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Prevalence of *blaZ* Gene and Performance of Phenotypic Tests to Detect Penicillinase in *Staphylococcus aureus* Isolates from Japan

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Guidelines recommend that clinical laboratories perform phenotypic tests (nitrocefin-based test and penicillin 10-U [P10] or 1-U [P1] zone edge tests) to detect penicillinase in *Staphylococcus aureus* isolates. This study aimed to assess the prevalence of *blaZ* encoding penicillinase and perform various phenotypic tests in *S. aureus* isolates from Japan. We prospectively collected 200 methicillin-susceptible *S. aureus* isolates from June 2015 to January 2016 and performed six phenotypic tests (nitrocefin-based test, P10 zone edge test/P10 diffusion test, penicillin 2-U [P2] zone edge test/P2 diffusion test, and cloverleaf test) on each sample. We confirmed the presence of *blaZ* (two *blaZ*-positive isolates) using PCR. Using *blaZ* PCR as a standard, we observed a low sensitivity (50%) and positive predictive value (PPV, 50%) of the nitrocefin-based test, low PPV (18.2%) of the P10 zone edge test, low sensitivity (50%) of the P10 diffusion test, low PPV (50% and 22.2%) of the P2 zone edge test and P2 diffusion test, respectively, and low sensitivity (50%) of the cloverleaf test. These data suggest a low performance (sensitivity and PPV) of these six phenotypic tests because of the low prevalence (1%) of *blaZ* in *S. aureus* isolates from Japan.

Key Words: *blaZ*, Phenotypic tests, Penicillinase, *Staphylococcus aureus*, Japan

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Some *Staphylococcus aureus* strains remain susceptible to penicillin G (Pc), although resistance rapidly emerged after introducing Pc. According to the Japan Nosocomial Infections Surveillance report in 2015 (http://www.nih-janis.jp/), 43.8% of 119,343 methicillin-susceptible *S. aureus* (MSSA) isolates from all registered medical institutes were susceptible to Pc. Thus, this antimicrobial agent remains the treatment of choice for patients infected with Pc-susceptible isolates, as Pc is considered superior to oxacillin against penicillinase-negative isolates. Reliable detection of penicillinase production is important, but the detection and reporting of Pc susceptibility and resistance remains difficult.

Two mechanisms contribute to Pc resistance in *S. aureus*; first, involving the production of penicillinase encoded by *blaZ*, which can inactivate Pc by hydrolyzing the β-lactam ring [1], and second, involving an altered Pc-binding protein, PBP2a, encoded by *mecA* [2, 3]. *blaZ* is an 846-bp gene controlled by two regulatory genes (antirepressor *blaR1* and repressor *blaI*) [3]. After exposure to β-lactams, *blaR1* (transmembrane sensor–transducer) undergoes autocatalytic cleavage, promoting the cleavage of *blaI* and leading to the transcription of *blaZ* [1, 4]. Serotype analysis has reported four types (Ambler class A) of penicillinase: A, C, and D are located on plasmids, while B is located on the chromosome [5, 6].

Guidelines of the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommend that micro-

biological laboratories perform phenotypic tests (nitrocefin-based test and Pc 10-U [P10] or 1-U [P1] zone edge tests) to detect penicillinase in *S. aureus* strains [7, 8]. Nitrocefin-based tests are reportedly less sensitive than Pc-disc zone edge determinations [9-11]. This study aimed to assess the prevalence of *blaZ* and performance of various phenotypic tests using *S. aureus* isolates from Japan.

We prospectively collected and randomly selected non-duplicate 200 MSSA isolates with Pc minimum inhibitory concentrations (MICs) of ≤0.12 μg/mL (breakpoint judged as susceptible to Pc) based on the CLSI broth microdilution (BMD) method with the MicroScan WalkAway System (Beckman Coulter Inc., Tokyo, Japan) at one laboratory (Byotai-Seiri Laboratory), from June 2015 through January 2016 [7]. Patient information (including gender, age, and clinical specimens) was recorded. We performed six phenotypic tests (nitrocefin-based test, P10 zone edge test/P10 diffusion test, Pc 2-U [P2] zone edge test/P2 diffusion test, and cloverleaf test) for each screened isolate. P2 was applied as the minimum U of Pc because the recommended P1 was not available in Japan. Briefly, a BBL Cefinase Paper disc (Becton, Dickinson and Company, Tokyo, Japan) was used in the nitrocefin-based test [7]. This test was conducted using inoculum directly from the margin zone surrounding a cefoxitin disc (30 μg) placed on Mueller-Hinton agar (MHA) after 16–18 hours of incubation to induce penicillinase production in the isolates. When the disc showed a pink color at room temperature (approximately 20°C) within 1 hour of the reaction, the isolate was considered penicillinase-positive. Two different disc dif-

Table 1. Relationship between results of phenotypic penicillinase tests and *blaZ* PCR as the standard

Isolate No.	Time of isolation (year/month)	Source	Nitrocefin-based test	P2 zone inhibition test (mm)*	P2 zone edge test	P10 zone inhibition test (mm)†	P10 zone edge test	Cloverleaf test	*blaZ* PCR using primer set stau-*blaZ*-fwd/stau-*blaZ*-rev	*blaZ* PCR using primer set 486/488
2	2015/Jun	Pus	-	24 (R)	Fuzzy	44 (S)	Fuzzy	-	-	-
3	2015/Jun	Sputum	-	25 (R)	Fuzzy	40 (S)	Fuzzy	-	-	-
6	2015/Jun	Skin	-	25 (R)	Fuzzy	44 (S)	Fuzzy	-	-	-
8	2015/Jun	Periunguinal region	-	25 (R)	Fuzzy	42 (S)	Fuzzy	-	-	-
10	2015/Jun	Sputum	-	25 (R)	Fuzzy	39 (S)	Fuzzy	-	-	-
16	2015/Jul	Pus	-	25 (R)	Fuzzy	44 (S)	Fuzzy	-	-	-
28	2015/Jul	Sputum	-	25 (R)	Sharp	32 (S)	Sharp	-	+	+
31	2015/Jul	Pus	-	31 (S)	Fuzzy	44 (S)	Sharp	-	-	-
33	2015/Jul	Sputum	+	15 (R)	Sharp	20 (R)	Sharp	+	+	+
63	2015/Jul	Decubitus	-	31 (S)	Fuzzy	37 (S)	Sharp	-	-	-
65	2015/Jul	Sputum	-	31 (S)	Fuzzy	37 (S)	Sharp	-	-	-
79	2015/Aug	Ear discharge	-	31 (S)	Sharp	34 (S)	Sharp	-	-	-
85	2015/Sep	Stool	-	32 (S)	Sharp	37 (S)	Sharp	-	-	-
88	2015/Sep	Blood	-	40 (S)	Fuzzy	46 (S)	Sharp	-	-	-
93	2015/Sep	Sputum	-	31 (S)	Fuzzy	36 (S)	Sharp	-	-	-
95	2015/Sep	Blood	-	31 (S)	Fuzzy	37 (S)	Sharp	-	-	-
121	2015/Oct	Pus	-	32 (S)	Fuzzy	40 (S)	Sharp	-	-	-
178	2015/Dec	Sputum	-	22 (R)	Fuzzy	31 (S)	Fuzzy	-	-	-
187	2015/Dec	Skin	+	30 (S)	Fuzzy	42 (S)	Fuzzy	-	-	-
ATCC 25923	NA	NA	-	28 (S)	Fuzzy	34 (S)	Fuzzy	NA	-	-
ATCC 29213	NA	NA	+	17 (R)	Sharp	19 (R)	Sharp	+	+	+

ATCC 25923 and ATCC 29213 were applied as penicillinase-negative and -positive controls, respectively.
*P2 zone diameters were interpreted according to the EUCAST criteria (isolates with P1 diameter of ≤25 mm were considered resistant) [11], and its interpretations are given in parentheses; †P10 zone diameters were interpreted according to the CLSI criteria (isolates with P10 diameter of ≤28 mm were considered resistant) [11], and its interpretations are given in parentheses.
Abbreviations: R, resistant; S, susceptible; NA, not applicable; +, positive; -, negative.

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fusion tests were performed on MHA by applying both P10 (BD Sensi-Disc, Becton, Dickinson and Company) and P2 (SP Check, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). P10 zone diameters were interpreted according to the CLSI criteria (isolates with a P10 diameter of ≤28 mm were considered resistant) [7], and P2 zone diameters were interpreted according to the EUCAST criteria (isolates with a P1 diameter of ≤25 mm were considered resistant) [8]. The Kirby–Bauer Pc disc diffusion zone of inhibition was virtually assessed as "sharp" or "fuzzy" [7]. A "sharp" edge at the inhibition zone around the disc suggested penicillinase production, whereas a "fuzzy" edge suggested no production. Both the zone diameter and appearance of the zone edge were recorded independently by two investigators. The cloverleaf test was also conducted to detect penicillinase [10]. Any deviation from a complete circle was considered positive for penicillinase. All isolates were stored at –80°C until genetic analyses. The study protocol was examined and approved by the committee of the institution (Byotai-Seiri Laboratory).

After completing the phenotypic tests, all the stored isolates were sent to one laboratory (Kitasato Institute for Life Sciences) for further analyses. Two different primer sets of stau-*blaZ*-fwd/stau-*blaZ*-rev 488 were used to amplify *blaZ* by PCR from a mixture of 100–200 ng of extracted genomic DNA [10]. PCR by stau-*blaZ*-fwd/stau-*blaZ*-rev was selected as the standard, as there were no mutations in its primer sequences and the amplicon generated by this set was within the *blaZ* coding region. Amplification products (421-bp and 674-bp) were resolved on a 1.5% agarose gel, stained with ethidium bromide, and visualized using an ultraviolet transilluminator. We also confirmed the correct sequences of the amplicons of all positive isolates by PCR. ATCC 29213 (penicillinase-positive) and ATCC 25923 (penicillinase-negative) were used as quality control strains for the BMD method, phenotypic tests, and PCR methods [11].

Two hundred isolates were recovered from 13 sterile specimens (11 blood/1 joint fluid/1 pleural effusion) and 187 nonsterile specimens (120 respiratory tract-origin/52 skin-origin/14 urine/1 stool) of patients (109 men/91 women; median age 76 years, range 0–102 years). The relationship between phenotypic test data and *blaZ* PCR results is shown in Table 1. All isolates showed Pc MICs of ≤0.03 μg/mL based on the CLSI BMD method [7]. The isolates excluded from Table 1 showed negative results for the six phenotypic tests and two *blaZ* PCR methods. We observed only two *blaZ*-positive isolates (No. 28 and 33) with correct sequences, which were amplified by both primer sets stau-

Table 2. Sensitivity, specificity, and positive and negative predictive values of penicillinase tests using *blaZ* PCR as the standard

Penicillinase test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Nitrocefin-based test	50	99.5	50	99.5
P2 zone diameter interpretation*	100	96.5	22.2	100
P2 zone edge test	100	99	50	100
P10 zone diameter interpretation†	50	100	100	99.5
P10 zone edge test	100	95.5	18.2	100
Cloverleaf test	50	100	100	99.5

*P2 zone diameters were interpreted according to the EUCAST criteria (isolates with a P1 diameter of ≤25 mm were considered resistant) [11]; †P10 zone diameters were interpreted according to the CLSI criteria (isolates with a P10 diameter of ≤28 mm were considered resistant) [11].

Table 3. Prevalence of *blaZ* among *Staphylococcus aureus* isolates in previous reports and the current study

Year of report (reference)	Country of isolation (total N of collected isolates)	Percentage (%) of *blaZ* gene prevalence among all isolates	Primer set used to amplify *blaZ*	Amplicon size (bp)
2008 [9]	Germany (197)	14.2	stau-*blaZ*-fwd/stau-*blaZ*-rev	421
2011 [12, 13]	Japan (450)	2.7	stau-*blaZ*-fwd/stau-*blaZ*-rev	421
2012 [10]	United States (105)	9.5	stau-*blaZ*-fwd/stau-*blaZ*-rev, 487/373, & 486/488	421, 377, & 674
2014 [11]	Australia (157)	24.2	blaZ-F/blaZ-R	326
2014 [14]	Japan (170)	3.5	ND	ND
2017 [15]	Switzerland (215)	40.9	*blaZ*-fwd/*blaZ*-rev & blaZ F1/blaZ R1	418 & 533
This report	Japan (200)	1	stau-*blaZ*-fwd/stau-*blaZ*-rev & 486/488	421 & 674

Abbreviation: ND, not described.

blaZ-fwd/stau-*blaZ*-rev and 486/488 (Table 1).

Sensitivity, specificity, and positive and negative predictive values of phenotypic tests using *blaZ* PCR as the standard are indicated in Table 2. We found a low sensitivity (50%) and positive predictive value (PPV, 50%) of the nitrocefin-based test, low PPV (18.2%) of the P10 zone edge test, low sensitivity (50%) of the P10 diffusion test, low PPV (50% and 22.2%) of the P2 zone edge test and P2 diffusion test, and low sensitivity (50%) of the cloverleaf test.

Table 3 summarizes the prevalence of *blaZ* among *S. aureus* isolates, various primer sets to amplify *blaZ*, and different amplicon sizes, used in previous studies and the current study [9-15]. *blaZ* prevalence in Japan (2.7%, 3.5%, and 1%) was lower than that observed in Germany, the United States, Australia, and Switzerland (14.2%, 9.5%, 24.2%, and 40.9%, respectively), although it remains unclear why the prevalence was low in Japan. The use of only one PCR primer set and lack of amplicon sequencing may have caused false-positive or false-negative results because of polymorphisms within the *blaZ* sequence (including the PCR primer sequence regions) [5, 16] or targeting a genetic region peripheral to *blaZ* [10]. False-positive results are induced by non-functional mutant genes that are inadequately counted, and false-negative results occur when functional mutant genes are missed [11]. Therefore, we applied two primer sets for sequencing.

In conclusion, these data suggest the low performance (sensitivity and PPV) of the six phenotypic tests because of the low prevalence (1%) of *blaZ* in *S. aureus* isolates from Japan. The decreased sensitivities of the phenotypic tests in this study may be related to the use of a highly selective collection of isolates, all of which had low Pc MICs (≤0.03 μg/mL). Therefore, additional studies on isolates with borderline MICs (0.06 and 0.12 μg/mL) are needed.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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