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RESEARCH NOTE

spa typing directly from a *mecA*, *spa* and *pvl* multiplex PCR assay—a cost-effective improvement for methicillin-resistant *Staphylococcus aureus* surveillance

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ABSTRACT

Rapid detection and typing of methicillin-resistant *Staphylococcus aureus* are important components of infection control programmes. A protocol is described that enables sequencing of the *spa* gene fragment directly from a multiplex PCR targeting the clinically relevant *mecA*, *pvl* and *spa* genes, resulting in high-throughput characterisation of *S. aureus*. Implementation of the method in the Danish national reference laboratory has markedly reduced the use of reagents and the requirement for hands-on time, and has also provided fast typing results. In addition, the method reduces the risk of sample mishandling.

Keywords Detection, direct sequencing, MRSA, multiplex PCR, *spa* typing, typing

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The emergence and spread of strains of methicillin-resistant *Staphylococcus aureus* (MRSA) is a major healthcare concern in many parts of the world. MRSA surveillance should include, as a minimum, detection of the *mecA* gene and typing of the isolates, and detection of the *pvl*

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genes encoding Panton–Valentine leukocidin is often of interest [1,2]. In Denmark, all MRSA isolates are referred to the Statens Serum Institut (SSI), Copenhagen, for typing. In 2007, *spa* typing replaced pulsed-field gel electrophoresis at SSI as the primary typing method for MRSA because of its high discriminatory power and other advantages with respect to speed, interpretation and communicability. The present study describes a method that, in addition to conventional *spa* typing, detects the presence of the *mecA* and *pvl* genes in a single multiplex PCR. As *spa* typing involves DNA sequencing, the method also introduces the concept of sequencing one PCR fragment directly from among a mixture of PCR amplicons. Direct sequencing from the multiplex PCR minimises the number of PCRs required and the risk of sample mishandling.

DNA templates were prepared according to Kumari *et al.* [3]. Each PCR contained 0.45 μM *mecA* primers (*mecA* P4, 5′-TCCAGATTACA ACTTCACCAGG; *mecA* P7, 5′-CCACTTCA TATCTTGTAACG) [4], 0.18 μM *spa* primers (*spa*-1113f, 5′-TAAAGACGATCCTTCGGTGAGC; *spa*-1514r, 5′-CAGCAGTAGTGCCGTTTGCTT) [5], 1 μM *pvl* primers (*pvl*-FP, 5′-GCTGGACAAA ACTTCTTGGAATAT; *pvl*-RP, 5′-GATAGGAC ACCAATAAATTCTGGATTG) [6], 1 × Multiplex PCR Master Mix (Qiagen, Valencia, CA, USA), 0.5 × Q-Solution (Qiagen) and 1 μL of DNA template preparation. Amplification was performed in a DNA Engine DYAD (Bio-Rad, Hercules, CA, USA), with 15 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 59°C and 1 min at 72°C, with a final 10 min at 72°C. PCR products were visualised on E-Gels 2% w/v (Invitrogen, Carlsbad, CA, USA). The PCR products were vacuum-purified using NucleoFast 96 PCR plates (Macherey-Nagel, Easton, PA, USA) according to the manufacturer's instructions. Sequencing of the amplicons with the PCR primers was performed as described previously using an ABI 3130 sequencer [5]. Ridom StaphType (Ridom, Münster, Germany) and BioNumerics v.4.6 (Applied Maths, Sint-Martens-Latem, Belgium) software, together with the multilocus sequence typing database (http://www.MLST.net), were used for analysis and annotation of the sequences generated from the isolates.

The protocol was validated against 70 known MRSA isolates, 25 isolates of coagulase-negative staphylococci, and the three reference strains ATCC 6538 (methicillin-susceptible *S. aureus*), ATCC 33591 (MRSA) and ATCC 51625 (methicillin-resistant *Staphylococcus epidermidis*). Thirty-five of the 70 MRSA isolates had previously been confirmed as *pvl*-positive according to the PCR method described by Lina *et al.* [7]. The multiplex assay was initially optimised as a duplex PCR containing the *mecA* and *spa* primers, and was later expanded to include the *pvl* primers. Both PCRs were validated with the collection of isolates described above.

The MRSA isolates had been assigned previously to clonal complexes (CCs) by pulsed-field gel electrophoresis, *spa* typing and, in some instances, multilocus sequence typing. The isolates belonged to 11 CCs (CC5, CC8, CC9, CC22, CC30, CC45, CC59, CC72, CC78, CC80 and CC152) and harboured SCC*mec* types I–VI. The known *pvl*-positive isolates belonged to nine different CCs, including the USA300-0114 (ST8-MRSA-IV), European CA-MRSA (ST80-MRSA-IV) and Southwest Pacific (ST30-MRSA-IV) clones (Fig. 1). Of the coagulase-negative staphylococcus isolates tested (11 *S. epidermidis*, seven *Staphylococcus hominis*, two *Staphylococcus lugdunensis*, two *Staphylococcus capitis*, two *Staphylococcus haemolyticus* and one *Staphylococcus warneri*), 11 were *mecA*-positive. The *mecA* status and species identifications were confirmed using the EVIGENE MRSA detection kit (SSI, Copenhagen, Denmark), and the RapidStaph32 system (bioMérieux, Marcy-l'Étoile, France).

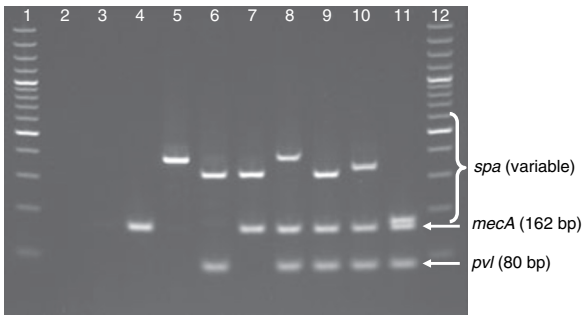

Fig. 1. Examples of isolates typed by the *spa*, *mecA* and *pvl* multiplex PCR. Lanes: 1 and 12, 100-bp ladder; 2, H_2O control; 3, *Staphylococcus epidermidis* DK-14; 4, *S. epidermidis* ATCC 51625; 5, methicillin-susceptible *Staphylococcus aureus* (MSSA) ATCC 6538; 6, MSSA DK-E2211; 7, methicillin-resistant *Stapylococcus aureus* (MRSA) ATCC 33591; 8, MRSA USA300-0114; 9, MRSA CC80 (282-01); 10, MRSA CC30 (3250-01); 11, MRSA DK-55599 (one *spa* repeat).

The results of the initial validation showed complete concordance with the results obtained previously according to *mecA*, *spa* and *pvl* PCRs performed individually. The *mecA* and *pvl* amplicons from the multiplex PCR were sequenced to confirm the identity of the amplicons and to test the feasibility of direct sequencing of a particular amplicon from a multiplex PCR mixture.

Following the initial validation of the PCR method with only the *mecA* and *spa* primers, the duplex protocol was introduced routinely at SSI during January 2007. Testing of 759 routine samples identified 686 MRSA and 73 methicillin-susceptible *S. aureus* isolates. Sequencing revealed 102 different *spa* types among the MRSA isolates, of which ten were identified as new types according to the Ridom *spa* server (www.ridom.de). Of the 102 *spa* types, 79 were assigned to 16 different CCs, while 23 *spa* types (42 isolates) could not be assigned to CC groups. Isolates containing only one *spa* repeat (*spa* type t693; r07) were still detected correctly (Fig. 1, lane 11). Nine isolates failed to yield the *spa* gene fragment, but were confirmed as *S. aureus* by coagulase tests and by detection of the *nuc* and *femA* genes by PCR [8–10].

The multiplex method described in this report provides an efficient protocol for detecting three of the most important determinants used in surveillance of MRSA. The method confirms the presence of the *mecA* gene and detects the *pvl* genes in the same PCR used for conventional *spa* typing, which makes the procedure less expensive and laborious than performing separate reactions. Like conventional *spa* typing, the method is a fast, sequence-based typing method for *S. aureus*. Typing results are currently available in 2 working days, but could be made available in 1 day if facilities allow. Since January 2007, typing results have been reported twice-weekly from the laboratory, thereby providing timely results for local infection control purposes. Although the *pvl* genes are not restricted solely to community-acquired MRSA, *pvl* is present in all major community-acquired MRSA lineages and enables, e.g., distinction of USA300 isolates from other ST8 isolates [1,2,11].

To our knowledge, *spa*-sequencing directly from the products of a multiplex PCR has not been described previously. Other MRSA sequence typing protocols have involved other genes encoding surface proteins, e.g., *clfB*, in addition to *spa*, in order to improve the discriminatory power [12]. The concept presented here of direct sequencing of a single cohort of PCR products from among a mixture of different amplicons can be transferred easily to such protocols, thus enabling, e.g., *spa* and *clfB* to be sequenced from the same multiplex PCR.

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RESEARCH NOTE

Comparison of phenotypic methods for penicillinase detection in *Staphylococcus aureus*

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ABSTRACT

Penicillinase testing is required for *Staphylococcus aureus* isolates with a penicillin MIC of ≤0.12 mg/L. This study compared five phenotypic assays with a PCR for *blaZ* when testing 197 *S. aureus* isolates. The starch–iodine plate method and nitrocefin tests had low sensitivities of 42.9% and 35.7%, respectively. The cloverleaf assay and the penicillin zone-edge determination method had sensitivities of 67.8% and 71.4%, respectively, and these methods might be appropriate in many circumstances, but were not as sensitive as *blaZ* PCR.

Keywords *blaZ*, PCR, penicillinase, phenotypic assays, sensitivity testing, *Staphylococcus aureus*

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Resistance to penicillin in *Staphylococcus aureus* is mediated by production of a penicillinase, encoded by *blaZ*. Four types of penicillinase (termed A–D) have been described in *S. aureus*, all of which are Ambler class A β-lactamases [1]. Reliable detection of penicillinase production is important because penicillin is considered to be superior to oxacillin against isolates that do not produce a penicillinase. An erroneus report of penicillin susceptibility could result in potentially inadequate therapy of *S. aureus* infections. A penicillin MIC of ≤0.12 mg/L is formally in the sensitive range, but the CLSI recommends that additional testing should be performed [2]. The aim of the present study was to compare five phenotypic methods for penicillinase detection, i.e., penicillin disk-diffusion, penicillin zone-edge determination, a cloverleaf assay, nitrocefin tests and a starch–iodine plate method, against a PCR assay for *blaZ* when testing the sub-population of isolates with a penicillin MIC in the formally sensitive range according to the Vitek 2 system (bioMérieux, Durham, NC, USA).

Consecutive non-duplicate isolates of *S. aureus* (n = 197) were collected if their penicillin MIC according to Vitek 2 was ≤0.12 mg/L. Disk-diffusion tests for penicillin were performed according to CLSI recommendations [2] (10-U disk; Oxoid, Basingstoke, UK). The penicillin zone-edge was checked for a sharp edge with discrete full-sized colonies right at the edge, or for a tapered edge with a gradual decrease in growth [3]. A sharp edge was considered to be indicative of penicillinase production. Zone inhibition diameters were measured, with a diameter of ≤28 mm for penicillin being considered to be penicillinase-positive [2]. The cloverleaf assay was performed as described previously [4], except that *S. aureus* ATCC 25923 was used as the indicator strain on Mueller–Hinton agar (Merck, Darmstadt, Germany). Testing with nitrocefin sticks (Oxoid) was performed according to the manufacturer's instructions, with colonies located at the penicillin zone-edge, and was read after 5, 15 and 60 min. The starch–iodine plate method was performed as described previously [4], except that oxacillin (Sigma, Taufkirchen, Germany) 0.2 mg/L was used for penicillinase induction instead of methicillin. Decolourisation under and around the colonies was considered to be a positive result.

Primers for amplification of *blaZ*, designed according to *blaZ* sequences published previously, were stau-blaZ-fwd (5′-CAAAGATGA TATAGTTGCTTATTCTCC) and stau-blaZ-rev

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