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Original Article

Characterization of oxacillin-resistant *Staphylococcus lugdunensis* isolated from sterile body fluids in a medical center in Taiwan: A 12-year longitudinal epidemiological study

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Abstract *Background:* In this study, our objective was to characterize *Staphylococcus lugdunensis* isolated from sterile body fluids (SBFs) in a medical center in Taiwan between 2009 and 2020.
Methods: We used MALDI-TOF MS, disk diffusion testing, agar dilution assay, SCC*mec* typing, and antibiotic resistance gene screening to identify and investigate the characteristics of oxacillin-resistant *S. lugdunensis* (ORSL).
Results: A total of 438 *S. lugdunensis* isolates were collected and 146 (33.3%) isolates were identified as ORSL. SCC*mec* type V was dominant (65.7%) in our ORSL isolates, followed by SCC*mec* type II (18.5%), and type IV (8.9%). After 2013, a slight increase in SCC*mec* types IV and V was revealed. Moreover, all ORSL isolates with type II and untypable SCC*mec* were highly resistant to oxacillin (MIC >32 μg/mL), compared to ORSL that had SCC*mec* types IV, V, and VT. All 146 ORSL isolates were resistant to penicillin and susceptible to teicoplanin and vancomycin. High resistance rates

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of ORSL to clindamycin (43.2%), erythromycin (43.2%), gentamicin (78.1%) and tetracycline (46.6%) was observed. Moreover, only two (1.4%) and six (4.1%) ORSL isolates were resistant to trimethoprim/sulfamethoxazole and ciprofloxacin, respectively. The erythromycin-resistant ORSL isolates mostly exhibited constitutive MLS_B resistant phenotype (61/63, 96.8%) and contained either *ermC* alone (27/63, 42.9%) or a combination of *ermC* with *ermA* (28/63, 44.4%).
Conclusion: Our present study showed a stable rate of ORSL from SBFs during 2009—2020. Moreover, teicoplanin, vancomycin, trimethoprim/sulfamethoxazole, and ciprofloxacin were shown to be highly efficient for the treatment of ORSL *in vitro*.
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Introduction

Staphylococcus lugdunensis is coagulase-negative staphylococcus (CoNS) commensal on human skin, but can also be pathogenic, as it causes life-threatening invasive infections such as endocarditis, periprosthetic joint infections, and bacteremia.[1] In addition, clinical attention has been paid to *S. lugdunensis* in recent years due to its ability to resist β-lactam antibiotics such as oxacillin (oxacillin-resistant *S. lugdunensis*, ORSL).[2] Resistance to oxacillin is usually mediated by the acquisition of *mecA* gene that is carried on a mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) which encodes for penicillin-binding protein 2a (PBP2a) among *Staphylococcus* spp.[3]

Currently, a total of 14 types of SCC*mec* have been used as a tool for the molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA), as well as the evolution investigation of other species of *Staphylococcus*.[4] We previously reported the emergence of ORSL carrying SCC*mec* type V that causes bacterial infections in Taiwan between 2002 and 2013.[5,6] In the present study, we characterized *S. lugdunensis* isolated from sterile body fluids (SBFs) on a wider scale between 2009 and 2020. We also aimed to highlight the importance of monitoring *S. lugdunensis* antibiotic resistance to implement control measures and reduce the risk of healthcare- and community-acquired infections.

Materials and methods

Clinical setting

This study was conducted at Linkou Chang Gung Memorial Hospital (CGMH) in Taoyuan, Taiwan. The hospital is a 3700-bed university-affiliated hospital and tertiary referral medical center in northern Taiwan. All *S. lugdunensis* isolates were obtained from SBFs, including ascitic fluid, blood, and cerebrospinal fluid, from our clinical microbiology laboratory between 2009 and 2020.

Isolation and identification of *S. lugdunensis*

We collected 438 *S. lugdunensis* isolates from SBFs, 2009 to 2020. *S. lugdunensis* isolates were first identified by Gram staining, biochemical methods (catalase-positive, coagulase-negative, pyrrolidonyl arylamidase-positive, and ornithine decarboxylase-positive results), and rapid PCR gene amplification.[7] 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 according to the manufacturer's instruction. *S. lugdunensis* isolates were stored in tryptic soy broth with 20% glycerol at −80 °C until further experiments.

Antimicrobial susceptibility testing

In our previous study, we showed that the oxacillin agar dilution (OAD) test was more accurate for testing resistance to oxacillin in *S. lugdunensis*, compared to the oxacillin disk diffusion (ODD) test.[8] Therefore, the minimal inhibitory concentration (MIC) values were determined for oxacillin by OAD tests to detect the ORSL isolates following CLSI recommendations.[9] *S. lugdunensis* isolates with an OAD MIC ≥4 μg/mL were defined as ORSL according to CLSI guidelines.[9] We also tested the susceptibility of ORSL isolates to ten antibiotics (oxacillin, penicillin, clindamycin, erythromycin, trimethoprim/sulfamethoxazole, teicoplanin, vancomycin, gentamicin, tetracycline, and ciprofloxacin) by disk diffusion tests.[9] D-zone test was performed for erythromycin-resistant *S. lugdunensis* strains according to CLSI guidelines.[9] Resistance to both clindamycin and erythromycin indicates a constitutive MLS_B ($cMLS_B$) resistant phenotype. Strains resistant to erythromycin but sensitive to clindamycin were further classified into inducible MLS_B ($iMLS_B$) (D-zone positive) resistant phenotype and MS (D-zone negative) phenotype. *S. aureus* ATCC 29213 was used as a control strain. Antimicrobial susceptibility testing was performed in duplicate to ensure reproducibility.

SCC*mec* genotyping and screening of macrolide resistance genes

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. All ORSL isolates were subjected to SCC*mec* typing and *mecA* detection by using a multiplex PCR assay to amplify the *ccr* and *mec* genes as previously described.[10,11] The presence of macrolide-resistant genes among erythromycin-resistant isolates were examined by PCR using the primers listed in Table 1. PCR conditions for the detection of *ermA* and *ermB* genes were performed as previously described.[12,13] PCR

Table 1 PCR primers used to identify macrolide resistance genes.

Gene	Primer name	Sequence (5' - 3')	Reference
msrA	msrA-F	CCTATGCATACAACCGACAG	This study
	msrA-R	CTACACCATTTGCACCTACG	
mphC	mphC-F	GAGACTACCAAGAAGACCTGACG	This study
	mphC-R	CATACGCCGATTCTCCTGAT	
ermA	ermA-F	TCTAAAAAGCATGTAAAAGAA	12
	ermA-R	CTTCGATAGTTTATTAATATTAGT	
ermB	ermB-F-359-F	CCGTTTACGAAATTGGAACAGGTAAAGGGC	13
	ermB-F-359-R	GAATCGAGACTTGAGTGTGC	
ermC	ermC-F	GGTGTAATTTCGTAACTGCC	This study
	ermC-R	TAATGCCAATGAGCGTTTTG	

assays were developed for the detection of *ermC*, *mphC*, and *msrA* genes (Table 1). *Staphylococcus haemolyticus* and *S. aureus* isolates positive for PCR assays and confirmed by sequencing were used as positive controls. Phusion Flash High- Fidelity PCR Master Mix (ThermoFisher Scientific) was used for PCR using 10 mM of each primer under the following conditions: 5 min at 95 °C, followed by 35 cycles of 5 s at 95 °C for denaturation, 10 s at 55 °C for primer annealing, and 20 s at 72 °C for extension, and 5 min at 72 °C for final extension.

Statistical analysis

Student *t*-tests were used to compare categorical variables. All statistical analyses were performed using GraphPad Prism software version 8.0.2 (San Diego, California, USA). A *p*-value <0.05 was taken as a significant difference.

Results

The frequency of oxacillin-resistant *S. lugdunensis* isolated from sterile body fluids

A total of 438 *S. lugdunensis* isolates from SBFs were collected from 2009 to 2020 and 146 (33.3%) isolates were identified as ORSL determined by OAD tests (Fig. 1). The frequencies of ORSL were stable during our study period. The highest frequency of ORSL was observed in 2014 (45.5%), followed by 2009 (44.4%) and 2019 (41.5%). The frequencies of ORSL were relatively low in 2013 (21.9%) and 2017 (22.4%) (Fig. 1).

Distribution of SCC*mec* types and their association with oxacillin MICs in ORSL isolates

All 146 ORSL isolates were subjected to SCC*mec* typing to investigate the distribution of SCC*mec* types during 2009—2020. We found that SCC*mec* type V was dominant (96/146, 65.8%) in our ORSL isolates, followed by SCC*mec* type II (27/146, 18.5%), and type IV (13/146, 8.9%) (Table 2). However, the SCC*mec* of eight ORSL isolates (5.5%) was untypable (Table 2). An increase in ORSL isolates carrying SCC*mec* type V was observed between 2009 and 2020 (Table 2). Moreover, only one ORSL with SCC*mec* type IV was detected between 2009 and 2013, and a slight increase in SCC*mec* type IV was revealed after 2013 (Table 2). Importantly, all ORSL isolates that had SCC*mec* type II or untypable SCC*mec* were highly resistant to oxacillin (MIC >32 μg/mL), compared to ORSL isolates that had SCC*mec* types IV, V, or VT ($p < 0.0001$) (Fig. 2).

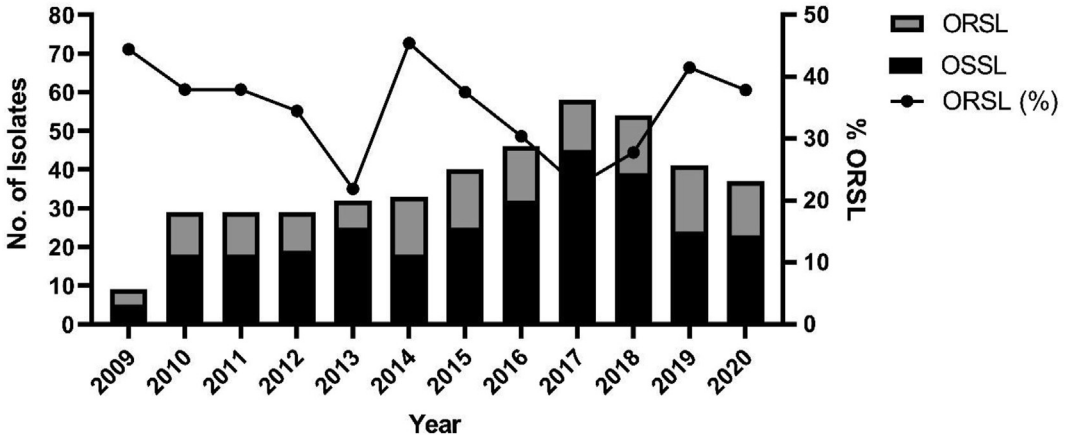

Figure 1. Distribution of oxacillin-resistant (ORSL) and oxacillin-susceptible (OSSL) *S. lugdunensis* isolated from sterile body fluids during 2009—2020.

Table 2 The association of oxacillin MIC and SCC*mec* types in ORSL, 2009—2020.

Year (No. of ORSL)	SCC*mec* type	Oxacillin MIC, n (μg/mL)				
		4	8	16	32	>32
2009 (4)	II	—	—	—	—	1
	V	3	—	—	—	—
2010 (11)	II	—	—	—	—	4
	V	—	3	—	2	1
	Untypable	—	—	—	—	1
2011 (11)	II	—	—	—	—	3
	V	1	—	1	1	4
	VT	1	—	—	—	—
2012 (10)	II	—	—	—	—	5
	IV	—	—	1	—	—
	V	2	—	—	1	—
	VT	—	—	—	—	1
2013 (7)	II	—	—	—	—	1
	V	1	3	—	2	—
2014 (15)	II	—	—	—	—	4
	IV	1	—	—	—	1
	V	1	2	2	2	—
	Untypable	—	—	—	—	2
2015 (15)	II	—	—	—	—	1
	IV	—	—	1	—	—
	V	1	1	5	3	3
2016 (14)	II	—	—	—	—	1
	IV	1	—	1	—	—
	V	—	2	4	4	—
	Untypable	—	—	—	—	1
2017 (13)	II	—	—	—	—	2
	IV	1	—	—	—	—
	V	2	1	3	3	—
	Untypable	—	—	—	—	1
2018 (15)	II	—	—	—	—	2
	IV	—	3	—	—	—
	V	2	2	3	1	2
2019 (17)	IV	—	1	—	—	—
	V	2	9	5	—	—
2020 (14)	II	—	—	—	—	2
	IV	—	—	2	—	—
	V	—	4	—	1	2
	Untypable	—	—	—	—	3

Antimicrobial susceptibility of 146 ORSL isolates

We observed a discrepancy when we compared the result of ODD to oxacillin MIC determined by OAD tests. Twenty-five isolates showed susceptibility to oxacillin using ODD tests, but had an oxacillin MIC value $\geq$ 4 μg/mL by OAD tests (Table 3). Moreover, five isolates showed resistance to oxacillin using ODD tests, but had an oxacillin MIC value $<$ 4 μg/mL by OAD tests. All 146 ORSL isolates were resistant to penicillin and susceptible to teicoplanin and vancomycin (Table 3). In addition, high resistance rates of ORSL to clindamycin (63/146, 43.2%), erythromycin (63/146, 43.2%), gentamicin (114/146, 78.1%) and tetracycline (68/146, 46.6%) were observed. Among 63 erythromycin-resistant isolates, the constitutive MLS_B, inducible MLS_B,

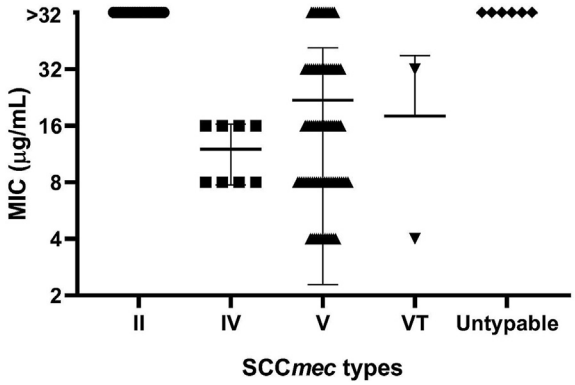

Figure 2. The distribution of oxacillin MICs in ORSL with different types of SCC*mec*. A scatter dot plot shows the mean oxacillin MICs ± SD for each SCC*mec* type. Each dot represents one individual strain, and error bars represent mean with SD. Statistical analysis was performed using Student's t-test.

and MS phenotypes were found in 61 (96.8%), 1 (1.6%), and 1 (1.6%) isolates, respectively (Table 3). Only two (1.4%) and six (4.1%) ORSL isolates were resistant to trimethoprim/sulfamethoxazole and ciprofloxacin, respectively (Table 3). *ermC* (57/63, 90.5%) was the most common gene conferring erythromycin-resistant phenotype in 63 erythromycin-resistant ORSL isolates, followed by *ermA* (34/63, 54.0%). The *ermB* gene was not detected in erythromycin-resistant isolates. The erythromycin-resistant isolates were found to contained either *ermC* alone (27/63, 42.9%) or a combination of *ermC* with *ermA* (28/63, 44.4%), *msrA* (1/63, 1.6%) or *mphC* (1/63, 1.6%). Additionally, 6 (9.5%) isolates contained only *ermA* (Table 4).

Discussion

The role of *S. lugdunensis* in human diseases is a major problem, especially in hospital settings, as it can induce a variety of infectious diseases, including skin and soft tissue infection, infectious endocarditis, and joint infections.[14] In the past years, the frequency of *S. lugdunensis* is still low. Only five (0.7%) of the 670 CoNS isolates were identified as *S. lugdunensis* in mainland China collected for 1 month in 2010.[15] Additionally, a retrospective study collected 129 *S. lugdunensis* clinical isolates between 2003 and 2014, and 58 (45%) of them were isolated from blood samples in Taiwan.[16] However, the characteristics of *S. lugdunensis* isolated from different specimens were rarely reported and deserve investigating. Antibiotic resistance acquired by *S. lugdunensis* also requires immediate clinical attention. Although our data suggest that the frequency of ORSL from SBFs was stable during 2009—2020, the distribution of ORSL should be continually monitored.

CoNS had been recognised as a reservoir of SCC*mec* available for *S. aureus*.[17] Various types of SCC*mec* have been reported among ten selected CoNS species. *Staphylococcus epidermidis*, *S. haemolyticus*, and *Staphylococcus capitis* had the widest distribution of SCC*mec* types (types I to VI). However, only one type of SCC*mec* was reported in *Staphylococcus chromogenes* (IV), *Staphylococcus lentus* (III), and *Staphylococcus cohnii* (untypable).[18] Therefore,

Table 3 Antibiotic susceptibility of 146 ORSL during 2009—2020.

Year (No. of ORSL)	Antibiotic susceptibility n (%)													
	Oxacillin		Clindamycin		Erythromycin		Trimethoprim/sulfamethoxazole		Gentamicin		Tetracycline		Ciprofloxacin	
	NS	S[a]	NS	S	NS	S	NS	S	NS	S	NS	S	NS	S
2009 (4)	3	1	1	3	1	3	0	4	3	1	2	2	0	4
2010 (11)	8	3	5	6	5	6	0	11	10	1	3	8	1	10
2011 (11)	8	3	3	8	3	8	0	11	10	1	6	5	0	11
2012 (10)	8	2	8	2	8	2	0	10	7	3	4	6	0	10
2013 (7)	7	0	1	6	1	6	0	7	6	1	2	5	0	7
2014 (15)	14	1	5	10	5	10	0	15	12	3	7	8	0	15
2015 (15)	13	2	7	8	8[b]	7	0	15	11	4	7	8	0	15
2016 (14)	11	3	6	8	5	9	0	14	12	2	9	5	1	13
2017 (13)	12	1	8	5	8	5	0	13	9	4	7	6	2	11
2018 (15)	10	5	7	8	7[c]	8	1	14	11	4	8	7	2	13
2019 (17)	13	4	2	15	2	15	0	17	13	4	9	8	0	17
2020 (14)	14	0	10	4	10	4	1	13	10	4	4	10	0	14
Total (146)	121 (83.6)	25 (17.1)	63 (43.2)	83 (56.8)	63 (43.2)	83 (56.8)	2 (1.4)	144 (98.6)	114 (78.1)	32 (21.9)	68 (46.6)	78 (53.4)	6 (4.1)	140 (95.9)

[a] Isolates showed susceptibility to oxacillin by disc diffusion test but were resistant to oxacillin determined by agar dilution (MIC $\geq$4 μg/mL).
[b] One erythromycin-resistant isolate in 2015 exhibited the iMLS resistance phenotype.
[c] One erythromycin-resistant isolate in 2018 exhibited the MS phenotype.
All isolates were resistant to penicillin and susceptible to teicoplanin and vancomycin.
NS, non-susceptible; S, susceptible.

Table 4 Distribution of macrolide resistance genes among 63 erythromycin-resistant *S. lugdunensis* isolates.

The presence of macrolide resistance genes					No. of isolates (%)
ermA	ermB	ermC	msrA	mphC	
+	−	+	−	−	28 (44.4)
−	−	+	−	−	27 (42.9)
+	−	−	−	−	6 (9.5)
−	−	+	+	−	1 (1.6)
−	−	+	−	+	1 (1.6)
34 (54.0)	0 (0)	57 (90.5)	1 (1.6)	1 (1.6)	

particular CoNS species may only possess that particular SCC*mec* types. In contrast, healthcare-associated methicillin-resistant *S. aureus* isolates mainly carried SCC*mec* types I, II, and III, although SCC*mec* types IV and V in healthcare-associated settings in many countries have also been reported in previous studies.[19] Our SCC*mec* typing revealed that most ORSL carried SCC*mec* type V. These findings were consistent with previous studies that also characterized ORSL by SCC*mec* typing.[20—22] Cheng et al. reported that most of the pulsotype A isolates (an endemic clone in Taiwan) were resistant to oxacillin and carried type V SCC*mec*.[20] Ho et al. investigated methicillin-resistant *S. lugdunensis* (MRSL) among patients undergoing long-term renal replacement therapy and their results showed that 18 of 21 MRSL isolates had SCC*mec* type V.[21] Moreover, most ORSL harbored SCC*mec* type V which strongly suggests that the epidemiological origin of these ORSL infections was community-acquired.[23,24] Thus, the clonal spread of SCC*mec* type V isolates is less likely to occur in the hospital. We previously reported that type II SCC*mec* was identified in an endemic ST6 *S. lugdunensis* clone in the hospital.[25] Therefore, it is worth investigating the clonality of isolates that have the same SCC*mec* type in the future.

Our study also observed an increase in SCC*mec* type IV after 2013. In addition, this study revealed eight isolates carrying untypable SCC*mec*. SCC*mec* types among non-*S. aureus staphylococci* are often untypable due to the lack of an identified *ccr* and/or *mec* gene complex, detection of more than one *ccr* complex, or detection of novel combinations of *ccr* and *mec* complex genes that cannot be assigned to previously described SCC*mec* types.[26] Previous studies have shown that ST6 ORSL isolates contained SCC*mec* type II or untypable SCC*mec*. In contrast, ST3 ORSL isolates contained SCC*mec* types V, VT, and IV, respectively.[8] Interestingly, ORSL isolates had type II and untypable SCC*mec* showed high resistance to oxacillin, compared to ORSL isolates that had types IV, V, and VT SCC*mec*. Our unpublished results also showed the high similarity of SCC*mec* structure between type II and untypable SCC*mec*. However, the characteristics of these untypable SCC*mec* remain to be determined.

Furthermore, this study noted a discrepancy between ODD and OAD tests. Our previous study highlighted the fact that OAD remains to be accurate and is considered the gold standard for testing resistance to oxacillin.[8] However, the mechanisms behind the five isolates in this study showed resistance to oxacillin by ODD tests, but had an oxacillin MIC value < 4 μg/mL by OAD tests, which are still unclear.

Conclusion

In summary, the frequency of ORSL isolated from SBFs remains stable during 2009—2020. A slight increase in ORSL with SCC*mec* type IV and type V after 2013 was observed. Moreover, ORSL isolates were highly susceptible to teicoplanin, vancomycin, and trimethoprim/sulfamethoxazole *in vitro*.

Authors' contributions

SCC, CYK, and JJL conceptualized the study. SCC, JHH, YHO, and LCL conducted the experiments. CYK, JHH, SCC, and JJL analyzed the data, generated the tables and figures, and contributed to the first draft of 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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