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Antimicrobial resistance and molecular characterization of methicillin-resistant coagulase-negative staphylococci from public shared bicycles in Tianjin, China

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ABSTRACT

Objectives: Methicillin-resistant coagulase-negative staphylococci (CoNS) have emerged as one of the major nosocomial pathogens, and antimicrobial resistance has aggravated the problem. Sixteen million public shared bicycles (PSBs) were launched in China, and 106 million users were recorded. However, no standard clean strategy has been applied to PSBs in China, and no formal surveillance has been carried out. The objective of this study was to understand the diversity and antimicrobial resistance of staphylococcal species from PSBs.

Methods: Seventy-nine specimens and 128 isolates were collected from 79 PSBs in Tianjin city, China. Antimicrobial susceptibility and molecular testing were performed to understand the species, antimicrobial resistance, carriage of *mecA* gene, and clonal lineage.

Results: Thirty-five staphylococcal isolates were identified, and 80% of staphylococci were resistant to at least one antibiotic. Seventeen (49%) staphylococci were *mecA*-positive. SCC*mec* type V (n = 5), SCC*mec* type 1A (n = 5), SCC*mec* type I (n = 3), SCC*mec* type III (n = 2), 1 SCC*mec* type II (n = 1), and SCC*mec* type IV (n = 1) were determined. In addition, *Staphylococcus epidermidis* ST5, ST35, ST234 and ST419 were determined, and a new ST (ST831) was also found.

Conclusions: The *Staphylococcus epidermidis* group was prevalent among PSBs, and multiple resistant staphylococci were determined in this study. The diversity of SCC*mec* elements was observed, and PSBs may act as the reservoir for antimicrobial pathogenic bacteria. Moreover, additional studies are necessary to systematically understand the prevalence and molecular epidemiology of methicillin-resistant staphylococci in PSBs.

1. Introduction

Coagulase-negative staphylococci (CoNS) have emerged as the most common causes of nosocomial-associated infections, and the health burden of CoNS is increasing and far from resolved [1]. *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus hominis* (*S. hominis*) and *Staphylococcus haemolyticus* (*S. haemolyticus*) are three vital species of CoNS [2]. Moreover, *S. epidermidis*, *S. hominis* and *S. haemolyticus* belong to the *S. epidermidis* group, and the *S. epidermidis* group accounts for >70% documented infections; two thirds of these isolates are resistant to oxacillin [3].

Molecular characterisation of staphylococci has been widely reported in both hospital-associated and community-associated isolates. Several recent studies have focused on environmental staphylococci. In 2009, Soge et al. first reported the recovery of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant CoNS (MRCoNS) from public marine water and intertidal beach sand [4]. Shortly after this, Simoes et al. and Roberts et al. reported the discovery of MRSA from public buses in Portugal [5] and a university campus in USA, respectively [6]. Mkrtchyan et al. reported molecular characterisation of MRSA and MRCoNS that were recovered from restrooms and public washrooms in the UK [7,8]. More recently, Xu et al. systematically reported the diversity and molecular character of methicillin-resistant staphylococcal species that were recovered from libraries, restaurants, supermarket, public transportation facilities, hotel, handbags, and baby care facilities [9]. The dissemination of MRSA

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and MRCoNS in a non-healthcare-associated environment poses a great public health threat.

The internet-based public shared bicycle (PSBs) system was implemented in China in 2016, which solves the last mile traffic gap for citizens. Sixteen million shared bicycles were launched in China, and 106 million users were recorded [10]. It is believed that there is no standard cleaning strategy applied to PSBs in China, and little is known about the hygiene of the shared bicycles. Therefore, the current study undertook a pilot study to understand the diversity, antimicrobial resistance, and genotype of staphylococci that were recovered from PSBs.

2. Material and methods

2.1. Isolation

Environmental samples were collected by dry sterilised cotton swabs (Yangzhou Yangsheng Medical Science & Technology Co., Ltd, China) from the hand rails of PSBs. The PSBs were located at a medical university and two public hospitals in Tianjin city; the collection dates were from December 2017 to March 2018. All specimens were transferred back to the laboratory within 24 h and plated on Mannitol Salt Agar (Oxoid, Basingstoke, UK), and then incubated aerobically at 37 ℃ for 24–72 h. The colonies were then purified on Nutrient Agar (Oxoid, Basingstoke, UK).

2.2. Identification

The DNA was extracted with a DNA extraction kit (Solarbio Co., Ltd, China) according to manufacturer's instructions. The 16S rDNA genes of the isolates were amplified by thermocycler T100 (Biorad, Ltd), and sequenced by Sangon biotech (Shanghai, China). The sequences were then blasted in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) database [8].

2.3. Antimicrobial susceptibility test

A panel of five antibiotics was used to determine the antibiotic susceptibility of all the isolates. The standard disk diffusion method was used to test gentamicin (10 μg), erythromycin (15 μg), cefoxitin (30 μg), penicillin (10 units), and tetracycline (30 μg). The susceptibility interpretation was determined by the Clinical and Laboratory Standards Institute (CLSI) Performance standard of Antimicrobial Susceptibility Testing (CLSI: 24th edition) [11].

2.4. mecA *gene determination*

The *mecA* gene was amplified with mA1 and mA2 primers: mA1 (5'-TGCTATCCACCCTCAAACAGG-3') and mA2 (5'-AACGTTGTAACCACCCCAAGA-3'). The reaction mixtures and PCR program was described by Kondo et al., and the PCR product was 286 bp [12].

2.5. *SCC*mec *typing*

The SCC*mec* types were determined by the combination of the *mec* gene complex and *ccr* gene complex. The *mec* and *ccr* gene complexes were determined by eight pairs of primers that were described by Kondo et al. [12].

2.6. Multi-locus sequence typing

Multi-locus sequence typing (MLST) was used to determine the sequence types of *S. epidermidis*. Seven housekeeping genes were amplified according to Thomas et al. [13], and the PCR products were then sequenced by Sangon Biotech (Shanghai, China). Sequence types were assigned using the *S. epidermidis* database (www.mlst.net).

2.7. Statistical analysis

The χ^2 test was used to analyse the quantitative variables. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Isolates

Seventy-nine specimens were collected from 79 PSBs that were located in Tianjin city, north China. Twenty specimens were recovered from a medical university campus, and 59 specimens were collected from PSBs that were located outside the hospitals. A total of 128 isolates were purified, and 45 isolates were selected for further analysis according to their morphology.

3.2. Identification

Thirty-five isolates were determined to be *Staphylococcus* species: 10 (29%) *S. haemolyticus*; 10 (29%) *S. hominis*; five (14%) *S. epidermidis*; five (14%) *S. equorum*; two (6%) *S. pasteuri*; two (6%) *S. capitis*; and one (2%) *S. vitulinus*.

3.3. Antimicrobial susceptibility results

The resistance ratio towards five antibiotics was as follows: gentamicin (n = 1, 3%), erythromycin (n = 17, 49%), cefoxitin (n = 18, 51%), penicillin (n = 21, 60%), and tetracycline (n = 13, 37%) (Tables 1 and 2).

3.4. mecA *gene determination of staphylococci*

The *mecA* gene was determined in 17 (49%) *Staphylococcus* isolates: two (6%) *S. capitis*; five (14%) *S. epidermidis*; one (3%) *S. haemolyticus*; and nine (26%) *S. hominis* (Table 1). All *mecA*-positive staphylococci were phenotypically resistant to cefoxitin. Except for one *mecA*-negative staphylococcal isolate, the remaining isolates were susceptible to cefoxitin.

Table 1
Molecular characterisation and antimicrobial susceptibility of methicillin-resistant staphylococci.

ID	Location	Species	CN	E	FOX	P	T	*mecA*	*ccr*	*mec*	SCC*mec*
1	H	*S. capitis*	S	R	R	R	S	+	1	B	I
2	H	*S. capitis*	S	R	R	R	S	+	3	A	III
3	H	*S. epidermidis*	S	I	R	R	S	+	5	C	V
4	H	*S. epidermidis*	S	S	R	R	S	+	5	C	V
5	H	*S. epidermidis*	S	S	R	R	S	+	5	C	V
6	H	*S. epidermidis*	S	S	R	R	S	+	5	C	V
7	H	*S. epidermidis*	S	R	R	R	R	+	5	C	V
8	H	*S. haemolyticus*	S	R	R	R	R	+	3	A	III
9	H	*S. hominis*	S	R	R	R	R	+	1	A	1A
10	H	*S. hominis*	R	R	R	R	R	+	1	A	1A
11	H	*S. hominis*	S	R	R	R	R	+	1	A	1A
12	H	*S. hominis*	S	R	R	R	R	+	1	A	1A
13	H	*S. hominis*	S	R	R	R	I	+	1	A	1A
14	H	*S. hominis*	S	R	R	R	S	+	1	B	I
15	H	*S. hominis*	S	R	R	R	S	+	1	B	I
16	H	*S. hominis*	S	R	R	R	S	+	2	A	II
17	C	*S. hominis*	S	R	R	R	S	+	2	B	IV

H, hospital; C, university campus; CN, gentamicin (10 μg); E, erythromycin (15 μg); FOX, cefoxitin (30 μg); P, penicillin (10 unit); T, tetracycline (30 μg); *S.*, *Staphylococcus*; I, intermediate; R, resistance; S, susceptible.

Table 2
Antimicrobial susceptibility of *mecA*-negative staphylococci isolates.

ID	Location	Species	CN	E	FOX	P	T	*mecA*
18	C	*S. equorum*	S	S	S	S	R	-
19	H	*S. equorum*	S	I	S	R	S	-
20	H	*S. equorum*	S	I	S	S	R	-
21	H	*S. equorum*	S	S	S	S	S	-
22	C	*S. equorum*	S	I	S	S	R	-
23	C	*S. haemolyticus*	S	R	S	R	S	-
24	H	*S. haemolyticus*	S	I	R	S	R	-
25	H	*S. haemolyticus*	S	R	S	S	R	-
26	H	*S. haemolyticus*	S	S	S	S	R	-
27	H	*S. haemolyticus*	S	S	S	S	S	-
28	H	*S. haemolyticus*	S	S	S	S	S	-
29	H	*S. haemolyticus*	S	S	S	S	S	-
30	H	*S. haemolyticus*	S	S	S	S	R	-
34	H	*S. haemolyticus*	S	S	S	S	S	-
32	H	*S. hominis*	S	S	S	S	S	-
33	H	*S. pasteuri*	S	R	S	R	S	-
34	H	*S. pasteuri*	S	R	S	R	S	-
35	H	*S. vitulinus*	S	S	S	S	S	-

H, hospital; C, University campus; CN, gentamicin (10 μg); E, erythromycin (15 μg); FOX, cefoxitin (30 μg); P, penicillin (10 unit); T, tetracycline (30 μg); *S.*, *Staphylococcus*; I, ; R, ; S.

3.5. SCCmec *typing of staphylococci*

The SCC*mec* types were determined in all 17 *mecA*-positive *Staphylococcus* isolates: five isolates harboured SCC*mec* type V; five harboured SCC*mec* type 1A; three SCC*mec* type I; two SCC*mec* type III; one SCC*mec* type II; and one SCC*mec* type IV. Five SCC*mec* V elements were determined in *S. epidermidis*, and five SCC*mec* 1A were all found in *S. hominis*. Three SCC*mec* type I elements were determined in two *S. hominis* and one *S. capitis*; two SCC*mec* type III elements were found in one *S. capitis* and one *S. haemolyticus*. One SCC*mec* type II and one SCC*mec* type IV were found in two *S. hominis* (Table 1).

3.6. *Multi-locus sequence typing of staphylococci*

The MLST was performed for five *mecA*-positive *S. epidermidis* isolates, and four of them belonged to the following STs: ST5, ST35, ST234 and ST419. One unclassified ST type was also characterised, which was assigned as ST831 (Table 3).

4. Discussion

Staphylococci are major nosocomial-associated pathogens, and the emergence of methicillin-resistant staphylococci has created a huge public health threat [14]. The current pilot study was undertaken to determine the dissemination of methicillin-resistant staphylococcal species recovered from PSBs.

CoNS is the top pathogen that causes healthcare-associated infections, and the pathogenic role of CoNS cannot be ignored [15]. Seven *Staphylococcus* species were determined in this study: *S. capitis*, *S. epidermidis*, *S. equorum*, *S. haemolyticus*, *S. hominis*, *S. pasteuri*, and *S. vitulinus*. Except for *S. vitulinus*, all the other species were reported to have been recovered from the environment [9,16]. *Staphylococcus vitulinus* is known to be principally isolated from animals and meat products such as lamb, chicken, beef, horse, vole, and whales [17]; however, isolation of *S. vitulinus* from urinary tract infections has also been reported [17]. The major species in the current study were *S. hominis* (28.5%) and *S. haemolyticus* (28.5%), followed by *S. epidermidis* (14%) and *S. equorum* (14%). In a previous study, *S. epidermidis* and *S. hominis* were reported to be the predominant species that were recovered from human hands [9]; therefore, the microflora of PSBs was significantly influenced by human microbiomes.

Table 3
Multi-locus sequence typing (MLST) of *Staphylococcus epidermidis*.

ID	MLST	MLST profile							SCC*mec*
		arcC	*aroE*	*gtr*	*muts*	*pyr*	*tpi*	*yqil*	
3	ST35	2	1	2	2	4	1	1	V
4	ST831	37	24	6	4	29	10	1	V
5	ST5	1	1	1	2	2	1	1	V
6	ST234	31	1	1	6	2	1	1	V
7	ST419	52	16	9	5	8	16	12	V

Multi-drug resistant staphylococci have been widely reported in both nosocomial and non-nosocomial environments [9,18]. The current resistance rates ranged from lowest (3%, gentamicin) to highest (60%, penicillin); the resistance rate towards penicillin is consistent with other environmental reports [9].

The pathogenicity of staphylococci is aggravated by acquiring the methicillin-resistant determinant *mecA* gene [19]. Methicillin-resistant staphylococci pose a huge threat to public health, and cause significant economic and health consequences [20]. In the current study, 17 (49%) staphylococcal isolates harboured the *mecA* gene, which is significantly higher than the percentages that were reported by a previous study [9]. The current study found no MRSA, and no *S. equorum*, *S. pasteuri*, and *S. vitulinus* harboured the *mecA* gene. However, it is worth noting that all *S. epidermidis* (100%) recovered in the current study harboured the *mecA* gene, 90% of *S. hominis* were *mecA* gene-positive isolates, and 10% *S. haemolyticus* were *mecA*-gene positive. A previous environmental staphylococcal study reported that 12.5% of *S. epidermidis*, 5% of *S. hominis*, and 25% of *S. haemolyticus* harboured the *mecA* gene [9]. Limited amounts of staphylococcal isolates were recovered in the current pilot study; however, the high *mecA* ratio was an important finding. The resistance rates of gentamicin, erythromycin, cefoxitin, penicillin and tetracycline in *mecA*-positive isolates were generally higher than *mecA*-negative isolates, which is consistent with the pattern reported by Pai et al. [21]. Moreover, the resistant rate of erythromycin ($\chi^2 = 10.3$), penicillin ($\chi^2 = 22$) and cefoxitin ($\chi^2 = 31.2$) in *mecA*-positive isolates was significantly higher than *mecA*-negative isolates ($P < 0.05$).

Swenson et al. reported that the cefoxitin disc diffusion method is desirable for predicting *mecA* gene-mediated oxacillin resistance in *S. aureus* and CoNS [22]. In the current study, the *mecA*-positive staphylococci were all phenotypically resistant to cefoxitin, which suggests that cefoxitin may also be a reliable agent for identifying environmental *mecA*-positive CoNS. One *mecA*-negative, but cefoxitin-resistant, staphylococcal isolate was also determined. It is well known that cefoxitin is also an indicator for phenotypic detection of *mecC*-positive MRSA [23]; however, the conventional *mecA* gene PCR is incapable of determining the *mecC* gene [24]. The controversial isolate that was found in the current study may harbour the *mecC* gene, which cannot be detected by *mecA* gene PCR. A further study will need to be performed to explore *mecC*-MRSA among the isolates that were recovered from PSBs.

Staphylococcal cassette chromosome *mec* is a mobile genetic island with two essential components: the *mec* gene complex and *ccr* gene complex. The *mecA* gene is located on the *mec* gene complex and the *ccr* gene complex encodes recombinase that is responsible for its mobility [25]. To date, 11 SCC*mec* types have been identified (SCC*mec* type I to type XI) [26]. The SCC*mec* types I, II, III are reported to be hospital associated, whereas the SCC*mec* types IV and V are community associated [27]. In the current study, six (35%) isolates carried SCC*mec* types I, II, III, and six (35%) isolates harboured SCC*mec* IV and V. In comparison with previous environmental staphylococcal studies [9], the ratio of nosocomial-associated SCC*mec* elements was high among the current isolates that were recovered from PSBs. The PSBs that the sampling was performed on were located outsides two public hospitals and

in a medical university campus, and this may have contributed to the high ratio of hospital-associated SCC*mec* types. In addition to the assigned SCC*mec* types, SCC*mec* composed of *mec* complex A in association with *ccr* type 1 (30%) was determined in *S. hominis*. Bouchami et al. reported that the class A *mec* complex and type 1 *ccr* complex were prevalent among the *S. hominis* population [28]; the current study is consistent with this finding. There was a high prevalence of class A *mec* complex observed in the current study (47%), followed by class C *mec* complex (30%) and class B *mec* complex (23%). In addition, the high ratio of *ccr* type 1 (47%) was determined among staphylococci that were recovered from shared bicycles, followed by *ccr* type 5 (30%).

Multi-locus sequence typing is a typing method specifically for several pathogenic bacteria, including *S. aureus* and *S. epidermidis* [29]. ST5 and ST35 were two major nosocomial *S. epidermidis* clones [30]. Moreover, *S. epidermidis* ST234 was reported to be recovered from a urinary tract infection case in Gansu province, China (www.mlst.net). In the current study, five *mecA*-positive *S. epidermidis* were recovered from PSBs that were located outside the hospitals, and the shared bicycles were presumed to have been used by healthcare workers or patients. Pathogenic *S. epidermidis* clones were disseminated among PSBs, and may have been circulated among people who used the PSBs. In this study, a new MLST type (ST831) was determined in one *S. epidermidis* isolate, and this might suggest the genetic variability of the isolates that were recovered from PSBs.

5. Conclusion

In conclusion, human-associated *Staphylococcus* species were determined from PSBs, which indicate the influence of human microbes on shared bicycles. The resistant rate of penicillin and erythromycin in *mecA*-positive staphylococci was significantly higher than that of *mecA* gene-negative staphylococci. The diversity of SCC*mec* elements was observed, and pathogenic *S. epidermidis* clones were recovered from PSBs. The shared bicycles may act as a reservoir for methicillin-resistant staphylococci in the community; therefore, cleaning strategies for PSBs should be applied to manage the dissemination of methicillin-resistant staphylococci in the community. There are currently no standard cleaning strategies or formal surveillance for PSBs in China. More structured studies are necessary to understanding of the prevalence, risk factors, antimicrobial resistance and genetic diversity of methicillin-resistant staphylococci among PSBs.

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Competing Interests

None declared.

Ethical Approval

Not required.

Authors' contributions

ZX: acquisition of data, data analysis, manuscript preparation, study design, and laboratory work. SL: sample collection and data analysis. LC: data analysis and critically reviewing the paper. YL: sample collection and critically reviewing the paper. LT: data analysis. JS: conception and design of the study. WZ: critically reviewing the paper.

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