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Original Article

Accurate detection of oxacillin-resistant *Staphylococcus lugdunensis* by use of agar dilution

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Abstract *Background/purpose: Staphylococcus lugdunensis* is a Gram-positive coagulase-negative bacterium and is recognized as a critical pathogenic species recently. Here, we aimed to evaluate the cefoxitin disk diffusion (CDD), oxacillin agar dilution (OAD), and *mecA* PCR for detecting oxacillin-resistant *S. lugdunensis* (ORSL) isolates.
Methods: Multilocus sequence typing (MLST) analysis was performed to determine the clonality of 117 *S. lugdunensis* isolates isolated between May 2009 and Jul 2014. CDD, OAD, and *mecA* PCR were used to identify oxacillin-resistant *S. lugdunensis* (ORSL).
Results: MLST results showed that the most common sequence type (ST) of our *S. lugdunensis* isolates was ST6 (35.9%) followed by ST3 (28.2%), ST27 (17.9%), and ST4 (6.8%). CDD and OAD showed that 39 and 43 isolates were ORSL, respectively. 4 ST3 CDD-susceptible *S. lugdunensis* (OSSL) isolates had MIC values $\geq$ 4 for oxacillin. *mecA* PCR results showed that 43 OAD-resistant *S. lugdunensis* and 3 OAD-susceptible ST27 *S. lugdunensis* had the *mecA* gene. Therefore, OAD was used as the gold standard to evaluate the performance of CDD and *mecA* PCR for identifying ORSL. The overall sensitivity, specificity, and accuracy of CCD for ORSL detection was 90.7%, 100%, and 96.8%, respectively. The sensitivity, specificity, and accuracy of *mecA* PCR for identifying ORSL was 100%, 95.9%, and 97.44%, respectively.

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Conclusion: Our results indicate that OAD shows higher accuracy for ORSL detection compared with CDD and *mecA* PCR.

Introduction

Staphylococcus lugdunensis is a Gram-positive, catalase-positive, and coagulase-negative *Staphylococcus* (CoNS). *S. lugdunensis* infections in humans range from harmless skin colonization to life-threatening invasive infections such as endocarditis and bacteremia.[1—3] Previously, *S. lugdunensis* isolates were usually susceptible to a wide range of antimicrobial agents such as cefazolin, daptomycin, oxacillin, tetracycline, and vancomycin.[4] β-lactams, such as oxacillin, nafcillin, and methicillin, are commonly prescribed to treat *Staphylococcus* infections in hospitals; however, oxacillin-resistant *S. lugdunensis* (ORSL) has been widely reported recently.[5,6] Tan et al. showed that 5 out of 106 *S. lugdunensis* isolates (4.7%) were resistant to methicillin, and all 5 isolates harbored the *mecA* gene which encodes an alternative penicillin-binding protein 2 (PBP2a) with lower affinity to β-lactam antibiotics.[7] We previously reported that 42 out of 118 (35.6%) *S. lugdunensis* isolated in a tertiary medical centre in northern Taiwan carried the *mecA* gene and were resistant to oxacillin.[8] In addition, we found the frequency of ORSL isolates carrying SCC*mec* type V was emerging in central Taiwan, and the major endemic clone of ORSL in Taiwan was sequence type (ST) 6 determined by multilocus sequence typing (MLST).[8,9]

mecA is carried on a mobile genetic element (MGE) called staphylococcal cassette chromosome *mec* (SCC*mec*).[10] According to the International Working Group on the Classification of SCC Elements (IWG-SCC), 13 types (I-XIII) of SCC*mec* have been assigned for *S. aureus* (defined by a combination of *mec* gene complex class and cassette chromosome recombinase gene (*ccr*) allotype) (http://www.sccmec.org).[11—13]

Antibiotics are one of the greatest medical advances of the 20th century; however, these drugs are quickly becoming useless due to resistance that has been augmented by poor antibiotic stewardship and a void in novel antibiotic discovery.[14,15] Moreover, very few novel classes of antibiotics have been discovered since 1960, and the pipeline of antibiotics under development is very limited.[14,16] Therefore, precise and rapid diagnosis of antibiotic susceptibility of clinical isolates is a cost-effective strategy for improving antibiotic prescribing practice and to reduce multi-drug resistance.[17,18] Several phenotypic and genotypic methods such as oxacillin agar dilution (OAD), cefoxitin disk diffusion (CDD), and *mecA* PCR have been recommended by the Clinical and Laboratory Standards Institute (CLSI) for ORSL detection.[19] *S. lugdunensis* isolates with an oxacillin MIC ≥4 μg/mL are defined as resistant based on 2020 CLSI standard.[19] For disk diffusion tests, *S. lugdunensis* isolates with an cefoxitin (30 μg) zone diameter (mm) ≤21 mm are defined as resistant.[19] However, Ho et al. showed that oxacillin resistance in *mecA*-positive *S. lugdunensis* clonal complex 27 (CC27, including ST27 and ST42) cannot be reliably detected by the disk diffusion tests and MIC breakpoints.[20] Twenty-one CDD-susceptible isolates all contained *mecA* and had cefoxitin and oxacillin MIC ≤4 μg/mL and 1—4 μg/mL (MICs of ≤4 μg/mL and 1—4 μg/mL for cefoxitin and oxacillin), respectively.[20] However, the accuracy of phenotypic and genotypic methods for identifying ORSL isolates in different STs is still unclear. Here, we aimed to evaluate the accuracy of different methods for ORSL detection.

Materials and methods

Isolation and identification of *S. lugdunensis*

117 *S. lugdunensis* strains consecutively isolated between May 2009 and Jul 2014 were first identified by Gram staining, biochemical methods (catalase-positive, coagulase-negative, pyrrolidonyl arylamidase-positive, and ornithine decarboxylase-positive results), and rapid PCR detection.[21] All *S. lugdunensis* isolates were further confirmed by a Bruker Biotyper (database 2.0) matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) system. Each *S. lugdunensis* isolate was stocked in tryptic soy broth with 20% glycerol at −80 °C until use.

Multilocus sequence typing (MLST)

Multilocus sequence typing was performed on all *S. lugdunensis* isolates as previously described.[22] In brief, seven housekeeping genes, including *aroE*, *dat*, *ddl*, *gmk*, *ldh*, *recA*, and *yqiL*, were amplified and sequenced. The sequence data was imported to the *S. lugdunensis* MLST database for ST type determination (https://bigsdb.pasteur.fr/). Sequence types were identified based on the allele profiles.

Antimicrobial susceptibility testing

Susceptibility to β-lactams for *S. lugdunensis* isolates was interrogated by disk diffusion using 30 μg cefoxitin disks (Thermo Fisher Scientific Inc., Waltham, MA, USA) on BBL™ Mueller Hinton II agar (Cation-adjusted) with 2% sodium chloride (BD Difco™) according to the CLSI recommendations. MIC values were determined for oxacillin by agar dilution tests. The OAD and CDD were performed and interpreted according to CLSI guidelines.[19] *S. lugdunensis* isolates with an OAD MIC ≥4 μg/mL or CDD zone diameter

≤21 mm (disk diffusion) were defined as resistant. *S. aureus* ATCC 29213 was used as a control strain. Antimicrobial susceptibility testing was performed in duplicate to ensure reproducibility.

SCC*mec* typing and sequencing

All *S. lugdunensis* isolates were subjected to SCC*mec* typing and *mecA* detection by using a multiplex PCR assay to amplify the *ccr* and *mec* complex as previously described.[23] Primers mecA-F (5′-ACACATATCGTGAGCAATGAACTGA-3′) and mecA-R (5′- TGGACTCGTTACAGTGTCACTTTCA-3′) were used for determining *mecA* promoter and coding sequences by PCR-sequencing.[22]

PBP2a test

The penicillin-binding protein 2a (PBP2a) was detected using the MRSA-Screen test (Denka Seiken Co., Ltd., Japan). Colonies grown on 5% blood agar for 24 h were tested according to the manufacturer's instructions and controls (*S. aureus* USA300 and ATCC 29213) were included in this assay.

Results

MLST analysis, a standard nucleotide sequence-based approach, was performed on 117 isolates to characterize the clonality of these isolates, and the results showed that the most common ST among our isolates was ST6 (42/117, 35.9%), followed by ST3 (33/117, 28.2%), ST27 (21/117, 17.9%), and ST4 (8/117, 6.8%) (Table 1). We further performed CDD, OAD, and *mecA* PCR (SCC*mec* multiplex PCR) on 117 *S. lugdunensis* isolates to evaluate these methods to identify ORSL isolates and SCC*mec* types (Table 1). The results showed that 39, 43, and 46 isolates were ORSL, determined by CDD, OAD, and *mecA* PCR, respectively (Table 2).

In Table 1, our OAD results showed that 69.7% of ST3 (23/33) and 34.8% of ST6 (15/42) isolates were ORSL. Importantly, all 15 ST6 ORSL isolates showed high resistance to oxacillin (MIC > 32 μg/mL) (Table 1). In contrast, the range of MICs to oxacillin of 27 ST3 ORSL isolates (4 - >32 μg/mL) was lower than for the ST6 ORSL isolates (Table 1). Interestingly, 4 CDD-susceptible ST3 isolates showed MIC values ≥ 4 μg/mL for oxacillin which were near the susceptible breakpoint, and these 4 isolates were found to have SCC*mec* type V (Table 3). Moreover, strains 47, 71, and 82, had inconsistent CDD results (Table 3).

Chen et al. reported the polymorphisms in *mecA* genes of clinical *S. aureus* isolates are associated with their oxacillin MICs.[24] Therefore, we next determined the *mecA* promoter and coding sequences in our 21 isolates (Table 4). Four CDD-susceptible ST3 isolates (SL44, 47, 71, and 82) with MIC values ≥ 4 μg/mL had a C-33T substitution in the *mecA* promoter (Table 4), which had been shown to have low promoter activity and PBP2a production.[24] Moreover, we found a single nucleotide insertion in *mecA* coding sequence in SL71 (Table 4). In contrast, 2 ST6 isolates (SL90 and SL118) and 1 ST3 (SL138) had a −33C in the *mecA* promoter, showed high resistance to oxacillin (MIC, 16 - >32 μg/mL) (Table 4). In addition, a novel polymorphism C-30T with an unclear role in *mecA* expression was identified in 4 isolates (SL57, SL73, SL131, and SL134) (Table 4). PBP2a tests were carried out to determine the expression of PBP2a in our isolates to investigate the association between oxacillin susceptibility, *mecA* polymorphisms, and PBP2a production (Fig. 1). *S. aureus* USA300 and SA29213 were used as positive and negative controls, respectively. Compared to the positive control USA300, strains 44, 47, 71, and 82, showed weak PBP2a production (Fig. 1). Moreover, PBP2a tests showed the SL90 and SL118 expressed higher PBP2a (Fig. 1).

Table 1 Sequence types, disk diffusion results, MIC values, and SCC*mec* types of 117 isolates.

ST	No.	OXA disk diffusion		OXA MIC (μg/mL)										OXA MIC (R)	SCC*mec* type (no.)
		(R)	(S)	<0.25	0.25	0.5	1	2	4	8	16	32	>32		
ORSL															
ST3	27	23	4	0	0	0	0	0	12	4	2	4	5	27	IV (1), V (24), VT(2)
ST6	15	15	0	0	0	0	0	0	0	0	0	0	15	15	II (14), UT (1)
ST27	1	1	0	0	0	0	0	0	1	0	0	0	0	1	VT (1)
OSSL															
ST1	4	0	4	1	0	3	0	0	0	0	0	0	0	0	
ST2	1	0	1	1	0	0	0	0	0	0	0	0	0	0	
ST3	6	0	6	0	0	5	1	0	0	0	0	0	0	0	
ST4	8	0	8	2	0	6	0	0	0	0	0	0	0	0	
ST6	27	0	27	9	1	10	7	0	0	0	0	0	0	0	
ST9	3	0	3	2	0	1	0	0	0	0	0	0	0	0	
ST12	2	0	2	0	0	1	1	0	0	0	0	0	0	0	
ST15	1	0	1	0	0	1	0	0	0	0	0	0	0	0	
ST27	20	0	20	0	0	3	17	0	0	0	0	0	0	0	V (3), UT(1)
ST29	1	0	1	0	0	1	0	0	0	0	0	0	0	0	
UT	1	0	1	1	0	0	0	0	0	0	0	0	0	0	

ORSL and OSSL were defined by oxacillin agar dilution results.
MIC, minimal inhibitory concentration; OXA, oxacillin; UT, untypable; R, resistant; S, susceptible.

Table 2 Sensitivity, specificity, and accuracy of cefoxitin disk diffusion and *mecA* PCR for identifying oxacillin resistant *S. lugdunensis*.

	Cefoxitin disk diffusion	*mecA* PCR
TP (no.)	39	43
FN (no.)	4	0
TN (no.)	74	71
FP (no.)	0	3
[a]Sensitivity (%)	90.7	100
[b]Specificity (%)	100	95.9
[c]Accuracy	96.8	97.44

[a] Sensitivity = TP/TP + FN.
[b] Specificity = TN/TN + FP.
[c] Accuracy = (TP + TN)/(TP + TN + FP + FN).
ORSL and OSSL were defined by oxacillin agar dilution results. TP, true positive; FN, false negative; TN, true negative; FP, false positive.

SCC*mec* typing results showed that 14 ST6 OAD-resistant isolates (14/15, 93.3%) contained SCC*mec* type II and 1 ST6 OAD-resistant isolate contained untypable SCC*mec* (Table 1). In contrast, 24 (88.9%), 2 (7.4%), and 1 (3.7%) ST3 OAD-resistant isolates had SCC*mec* type V, VT, and IV, respectively (Table 1). All OAD-resistant isolates had *mecA*; however, 4 out of 78 (5.1%) OAD-susceptible isolates also contained SCC*mec*. SL29, 35, 79 had SCC*mec* type V with *mecA* gene and SL210 had untypable SCC*mec* without *mecA* gene (Tables 1 & 4). Moreover, MLST results showed that these 4 SCC*mec*-positive OAD-susceptible isolates belonged to ST27 (Table 1). These results suggest the decrease or loss of *mecA* expression in three *mecA*-positive OSSL ST27 isolates. In contrast to SL90, 118 and SL138, our sequencing results showed that OSSL isolates SL29, SL35, and SL79 had a C-33T substitution in the *mecA* promoter (Table 4) which had been shown to have low promoter activity and PBP2a production.[24] Surprisingly, PBP2a test results showed that 3 *mecA*-positive OAD-susceptible strains (strain 29, 35, and 79), expressed high PBP2a (Fig. 1).

Finally, we used OAD as the gold standard to evaluate the performance of CDD and *mecA* PCR for identifying ORSL isolates. The overall sensitivity, specificity, and accuracy of disk diffusion tests for the detection of ORSL was 90.7%, 100%, and 96.8, respectively (Table 2). In contrast,

Table 3 SCC*mec*, disk diffusion, agar dilution test of 4 disk diffusion-susceptible ST3 oxacillin resistant *S. lugdunensis* isolates.

Strain	SCC*mec* M1	SCC*mec* M2	SCC*mec* type	disk diffusion (mm)	disk diffusion	OXA MIC (μg/mL)	OXA MIC
44	ccr5, *mecA* (+)	C	V	25/25	S	4	R
47	ccr5, *mecA* (+)	C	V	22/21	S	4	R
71	ccr2, ccr5, *mecA* (+)	C	V	20/22	S	8	R
82	ccr5, *mecA* (+)	C	V	22/20	S	4	R

MIC, minimal inhibitory concentration; OXA, oxacillin; UT, untypable; R, resistant; S, susceptible.

Table 4 Polymorphisms in *mecA* genes of clinical isolates of different ST types, SCC*mec* types and their oxacillin MICs.

Disk diffusion	MLST	SCC*mec* type	Strain	Nucleotide polymorphism in *mecA* gene at position						OXA MIC (μg/mL)
				−33	−30	−7	675	1752 insert A	2005	
ORSL	ST6	II	SL90	C	C	G	T	−	T	>32
			SL118	C	C	G	T	−	T	>32
	ST3	IV	SL138	C	C	T	A	−	T	16
	ST27	V	SL149	T	C	G	A	−	T	32
	ST3	V	SL19	T	C	G	A	−	A	4
			SL70	T	C	G	A	−	T	4
			SL55	T	C	G	A	−	T	8
			SL72	T	C	G	A	−	T	8
			SL76	T	C	G	A	−	T	16
			SL57	T	T	G	A	−	T	32
			SL131	T	T	G	A	−	T	32
			SL134	T	T	G	A	−	T	32
			SL73	T	T	G	A	−	T	>32
			SL99	T	C	G	A	−	T	>32
OSSL	ST3	V	SL44	T	C	G	A	−	A	4
			SL47	T	C	G	A	−	T	4
			SL82	T	C	G	A	−	T	4
			SL71	T	C	G	A	+	T	8
	ST27	V	SL29	T	C	G	A	−	T	1
			SL35	T	C	G	A	−	T	1
			SL79	T	C	G	A	−	T	1

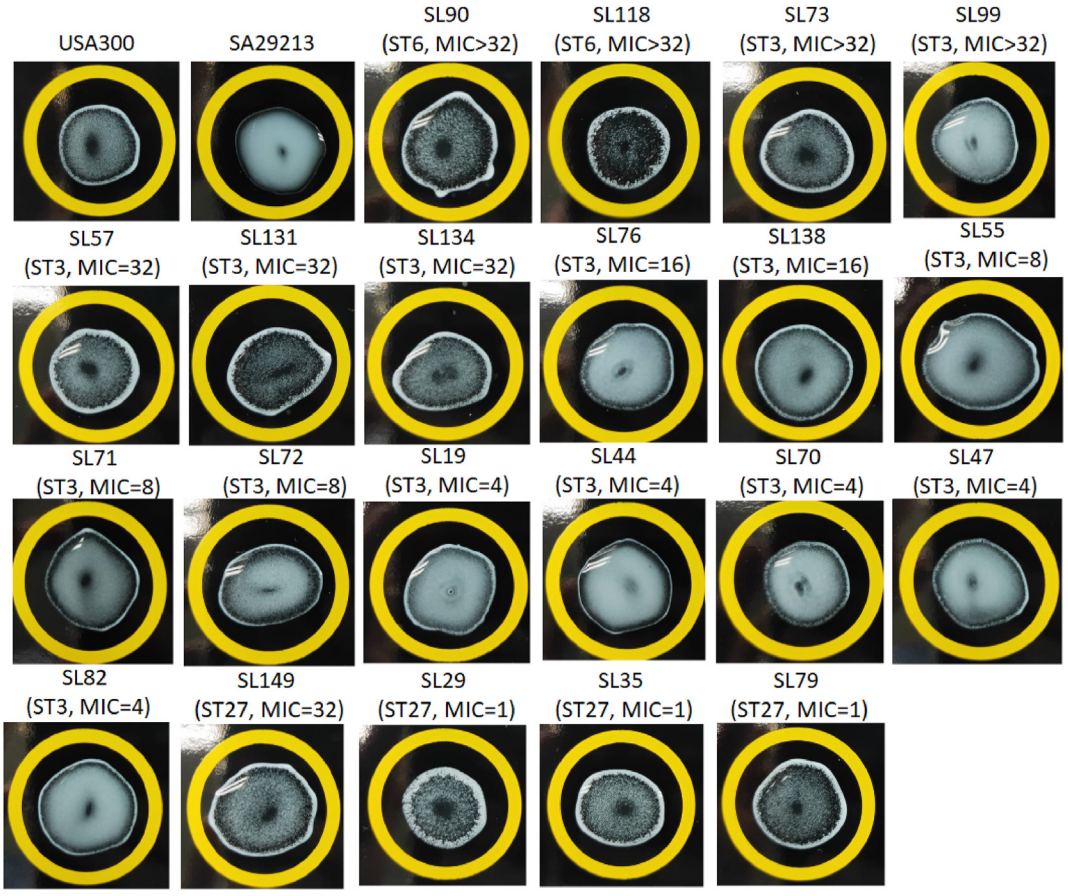

Figure 1. PBP2a agglutination tests for 18 clinical *S. lugdunensis* isolates. USA300 and SA29213 were used as positive and negative controls, respectively.

the sensitivity, specificity, and accuracy of *mecA* PCR for the detection of ORSL was 100%, 95.9%, and 97.44, respectively (Table 2).

Discussion

Our MLST results showed that the most common ST among our isolates was ST6 followed by ST3, ST27, and ST4 (Table 1). Chassain et al. reported that the most frequent STs were ST3 (18 isolates), ST2 (15 isolates), and ST1 (11 isolates) among 87 *S. lugdunensis* isolated in Europe.[22] These results suggest the genetic differences between geographical *S. lugdunensis* isolates. Moreover, our results showed that ST6 ORSL showed higher oxacillin MICs compared with ST3 ORSL (Table 1). However, whether *mecA* expression levels are different in ST3 and ST6 ORSL isolates and thus affect their oxacillin MIC values is unclear and worth investigating.

The overall sensitivity, specificity, and accuracy of CDD for the detection of ORSL was 90.7%, 100%, and 96.8, respectively, based on our gold standard OAD results (Table 2). In most clinical laboratories, disk diffusion tests are used routinely for antimicrobial susceptibility determination. Our results suggest CDD tests underestimate the frequency of ORSL isolates (especially for ORSL ST3 isolates which showed MIC values near the susceptible breakpoint), and thus OAD is more reliable for identifying ORSL compared to CDD (Table 1). However, whether factors such as the level of PBP2a production, are associated with the inconsistency of CDD and OAD for ORSL identification, remain to be investigated.

Ho et al. reported that all 19 ST27 OSSL carried SCC*mec* type V[20]; however, we found only 3 out of 20 (15%) ST27 OSSL carried SCC*mec* type V (Tables 1 & 4). These results indicate the diverse evolution of *S. lugdunensis* across different geographical regions. Our PBP2a test results showed that 3 *mecA*-positive OAD-susceptible strains (strain 29, 35, and 79), expressed high level of PBP2a (Fig. 1). The results were consistent with Ho's report.[20] Although we could not explain why strains have high PBP2a production were susceptible to oxacillin, these results showed that OAD was more reliable to identify ORSL, compared to *mecA*-PCR.

Additionally, we identified a novel polymorphism C-30T in the *mecA* promoter of 4 isolates (SL57, 73, 131, and 134) which showed high resistance to oxacillin (MIC $\geq$ 32 μg/mL). These results suggest that C-30T in *mecA* promoter may increase the expression level of *mecA* and ultimately lead to high oxacillin resistance in *S. lugdunensis*. However, the effects of polymorphism(s) in the *mecA* promoter and coding sequences to PBP2a production and activity remain to be studied in the identical genetic background.

Conclusion

Precise diagnosis of the antibiotic susceptibility of clinical isolates is a cost-effective strategy for improving antibiotic prescribing practice and to reduce drug resistance of pathogenic bacteria. Although disk diffusion tests are used routinely for antibiotic susceptibility determination in most clinical laboratories, it may lead to the underestimation of ORSL isolates. In conclusion, our results indicate that OAD is more reliable for identifying ORSL compared with CDD and *mecA* PCR.

Author contributions

CYK, SCC and JJL conceptualized the study. SCC, HHW, LCL and TPL conducted the experiment. CYK, SCC and JJL analyzed the data, generated the tables and figures, and contributed to the drafting of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.02.009.