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Real-Time PCR Assay for Detection of *blaZ* Genes in *Staphylococcus aureus* Clinical Isolates

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The Clinical and Laboratory Standards Institute recommends consideration of *blaZ* gene testing for cases of serious *Staphylococcus aureus* infection. Conventional PCR methods have demonstrated superior sensitivity and specificity to phenotypic tests. To our knowledge, this is the first description of real-time PCR detection of the *blaZ* gene.

Staphylococcus aureus bacteremia is a cause of significant morbidity and mortality, with 30-day mortality rates of up to 27% (1). Although rates of *S. aureus* penicillin susceptibility are generally low, ranging from 5 to 20% (2, 3), penicillin remains the treatment of choice for penicillin-susceptible *S. aureus* (PSSA) based on *in vitro* data indicating increased susceptibility to penicillin over penicillinase-resistant β-lactams (3–5).

Penicillin resistance in *S. aureus* is manifest predominantly via the production of β-lactamase encoded by the *blaZ* gene (6), with four variants of the β-lactamase (A, B, C, and D) identified using immunologic methods and substrate profile techniques (7, 8). A number of phenotypic methods for the detection of β-lactamase production in *Staphylococcus* species have been investigated (9–15), but compared to detection of the *blaZ* gene by PCR, all phenotypic methods had a sensitivity of less than 72% (15, 16). This has led to the Clinical and Laboratory Standards Institute (CLSI) recommending that *blaZ* gene detection be considered for PSSA isolates from cases of serious infection requiring penicillin therapy (4).

Various PCR methods have been described for the detection of *blaZ* gene in *Staphylococcus* species (5, 16), but to date there are no published real-time PCR methods. We describe a real-time PCR assay for the detection of the *blaZ* gene in *S. aureus*.

One hundred nonduplicate *S. aureus* bloodstream isolates (BSI) collected between September 2011 and December 2012 by the PathWest Laboratory Medicine WA Queen Elizabeth II Medical Centre Department of Microbiology were included in this study. Of these, 50 isolates were susceptible to penicillin and 50 were resistant to penicillin when tested by Kirby-Bauer disc diffusion according to CLSI guidelines (17). An additional 28 nonduplicate clinical *S. aureus* isolates which had the presence or absence of the *blaZ* gene determined by microarray analysis (Alere Technologies, Jena, Germany), as previously described (18), were also included. Of these, 14 isolates had *blaZ* detected by microarray, and 14 isolates did not. Previously characterized (8, 19–22) reference *S. aureus* type strains and β-lactamase strain variants were used as positive controls. These included the following: type A, PC1(pI254) and NCTC 9789; type B, 22260 and ST79/741; type C, 3804(pII3804), RN9(pII147), and V137; and type D, FAR10.

Colonies of *S. aureus* located at the penicillin zone edge of a 10-μg penicillin disc on Mueller-Hinton agar (Oxoid, Ltd., Hampshire, United Kingdom) were tested with nitrocefin-impregnated discs (BBL; BD, USA) according to the manufacturer's instructions. Zone edge assessment was made on all isolates and recorded as being either a sharp "cliff" edge suggestive of β-lactamase production or a tapered "beach" edge suggestive of absence of β-lactamase production. Interpretation of phenotypic tests was performed by two independent observers without reference to the PCR results. Although interobserver variability has been demonstrated previously in regard to interpretation of zone edge characteristics (15), in this study no interobserver discrepancies were demonstrated.

Bacterial DNA from *S. aureus* isolates cultured on horse blood agar for 24 h at 37°C in room air was extracted as follows. Two to five loopfuls of *S. aureus* were suspended in 1 ml sterile demineralized water and boiled for 10 min, and the suspension was centrifuged for 2 min. From this, 8 μl of the supernatant was used with 12 μl of either conventional or real-time PCR amplification mixes.

The conventional *blaZ* PCR was performed as described previously (16). The real-time *blaZ* PCR primer and probe sequences were designed in-house using Primer Express software (Applied Biosystems). Fluorophore-labeled oligonucleotide probes were synthesized by Applied Biosystems (MGB probes), and primers were synthesized by Integrated DNA Technologies (Fisher Biotec, Australia). Sequences for the *blaZ* gene available from GenBank were obtained by BLASTn search using accession no. FR714929 and aligned prior to primer and probe design. Anomalous bases were used in the primers and probe where necessary to allow detection of genetic variants of the *blaZ* target. The reaction mix contained PCR buffer (Applied Biosystems, USA), 4 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (Sigma, USA), 0.75u AmpliTaq Gold DNA polymerase (Applied Biosystems, USA), 0.2 μM forward primer (5′-GCTTTAAAAGAACTTATTGAGGCTTCA-3′), 0.2 μM reverse primer (5′-CCACCGATYTCKTTTATAATTT-3′), and 0.2 μM TaqMan probe (5′-FAM-AGTGATAATACAGCAAACAA-MGBNFQ-3′, where FAM is 6-carboxyfluorescein). The amplification was performed using RotorgeneQ real-time thermocyclers (Qiagen, Australia). Reactions

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TABLE 1 Phenotypic and real-time PCR tests compared to conventional PCR for detection of β-lactamase in *S. aureus* isolates

Testing method and result by *S. aureus* isolate group	No. of positive tests	No. of negative tests	% sensitivity	% specificity	PPV (%)[a]	NPV (%)[b]
Penicillin-sensitive (by disc diffusion) *S. aureus* BSI ($n = 50$)						
Nitrocefin disc test	0	50	0	100	0	96
Penicillin zone edge[c]	1	49	50	100	100	98
Real-time PCR	2	48	100	100	100	100
Conventional PCR	2	48				
Penicillin-resistant (by disc diffusion) *S. aureus* BSI ($n = 50$)						
Nitrocefin disc test	50	0	100		100	
Penicillin zone edge[c]	50	0	100		100	
Real-time PCR	50	0	100		100	
Conventional PCR	50	0				
S. aureus clinical isolates, *blaZ* status characterized by microarray ($n = 28$)						
Nitrocefin disc test	14	14	100	100	100	100
Penicillin zone edge[c]	14	14	100	100	100	100
Real-time PCR	14	14	100	100	100	100
Conventional PCR	14	14				
S. aureus blaZ-positive reference strains ($n = 6$)						
Nitrocefin disc test	6	0	100		100	
Penicillin zone edge	6	0	100		100	
Real-time PCR	6	0	100		100	
Conventional PCR	6	0				
All *S. aureus* isolates tested ($n = 134$)						
Nitrocefin disc test	70	64	97.2	100	100	96.9
Penicillin zone edge[c]	71	63	98.6	100	100	98.4
Real-time PCR	72	62	100	100	100	100
Conventional PCR	72	62				

[a] PPV, positive predictive value.
[b] NPV, negative predictive value.
[c] A beach edge indicates lack of β-lactamase (negative test), whereas a cliff edge indicates its presence (positive test).

were run under the following conditions: 95°C for 10 min, followed by 50 cycles of 12 s at 94°C, 15 s at 55°C, and 20 s at 72°C. Probe emission signals were acquired during the 20-s extension step of the cycling program.

Amplified DNA produced by the conventional and real-time PCR was sequenced using the AB BigDye Terminator version 3.1 sequencing kit on the AB 3130xl genetic analyzer (Applied Biosystems). Sequences were compared to those of the *blaZ* gene available in GenBank using BLASTn software.

The conventional PCR method was verified by testing with the positive-control reference strains and the clinical isolates previously characterized by microarray analysis. All positive-control reference strains had *blaZ* detected by both conventional PCR and real-time PCR. The 14 clinical isolates that had *blaZ* detected by microarray analysis, demonstrating cliff penicillin zone edges, were nitrocefin test positive and had *blaZ* detected by both conventional and real-time PCR. The 14 clinical isolates that did not have *blaZ* detected by microarray analysis, demonstrating beach penicillin zone edges, were nitrocefin test negative and did not have amplified DNA produced by either conventional or real-time PCR.

The 50 *S. aureus* BSI that tested penicillin resistant by disc diffusion according to CLSI criteria had *blaZ* detected by both the conventional PCR and real-time PCR. For these isolates, all had either no zone around the penicillin disc or a cliff zone edge, and all had a positive nitrocefin test; results are shown in Table 1. For the 50 phenotypically penicillin-susceptible *S. aureus* BSI, the sensitivity, specificity, and positive and negative predictive values of the real-time PCR, the nitrocefin disc test, and the characterization of zone edge, compared to those of the conventional PCR reference method, are shown in Table 1. Two of these had *blaZ* detected by both the conventional and real-time PCR methods, and the results were confirmed by sequencing. Sequences of the 2 isolates demonstrated 100% homology with *blaZ* types in GenBank (accession no. DQ016067 and GQ980074). There was no genetic mutations identified that would account for the discrepant phenotypic and genotypic results; however, expression of the *blaZ* gene may be affected by mutations in DNA encoding promoter or repressor regions (23). These 2 isolates had a negative nitrocefin test, and one of the isolates had a penicillin zone edge characterized as beach edge and the other a cliff edge. All of the remaining 48 BSI that did not have *blaZ* detected by PCR had phenotypic test results consistent with the absence of β-lactamase.

The real-time PCR for detection of the *blaZ* gene was developed in response to current CLSI recommendations. Conventional PCRs have previously been described and have been used as

the gold standard for β-lactamase detection when comparing phenotypic tests (6, 13, 16, 24, 25). The advantages of the real-time PCR over conventional PCR include a faster turnaround time, less specimen handling with subsequent reduced workload and risk of specimen contamination, lower cost, and equivalent sensitivity (100%) and specificity (100%). These advantages would facilitate more routine testing of the *blaZ* gene in accordance with CLSI recommendations. The clinical impact of introducing routine real-time PCR testing will require further study.

To our knowledge, this is the first published study to describe a real-time PCR for the detection of the *blaZ* gene in *S. aureus*. This real-time PCR is able to detect all four variants of *S. aureus* β-lactamase facilitating rapid and accurate confirmation of the presence of the *blaZ* gene in *S. aureus* clinical isolates that test penicillin susceptibility by phenotypic methods.

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