Real-time evaluation of an optimized real-time PCR assay versus Brilliance chromogenic MRSA agar for the detection of meticillin-resistant Staphylococcus aureus from clinical specimens

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A total of 1204 meticillin-resistant Staphylococcus aureus (MRSA) screens (3340 individual swabs) were tested to evaluate a staphylococcal cassette chromosome mec (SCCmec) real-time PCR. In total, 148 (12.3 %) of the screens were MRSA-positive, where 146 (12.1 %) were MRSA-positive by the SCCmec real-time PCR assay. In contrast, 128 (10.6 %) screens were MRSA-positive by culture. One hundred and twenty-six (10.5 %) of the screens were positive by both culture and PCR; these samples were sequenced. In 14 of the cases, a homology search confirmed the sequence as SCCmec, indicating that these samples could be considered true positives. Two of the 1204 (0.2 %) screens were negative by culture but positive by PCR; these samples were sequenced. Twenty of the 1204 screens (1.66 %) were positive by PCR but negative by culture; these samples were sequenced. The mean turnaround time (TAT) for PCR-negative swabs was 6 h 12 min and for PCR-positive swabs was 6 h 48 min. In comparison, for culture-negative swabs the mean TAT was 29 h 30 min and for culture-positive swabs was 69 h. The cost per swab for routine culture was £0.41 (€0.48) and that of the real-time PCR assay was £2.35 (€2.75). This optimized, in-house, inexpensive real-time PCR test maintained a very high sensitivity and specificity when evaluated under real-time laboratory conditions. The TAT of this real-time PCR assay was substantially lower than that of chromogenic culture. It was also maintained throughout the entire process, which can be taken as an indirect measure of test performance. This study showed that implementation of a molecular test can be achieved with limited resources in a standard microbiology laboratory.

INTRODUCTION

Meticillin-resistant Staphylococcus aureus (MRSA) remains a leading cause of healthcare-acquired infection and affects the most vulnerable patients with significant morbidity and mortality (Harbarth et al., 1998; Cosgrove et al., 2003; Salgado et al., 2003; Cooper et al., 2004; Francois et al., 2007). Despite the lack of convincing evidence (Coia et al., 2006), it is now accepted that a major aspect of controlling the spread of MRSA is the prompt identification of patients at risk of MRSA carriage (Chaix et al., 1999; Cepeda et al., 2005; Malde et al., 2006; Cunningham et al., 2007). Increasing numbers of hospitals in the UK will be expected to perform MRSA screening of all elective hospital admissions and emergency admissions in the near future (Department of Health in England, 2008; Keshtgar et al., 2008). However, there is a possibility that, even if adequate infection control precautions are in place, the delay in obtaining results from screening swabs will allow transmission of MRSA from colonized patients to occur before carriage has been detected.

Screening using faster methods such as nucleic acid amplification can produce results within 2–4 h directly from clinical samples (Jeyaratnam et al., 2008; Renwick et al., 2008). However, the reality is that various external factors such as sample collection, transport, reception, documentation and reporting significantly increase the real reporting time, making the target of same-day reporting difficult to achieve (Harbarth et al., 1998; Jeyaratnam et al., 2008; Aldeyab et al., 2009). Commercially available methods have limitations, including detecting meticillin-sensitive S. aureus, high rates of inhibition (Huletsky et al., 2004; Conterno et al., 2007; Harbarth et al., 2008; Keshtgar et al., 2008; Robicsek et al., 2008) and no proven ability to
test pooled samples (Rossney et al., 2008; Gröbner et al., 2009; Kobayashi et al., 2009). Commercial assays are also inflexible to changes when alternative primers are required. The general perception among standard diagnostic laboratories in the National Health Service (NHS) is that implementation of molecular techniques is too technically demanding and unaffordable (Conterno et al., 2007; Robicsek et al., 2008).

In this study, an in-house real-time PCR for the detection of MRSA is described, which overcomes the technical problems observed with comparable commercially available methods, namely showing implementation of the assay into routine diagnostics to maintain robust performance, reporting consistent results from pooled samples, the feasibility of implementing a molecular test in a standard microbiology laboratory, development of quality-assurance schemes to maintain performance and the cost-effectiveness of reporting the assay in routine practice.

METHODS

Patients and samples. All the screening swabs sent to the Royal Infirmary of Edinburgh, UK, from NHS Lothian over a 1-month period (11 February 2008 to 12 March 2008) were tested simultaneously by culture and real-time PCR assay. A routine MRSA screen in our hospitals comprises a nose, throat, groin and/or ulcer site swab. All samples were received and labelled for registration as per routine protocols for MRSA screening. Both culture and molecular MRSA results were reported electronically via the hospital information system. The PCR assay MRSA results were suppressed and not reported until the culture result was available. Only patients who were routinely screened for MRSA were included. The study was carried out in full accordance with clinical governance as stated by NHS Lothian.

Collection and culture of specimens. All specimens were collected using a Transwab with plain and charcoal medium (Medical Wire & Equipment). All specimens were transported at room temperature to the Royal Infirmary of Edinburgh, UK, and tested within 48 h of collection. The same specimen was used for standard culture and for PCR. For identification of MRSA, the swabs were streaked directly onto Brilliance Chromogenic MRSA agar (Oxoid) and cultured for 18 h at 37 °C. Pure colonies were picked and a latex agglutination test for S. aureus surface antigens was carried out (Pastorex; Biostat) followed by a DNase test (DNase plate; Oxoid). For new MRSA-positives, antibiotic susceptibility was determined by VITEK test (bioMérieux) followed by Etests for cefoxitin resistance and oxacillin resistance. A latex agglutination test for penicillin-binding protein 2 (PBP2; Oxoid) was also tested in some cases. Screens that were culture-positive but PCR-negative were treated as if they were new positives.

MRSA PCR. One technical operator and support worker with no previous experience in molecular methods was trained over a 1-week period to conduct all the molecular tests. The screening swabs were plated for culture and expressed into 1 ml saline (E&O Laboratories), and samples from individual patients were pooled together, using 200 μl suspension from each of the swabs with a maximum of three pooled at once. The assay was performed as described by Renwick et al. (2008) and primers were as described by Huletsky et al. (2004) with the addition of an internal control as described by Kalpoe et al. (2004). When a positive pool was identified, the individual samples from the pool were reprocessed and analysed by MRSA PCR assay.

Briefly, samples and controls were extracted using a NucliSens easyMAG system (bioMérieux) (Huletsky et al., 2005; Loens et al., 2007). Lysis buffer contained phocine herpesvirus (PhoHV) as an internal control. Saline suspensions were pre-treated with proteinase K (Qiagen) and extracted according to the manufacturer’s instructions. Purified nucleic acid was eluted in 110 μl resuspension buffer. The PCR was performed in a volume of 25 μl, consisting of 10 μl extracted nucleic acid, 2.5 μl HotStarTag DNA polymerase, 200 μM each dNTP, 1.5 mM MgCl₂ (final concentration 5 mM; Qiagen), 0.5 μM each staphylococcal cassette chromosome mec (SCCmec) primer, 0.35 μM SCCmec probe, 0.3 μM each forward and reverse PhoHV primer and 0.05 μM PhoHV probe. Amplification, detection and analysis were performed in an ABI 7500 real-time PCR system (Applied Biosystems) under the following conditions: 1 cycle of 95 °C for 15 min, followed by 50 cycles of 95 °C for 15 s, 60 °C for 40 s and 72 °C for 30 s.

Reporting and data gathering. Results were reported on the laboratory information system (APEX; iSOFT) in real time, and the data gathered for each patient were as follows: age, sex, location, specimen type, culture result and real-time PCR result (including cycle threshold value, if positive). The laboratory turnaround time (TAT) for culture and PCR was calculated from the actual electronic record of the sample at reception and result authorization held in APEX. Time from sample collection from patient to reception and time from authorization to telephoning results (patient receiving results) were not collected.

Molecular epidemiology and surveillance of mecA variants. One hundred and sixty-one MRSA isolates from positive blood cultures collected from 1 July 2007 to 1 July 2008 identified by the local laboratory using standard identification methods were used to monitor for SCCmec variation. These isolates are part of an ongoing epidemiological surveillance programme. These isolates were used because they are likely to represent circulating strains, particularly those involved with severe or invasive disease. All isolates were tested for the presence of the mecA gene with the in-house PCR and subsequently sequenced.

SCCmec sequencing. Sequence analysis was set up using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer’s instructions. The products were analysed on an ABI 3730 DNA Analyser (Applied Biosystems) and the sequences were analysed using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al., 2007). Extracted DNA samples were amplified using an orfX primer and the same primer was also used as the sequencing primer. The sequences were aligned and assigned to their respective SCCmec types.

Statistical analysis. The sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) of the MRSA PCR assay were calculated by comparing the results of PCR with a combination of sequencing of SCCmec and the results of the standard culture method. A χ² test was also carried out using the Microsoft Excel data analysis tool to compare whether the difference in PCR and culture result was statistically significant (P<0.05).

RESULTS

Clinical validation of the SCCmec real-time PCR assay

Over the study period, 3340 swabs were received from 1204 patients. In total, 148 (12.3 %) of the screens were MRSA-positive, where 146 (12.1 %) were MRSA-positive by
SCC\textit{mec} real-time PCR assay. In contrast, 128 screens (10.6\%) were MRSA-positive by culture. One hundred and twenty-six (10.5\%) of the screens were positive by both culture and PCR. Twenty of the 1204 (1.66\%) screens were negative by culture and positive by PCR; these samples were sequenced. In 14 of the cases, a homology search confirmed the sequence as SCC\textit{mec}, indicating that these samples could be considered true positives and the sensitivity and specificity adjusted accordingly. Two of the 1204 (0.2\%) screens were positive by culture but negative by PCR. As a follow-up, Etests for cefoxitin and oxacillin resistance and an agglutination test for penicillin-binding protein were carried out to confirm these two screens during the study.

Compared with routine culture, the PCR-based assay had a sensitivity of 98.6\%, a specificity of 99.4\%, and a PPV and NPV of 95.9 and 99.8\%, respectively (Table 1). The PCR method was significantly more sensitive ($P<0.05$) than the culture method, detecting 0.7\% more MRSA screen positives within a 1-month period.

### Sites testing positive for MRSA by culture and PCR

One hundred and twenty-six of the screens were positive by both PCR and culture for MRSA. Of these, 103 PCR-positive screens and 93 culture-positive screens were full screens (combination of three swabs from nose, throat, groin, wound or other), whilst the rest were partial screens (two or fewer swabs). By PCR, nose swabs were positive in 88 screens (85\%), but additional positives were obtained by PCR from other sites: five from the throat (5\%), eight from the groin, wound or another site (8\%) and two from a combination of these sites (2\%) (Fig. 1). In contrast, by culture, nose swabs were positive in 72 screens (77\%), whilst additional positives were obtained by culture from the throat (five screens; 5\%), groin or wound (12 screens; 13\%) or a combination of these sites (four screens; 4\%) (Fig. 2).

### Total TATs

The mean TAT for the PCR-positive screen was 6 h 48 min and for the PCR-negative screen was 6 h 12 min. The mean TAT for the culture-positive screen was 67 h and for the culture-negative screen was 27 h 30 min. Therefore, a negative and a positive assay were approximately 22 and 60 h shorter, respectively, than those for the culture method.

### Cost

The consumables cost for MRSA culture screening was £0.41 per swab versus £2.35 per swab for the PCR assays, both incorporating the mean positive rate for swabs and the concomitant cost of full identification. Comparison of the projected costs for monthly screening for each of the methods was £5711 (£6674) and £14 329 (£16 744), respectively, as presented in Table 2.

### Table 1. Breakdown of the screen results: comparison of the SCC\textit{mec} real-time PCR assay with routine culture

Where discrepancies between the real-time PCR assay and the culture method occurred, sequencing of DNA extracts from the culture screens was used to determine true- or false-positive or negative culture results, and the sensitivity/specificity was adjusted accordingly.

<table>
<thead>
<tr>
<th>Culture and SCC\textit{mec} sequencing</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR-positive</td>
<td>140</td>
<td>6</td>
</tr>
<tr>
<td>PCR-negative</td>
<td>2</td>
<td>1056</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>98.6%</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>99.4%</td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>95.9%</td>
<td></td>
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<tr>
<td>NPV</td>
<td>99.8%</td>
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</table>

![Fig. 1. Venn diagram showing the number of PCR-positive isolates from various sites.](image1)

![Fig. 2. Venn diagram showing the number of culture-positive isolates from various sites.](image2)
The use of all blood culture MRSA isolates allowed large-scale screening programmes. This is because the high throughput of real-time PCR, with automated extraction and no requirement for extensive post-amplification analysis, also makes this assay plausible to use in large-scale screening programmes.

Sequencing results from blood culture samples

The 161 MRSA isolated from blood cultures by a routine culture method were all mecA PCR-positive. All isolates were SCCmec PCR-positive. Sequencing showed that there were 151 SCCmec II or IV (two forms with 254A/C and 263G/T), eight identical SCCmec III, one SCCmec I and one that did not give convincing sequence data. The sequence in relation to the reverse primer in SCCmec showed two strains with one nucleotide difference within the SCCmec II or IV types, and the primers were fully compatible with the MRSA PCR.

DISCUSSION

In this study, an in-house PCR assay for MRSA was shown to perform well in a routine diagnostic service. In order for an assay to be successful in a routine diagnostic laboratory, a high level of automation and robust straightforward protocols are required. Here, a staff member with minimal molecular skill was trained within a week to fully run the routine assay. The design of this assay with an internal control, which was co-extracted and co-amplified, ensured that there was a robust check for the nucleic acid extraction procedure and PCR inhibition. Of the 1204 screens tested, less than 2% of the screens needed to be repeated due to internal control failure and they were subsequently successfully retested from the freeze–thawed extract. The high throughput of real-time PCR, with automated extraction and no requirement for extensive post-amplification analysis, also makes this assay plausible to use in large-scale screening programmes.

The use of all blood culture MRSA isolates allowed monitoring of the variation in local SCCmec types over 1 year. This process was used as a surveillance tool to detect the emergence of variants of SCCmec that could render the PCR ineffective. Here, no significant variants were detected and the PCR detected all the isolates tested, so no additional changes of the target primers were necessary. The addition of a new primer into an in-house assay would be a small change but would be completely necessary to enable the assay to continue to improve and detect significant SCCmec variants. According to Hiramatsu et al. (2001) and Deurenberg et al. (2007), MRSA evolution in SCCmec is likely to happen and the monitoring of MRSA variation is necessary to maintain confidence in the assay.

The design of this assay gave an improved assay performance with sensitivity, specificity, PPV and NPV of 98.6, 99.4, 95.9 and 99.8%, respectively; in other studies on commercial PCR, the reported sensitivity varied between 95 and 97% (Jonas et al., 2002; Fang & Hedin, 2003; Francois et al., 2003, 2007; Bishop et al., 2006; Wren et al., 2006; Gröbner et al., 2009; Kobayashi et al., 2009). One possible explanation is that this assay included extraction, the Taqman probe and an internal control, which was co-extracted and co-amplified, all of which enhanced the performance of the assay. Overall, the real-time PCR assay detected 0.7% more MRSA-positive screens than the routine standard Brilliance Chromogenic MRSA agar culture method. It has been suggested that there is a need for enrichment culture (Fang & Hedin, 2003). However, the use of enrichment would increase the TAT, and this PCR method has been shown to perform well without enrichment (Niesters, 2004; Krishna et al., 2008). Any laboratory performing tests for MRSA needs to weigh up the patient benefit in relation to MRSA prevalence and the benefit of rapid results. In our study, there were two screens that were not detected by the PCR but were positive by culture. The most likely explanations include poor inoculation into the saline broth, a bacterial count on the swab below the level of detection by PCR or false-positive results. Sequence analysis of the cultured isolates from these two screens confirmed that they had the full SCCmec cassette present and a subsequent re-run of the isolates by PCR proved that the assay was capable of detecting these MRSA isolates. This problem could be avoided by using an improved swab collection system such as the flocked swab with liquid Amies medium (Chernesky et al., 2006); this would negate the need to express the swab in a liquid prior to PCR extraction.

With this improved assay and methodology, we were able to maintain the TAT in the laboratory to within an 8 h shift, i.e. within the same working day, whereas culture always required substantially longer. For the purposes of this study, TAT was calculated from booking-in time to authorization, as specimens were collected throughout the morning and the cut-off was set at 13:30 Monday to Friday. The TAT could be improved further by reducing delays in specimen collection from time of hospitalization, transport from the patient to laboratory and telephoning of results. In this study, it was found to take 12 h, on average, whether for PCR or culture. This could be achieved by developing a platform for testing in specific areas within the hospital.

Testing of pooled samples proved effective: it had a significant impact and helped decrease costs to the laboratory. Screening programmes currently recommend nasal swabs only; however, the risk is that a significant number of MRSA carriers will not be identified in time. As in Fig. 1, 15 cases (five positive only in throat, eight

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### Table 2. Comparison of the projected cost of screening per swab

<table>
<thead>
<tr>
<th></th>
<th>Routine screening</th>
<th>Real-time PCR assay</th>
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</thead>
<tbody>
<tr>
<td>Consumables</td>
<td>£0.41</td>
<td>£2.35</td>
</tr>
<tr>
<td>Staff</td>
<td>£0.78</td>
<td>£0.66</td>
</tr>
<tr>
<td>Overheads</td>
<td>£0.52</td>
<td>£1.28</td>
</tr>
<tr>
<td>Total cost per swab</td>
<td>£1.71</td>
<td>£4.29</td>
</tr>
<tr>
<td>Total cost for 3340 swabs</td>
<td>£5711.40</td>
<td>£14 328.60</td>
</tr>
</tbody>
</table>

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Testing of pooled samples proved effective: it had a significant impact and helped decrease costs to the laboratory. Screening programmes currently recommend nasal swabs only; however, the risk is that a significant number of MRSA carriers will not be identified in time. As in Fig. 1, 15 cases (five positive only in throat, eight
positive only in groin, wound or other, and two positive by a combination of the two) would have been missed by screening only nasal swabs. However, additional swabs add complexity and cost to the laboratory, and the requirement to process only nose swabs is driven by the need to have a simple, less costly programme for screening.

Molecular screening is more expensive than culture. However, the difference in cost for this assay was in consumables and not in staffing time. The machinery used is generic to other molecular platforms, which enables laboratories to use these platforms for a range of PCR assays. The assay described in this study cost £2.35 (€2.75) per test, which is considerably less than commercial options, most of which cost more than £10 (€11.69) per test. Further study needs to be carried out to see whether intervention within 1 day has an impact on hospital-wide costs, number of patients, hospital stay, etc.

Further work would include other improvements to the assay for automated liquid handling in the laboratory, liquid swabs and laboratory interface machines so the assay becomes as simple as pressing one button to obtain results, as well as reducing the laboratory time for extraction and PCR to enable test results to be available within 2 h.

This optimized, in-house, real-time PCR test maintained a very high sensitivity and specificity when evaluated under real-time laboratory conditions. The TAT of this real-time PCR assay was substantially lower than that of chromogenic culture. It was also maintained throughout the entire process, which can be taken as an indirect measure of the test performance. Thus, implementation of such a molecular test could be achieved with limited resources in a standard microbiology laboratory.

ACKNOWLEDGEMENTS

This study was funded by an unrestricted grant from the Scottish Executive. The funding source did not have any role in the study design, execution, analysis or writing of the manuscript conclusions.

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