 samples were MRSA-negative according to both detection methods. There were no culture-positive samples that were not detected by PCR, but 119 samples identified as MRSA-positive by the multiplex PCR were negative according to culture. Thus, the PCR method had a sensitivity of 100% and a specificity of 94%. The positive predictive value and negative predictive value of PCR were 43% and 100%, respectively.

As 5.4% of the 2194 clinical samples were apparently false-positive in comparison with culture, 20 of these clinical samples were investigated microbiologically, with the different species in each sample being separated and identified using the Vitek II instrument. Table 2 shows that each of the false-positive samples contained MSSA. Each of the original broths was very turbid, and had a high content of MSSA, as identified by culture, coagulase tests and cefoxitin susceptibility tests (results not shown), despite the fact that the TSB was designed to be selective for MRSA. This problem is analysed in more detail below.

Evaluation of the multiplex PCR using different bacterial strains and clinical isolates

The analytical sensitivity of the multiplex PCR was investigated by analysing 166 different MRSA isolates, of which 161 were PCR-positive and five were PCR-negative (Table 3), giving an analytical sensitivity of 97%, which is comparable to that observed in previous studies [17,18]. No cross-reactivity was detected with 27 different microbial species found in 50 'MRSA-negative' clinical samples (Table 4).

Table 2. Microbial content of 20 clinical samples that were false-positive according to PCR

Number of samples	Species in the sample	PCR result
12	MSSA	+
1	MSSA	+
	Staphylococcus epidermidis	–
2	MSSA	+
	Enterococcus faecalis	–
1	MSSA	+
	Candida parapsilosis	–
1	MSSA	+
	Candida inconspicua	–
1	MSSA	+
	Sphingomonas paucimobiles	–
1	MSSA	+
	Lactococcus lactis	–
1	MSSA	+
	Enterococcus gallinarum	–

MSSA, methicillin-susceptible *Staphylococcus aureus*.

Table 3. Analytical sensitivity of the multiplex PCR using previously characterised strains of methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA CC type	Number of isolates	PCR result
CC1	5	+
CC5	23	+
CC5 (*spa* type t002)	1	–
CC8	34	+
CC8 (*spa* type t059, t039)	2	–
CC15	1	+
CC22	54	+
CC30	11	+
CC45	13	+
CC59	3	+
CC59 (*spa* type t441)	1	–
CC78	3	+
CC80	10	+
CC80 (*spa* type t376)	1	–
CC97	2	+
ST152/377	1	+
Singleton	1	+

CC, clonal complex; ST, sequence type.

Characteristics of the multiplex PCR

Four different known MRSA strains were included in each PCR run as positive controls. Inter-assay variation was determined, based on the results of 22 independent samples analysed during 1 month, and showed a variation in the Ct values of 1.2–2.6% (Table 5). Likewise, the intra-assay variation was low (0.8–1.9%), based on the average of 16 Ct values obtained in two independent PCR runs.

The PCR efficiency and the detection limit for each control strain were measured using a serial dilution of 10^8–10^3 CFU/mL from an overnight culture (the CC8-t024 strain was only tested in the range 10^8–10^6 CFU/mL). The PCR efficiency varied between 75% and 116%, depending on the strain, which is acceptable for a multiplex PCR. The detection limit was 16 CFU/PCR for the CC30 strain, 156 CFU/PCR for the CC22 and CC5 strains, and 15 600 CFU/PCR for the CC8-t024 strain. The detection limit of 15 600 CFU/PCR for the CC8-t024 strain was rather high, but was compensated for by the overnight incubation period of *c.* 18 h.

As the multiplex PCR appeared to be generating false-positive reactions with MSSA (see above), several false-positive samples were analysed by testing each primer individually in the PCR (data not shown). Primers 85/1940 and 574 were both found to be associated with false-positive reactions. Primer 85/1940 was originally added to the assay to detect a specific CC8 MRSA strain present in Denmark. None of the primers in the assay described by Huletsky *et al*. [17], or the other candidate primer tested, could detect this specific CC8 strain (results not shown). Similarly, primer 574 detects a CC5 MRSA strain that has been identified in the County of Vejle. Therefore, omitting either of these two primers would reduce the number of false-positive reactions,

Table 4. Microbial species found in 50 clinical samples that were negative for methicillin-resistant *Staphylococcus aureus* according to the multiplex PCR

Species	No. of isolates	Species	No. of isolates
Staphylococcus aureus (MSSA)	6	*Acinetobacter baumannii* (G–)	1
Staphylococcus capitis (CoNS)	1	*Gemella morbillorum* (G+)	1
Staphylococcus epidermidis (CoNS)	18	*Enterococcus faecalis* (G+)	12
Staphylococcus haemolyticus (CoNS)	3	*Enterococcus faecium* (G+)	1
Staphylococcus hominis (CoNS)	1	*Enterococcus gallinarum* (G+)	1
Streptococcus alactolyticus (G+)	1	*Sphingomonas paucimobilis* (G–)	1
Streptococcus anginosus (G+)	4	*Lactococcus* spp. (G+)	8
Streptococcus mutans (G+)	1	*Leuconostoc* spp. (G+)	3
Streptococcus parasanguinis (G+)	3	*Moraxella* spp. (G–)	2
Streptococcus pneumoniae (G+)	1	*Corynebacterium* spp. (G+)	1
Streptococcus salivarius (G+)	13	*Candida albicans* (yeast)	4
Streptococcus sanguinis (G+)	8	*Candida parapsilosis* (yeast)	1
Streptococcus thoraltensis (G+)	1	*Candida inconspicua* (yeast)	1
Streptococcus vestubularis (G+)	1		

MSSA, methicillin-susceptible *S. aureus*; CoNS, coagulase-negative staphylococci; G+, Gram-positive; G–, Gram-negative.

Table 5. Characteristics of the multiplex PCR with four known strains of methicillin-resistant *Staphylococcus aureus*

	CC22	CC5	CC30	CC8-t024
Inter-assay variation (CV)	1.2% (Ct 33.0 ± 0.4)	2.6% (Ct 34.2 ± 0.9)	2.1% (Ct 30.6 ± 0.7)	1.8% (Ct 34.9 ± 0.6)
Intra-assay variation (CV)	1.3% (Ct 36.6 ± 0.5)	1.9% (Ct 38.9 ± 0.7)	0.8% (Ct 34.4 ± 0.3)	0.9% (Ct 37.7 ± 0.3)
PCR efficiency in the multiplex reaction	82%	76%	93%	107%
Determination limit (CFU/PCR)	156	156	16	15 600

CC, clonal complex; CV, coefficient of variation; Ct, PCR cycle threshold value.

but would increase the risk of false-negative reactions, which are potentially more serious.

DISCUSSION

MRSA is recognised as one of the most important nosocomial pathogens, being capable of causing a number of different clinical manifestations, ranging from mild skin infections to severe and sometimes life-threatening sepsis [20]. As MRSA is resistant to many antibiotics, treatment of an infection is often difficult and prolonged, resulting in increased hospitalisation periods and healthcare costs [3,21,22]. Accordingly, it is important to control and prevent the spread of MRSA, and many studies have shown that a screening and eradication programme is an optimal and cost-efficient solution when compared with the costs associated with the care of MRSA-infected patients [23–27]. Recommendations from Danish Health Authorities suggest that screening, and eradication after positive identification, should be the main strategy aimed at preventing the epidemic spread of MRSA.

The criteria for a successful screening programme are that the method used should be fast, reliable and capable of integration in the daily workflow of a routine laboratory. In this respect, the total analysis time for the real-time PCR method is *c.* 24 h, which includes an overnight incubation step, a semi-automated sample preparation step (capable of handling 96 samples simultaneously), and subsequent real-time PCR. Other published methods have omitted the overnight incubation period [18,28], but this approach resulted in an unacceptably low sensitivity in our hands. Furthermore, in order to benefit fully from a shorter analysis time, it would be necessary for the method to be available for 24 h daily, which may cause a problem with staffing resources. Focusing on the cost/benefit ratio, it seems that the real-time PCR screening method described in the present study has the advantages of being reliable, reasonably fast, easy to implement in the daily workflow, and able to handle a large number of samples simultaneously.

The method was evaluated by analysing 2194 clinical samples, and this revealed a sensitivity of 100% and a specificity of 94%. The specificity is affected by the number of false-positive reactions, which appear to be caused by a heavy load of MSSA. One explanation might involve non-*mecA*-containing SCC elements that have been reported to be present in some *S. aureus* strains [29,30]. Alternatively, the presence of DNA from dead MRSA in a sample could yield a positive PCR result and a negative culture result. This could be the case if the conditions in the TSB were too stringent, e.g., if the salt concentration was too high. Primers 574 and 85/1940 were particularly associated with false-positive results, and a search of the NCBI databases revealed that both primers have the possibility of annealing to DNA from MSSA (data not shown), which may explain why this and previous studies [17,28,31] have generated false-positive results using this molecular detection strategy. However, such false-positive samples do not lead to inappropriate treatment of patients, as all PCR-positive samples are further analysed microbiologically. Only when the MRSA-positive status is confirmed will medical treatment be initiated.

The analytical sensitivity of the assay was 97%, based on an analysis of 166 MRSA strains and isolates. The five strains that were not identified have not, to date, been found in the County of Vejle. Overall, taking into account the variation reported among SCC*mec* elements carried by MRSA strains [13,14,19], the PCR multiplex method detects a comprehensive selection of MRSA strains. It will be interesting to determine whether the method can also be used for detection of community-acquired MRSA, which may become an increasing problem in the near future [32,33].

In addition to sensitivity and specificity, the reproducibility of a method is also an important issue. One of the challenges associated with performing a multiplex PCR is that the primers may interact and have different optimum annealing temperatures, which normally requires an extended optimisation of the PCR conditions [34]. The present study found intra- and inter-assay variation of <2.6% for the control strains. The detection limit of the assay was generally slightly higher than that reported in other studies [17,18,35], but this was compensated for by the incubation step included in the assay. It was concluded that this semi-automated, high-throughput, real-time PCR-based screening method for MRSA has good reproducibility, 100% sensitivity and high specificity. The method has been implemented in the daily workflow of our laboratory, where it is currently used to analyse

300–400 clinical samples daily. More precisely, in the 6-month period from July to December 2006, 42 112 assays were performed for 24 687 individuals, with 393 assays for 114 individuals being MRSA-positive.

ACKNOWLEDGEMENTS

We would like to thank L. Frischknecht for assisting with the initiation of this project and H. Skytt for performing the Vitek II analysis. We acknowledge help from Ramcon (Birkerød, Denmark) in designing the protocol for the Biomek FX pipetting robot. The County of Vejle supported this work. No information has been provided by the authors concerning the existence or absence of conflicting or dual interests.

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