



NOTE

Public Health

First isolation of ST398 methicillin-resistant *Staphylococcus aureus* carrying staphylococcal cassette chromosome *mec* type IVd from pig ears in Japan

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ABSTRACT. The emergence and increasing prevalence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) are a global concern. To investigate the prevalence and characteristics of sequence type 398 (ST398) MRSA in pig ears, 102 pig's ears were collected from 102 animals shipped from 51 farms at an abattoir. Eight ST398 MRSA isolates were isolated from the ears of eight pigs shipped from seven farms. Of the eight ST398 isolates, seven had the staphylococcal cassette chromosome *mec* (SCC*mec*) type IVd and these were obtained from seven pigs shipped from six farms. Single nucleotide polymorphisms ranging from 13 to 26 were observed in the core-genome regions in the seven SCC*mec* type IVd isolates. We believe that this is the first report on the isolation of ST398 MRSA SCC*mec* type IVd in Japan.

KEYWORDS: methicillin-resistant *Staphylococcus aureus*, pig, sequence type 398

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major drug-resistant pathogen responsible for a variety of infections worldwide, ranging from mild skin infections to life-threatening invasive diseases. In recent decades, the emergence and increasing prevalence of livestock-associated MRSA (LA-MRSA), particularly sequence type 398 (ST398) in pigs, have become a global concern [11, 25–28]. Since ST398 MRSA was isolated from pigs in Japan in 2012, it has been isolated from pigs in the Kanto and Tohoku regions of Japan as well [20, 21]. Typing of both staphylococcal cassette chromosome *mec* (SCC*mec*) and region X of the protein A gene (*spa*) revealed that divergent types of MRSA are distributed in Japan. Two SCC*mec* types, IVa and V, of ST398 MRSA are dominant with *spa* types t034, t011, t3934, and t16450 in Japan [20, 21]. Moreover, as ST398 MRSA was found in imported pigs at animal quarantine stations in Japan [12], the divergence of ST398 MRSA may increase.

The MRSA isolation rate is reportedly higher in swabs of the skin behind the ear than in nasal swabs [2]. Because pig ears are edible parts and shipped from abattoirs for consumption, data on the prevalence and characteristics of LA-MRSA, particularly ST398, in pig ears are required to estimate the risk of transmission from contaminated ears to humans. This study aimed to investigate the prevalence and characteristics of ST398 MRSA in the ears of pigs slaughtered at abattoirs.

Sampling was conducted at an abattoir in the Kanto region for 10 days in December 2018. We collected ears of 102 pigs (two per farm) shipped from 51 farms. Of the 51 farms, 10 were located in the region of Tohoku, 40 in Kanto, and one in Kyushu. An ear was removed from the heads of slaughtered pigs and placed in a plastic bag. The collected samples were stored at 4°C and delivered to the National Institute of Health Sciences.

At the laboratory, MRSA isolation was conducted within 48 hr of sampling. One whole ear per pig was inoculated in 100 mL of Mueller Hinton (MH) broth (Kanto Chemical, Tokyo, Japan) containing 6.5% NaCl and incubated for 18–24 hr at 37°C for enrichment. After incubation, a loopful of the MH culture was plated onto CHROMagar™ MRSA medium (CHROMagar, Paris, France) and Pourmedia® MRSA II medium (Eiken Chemical, Tokyo, Japan) and incubated for 48 hr at 37°C. When suspected MRSA colonies

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were observed on these media, up to two colonies per sample were selected and subsequently identified as *Staphylococcus aureus* using commercial identification kits (CycleavePCR™ *Staphylococcus aureus* [DnaJ gene] Detection Kit; TaKaRa Bio, Kusatsu, Japan). The isolates were tested for the presence of the *pvl* and *hlg* genes encoding Pantone–Valentine leukocidin and γ -hemolysin, respectively, using polymerase chain reaction (PCR), as previously described [17]. The *S. aureus* ATCC strain BAA-1556 (USA 300 clone) was used as a control in the identification procedures. One MRSA isolate per sample was subjected to molecular typing and antimicrobial susceptibility testing. MRSA isolates were characterized via multilocus sequence typing [10] and *spa* typing [13]. SCCmec typing was also performed using two multiplex PCR assays [15, 29]. The isolates were tested for the presence of *czrC*, the gene encoding a protein that confers cadmium and zinc resistance, using PCR as previously described [5]. MRSA isolates were also tested for susceptibility to ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, trimethoprim, gentamicin, teicoplanin, tetracycline, oxacillin, and vancomycin. Oxacillin was used to confirm the identity of the isolate as MRSA. The minimal inhibitory concentrations (MICs) of these antimicrobials were determined using the broth microdilution method on dried plates (Eiken Chemical, Tokyo, Japan) according to the guidelines of the Clinical and Laboratory Standards Institute [6, 7]. *S. aureus* ATCC 29213 was used as the quality control strain. The MIC of zinc chloride was determined using a previously reported agar dilution assay for zinc chloride (0.25 to 16 mM) [1]. An MIC value >2 mM was used as the cut-off value to designate resistance in accordance with a previous report [1]. In addition, the presence of antimicrobial resistance genes and phylogenetic relationships in eight ST398 MRSA isolates was confirmed using draft whole-genome sequencing (WGS). Briefly, a DNA library was prepared using a QIAGEN QIAseq FX DNA library kit (QIAGEN, Hilden, Germany) and subjected to WGS using an Illumina iSeq sequencer (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The obtained data of sequence reads were submitted to ResFinder 4.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>). Core-genome phylogenetic analysis was performed according to our previous report [4] and *S. aureus* 08BA02176 genome (accession number: CP003808.1) was used as a reference. The draft-WGS read data of the eight ST398 MRSA isolated in this study were deposited in the DNA Data Bank of Japan Sequence Read Archive on accession numbers from DRR381280 to DRR381287.

MRSA was isolated from the ears of 16 (15.7%) pigs shipped from 13 (25.5%) farms (Table 1). On three farms (farm codes a, h, and k), MRSA was isolated from both tested pigs. All the isolates possessed γ -hemolysin genes but not *pvl*. The 16 isolates were subjected to molecular typing. Eight isolates were classified as ST398 and all of them were classified as *spa* type t011. ST398 MRSA

Table 1. Characteristics of methicillin-resistant *Staphylococcus aureus* isolated in this study

Prefecture code	Region	Farm code	Isolate	MLST		<i>spa</i>	SCCmec	Antimicrobial resistance profile	Zinc resistance		Other antimicrobial resistant genes
				CC	ST				<i>czrC</i>	MIC (mM)	
A	Kanto	a	a-1	398	398	t011	IVd	ABPC, TC, EM	+	4	<i>blaI, blaR1, blaZ, erm(C), mecA, tet(38), tet(M)</i>
			a-2	398	398	t011	IVd	ABPC, TC, CP, EM	+	4	<i>blaI, blaR1, blaZ, erm(C), fexA, mecA, str, tet(38), tet(M)</i>
A	Kanto	b	b-1	398	398	t011	IVd	ABPC, TC, CP, EM	+	4	<i>blaI, blaR1, blaZ, erm(C), fexA, mecA, qacG, str, tet(38), tet(M)</i>
B	Kanto	c	c-1	398	398	t011	IVd	ABPC, TC, CP, EM	+	4	<i>blaI, blaR1, blaZ, erm(C), fexA, mecA, qacG, tet(38), tet(M)</i>
C	Kanto	d	d-1	398	398	t011	IVd	ABPC, TC, EM	+	4	<i>blaI, blaR1, blaZ, erm(C), mecA, qacG, tet(38), tet(M)</i>
D	Kanto	e	e-1	398	398	t011	IVd	ABPC, TC, EM	+	4	<i>blaI, blaR1, blaZ, erm(C), mecA, str, tet(38), tet(M)</i>
E	Tohoku	f	f-1	398	398	t011	IVd	ABPC, TC, EM	+	4	<i>blaI, blaR1, blaZ, erm(C), mecA, qacG, tet(38), tet(M)</i>
A	Kanto	g	g-1	398	398	t011	V	ABPC, TC, CP, EM, TMP	+	4	<i>ant(6)-Ia, blaI, blaR1, blaZ, dfrG, erm(C), fexA, lnu(B), lsa(E), mecA, spw, tet(38), tet(K), tet(M)</i>
A	Kanto	h	h-1	5	5	t1560	V	ABPC, CPFX, EM	-	1	Not tested
			h-2	5	5	t1560	V	ABPC, CPFX, EM	-	1	Not tested
B	Kanto	i	i-1	5	5	t1560	V	ABPC, CPFX, EM	-	1	Not tested
F	Kanto	j	j-1	5	5	t1560	V	ABPC, CPFX, EM	-	1	Not tested
B	Kanto	k	k-1	5	5	t002	Atypical	ABPC	-	1	Not tested
			k-2	5	5	t002	Atypical	ABPC, CP	-	1	Not tested
E	Tohoku	l	l-1	8	8	t1767	IVa	ABPC, GM, KM,	-	1	Not tested
D	Kanto	m	m-1	8	8	t18785	IVa	ABPC, GM, KM, TC, CPFX, EM	-	1	Not tested

MLST: multilocus sequence typing, CC: clonal complex, ST: sequence type, SCCmec: staphylococcal cassette chromosome *mec*, ABPC: ampicillin, TC: tetracycline, TMP: trimethoprim, GM: gentamicin, EM: erythromycin, CP: chloramphenicol, MIC: minimal inhibitory concentration.

was isolated on five out of ten sampling days. The *SCCmec* type of seven ST398 isolates from pig ears from six farms (farms codes a to f) were IVd. The *SCCmec* type of one isolate from one of the farms (farms code g) was V. ST398 MRSA *SCCmec* type IVd isolates were obtained from the Kanto (prefecture codes A to D) and Tohoku (prefecture E) regions. All the ST398 isolates were resistant to ampicillin, tetracycline, and erythromycin. In addition, all the ST398 isolates possessed the *czrC* gene and were resistant to zinc chloride. Furthermore, six isolates were classified as ST5 and among them, four and two were classified as *spa* types t1560 and t002, respectively. ST5 MRSA was obtained from the Kanto region. The *SCCmec* type of ST5/t1560 was V. ST5/t002 isolates had the class A *mec* gene complex but were negative for *ccr* and were classified as atypical. In contrast, ST5/t1560 was resistant to ampicillin, ciprofloxacin, and erythromycin, whereas ST5/t002 was susceptible to both ciprofloxacin and erythromycin. Two isolates were classified as ST8 and *spa* types t1767 or t18785. The *SCCmec* type of ST8 was IVa. ST8 MRSA was resistant to ampicillin, gentamicin, and kanamycin. None of the ST5 and ST8 isolates possessed *czrC* and all were susceptible to zinc chloride.

In Europe and North America, ST398 of *SCCmec* type V accounts for the majority of ST398 MRSA isolated from pigs [8, 14, 18, 19]. In contrast, seven ST398 MRSA isolates in the present study possessed *SCCmec* type IVd and the remaining one ST398 MRSA possessed *SCCmec* V. To the best of our knowledge, the isolation of ST398 MRSA carrying *SCCmec* type IVd has not been reported in Japan or elsewhere. We speculate that we have isolated ST398 MRSA carrying *SCCmec* type IVd for the first time, at least in Japan. Recent studies indicate that the use of zinc compounds, such as zinc sulfate and zinc oxide, leads to the selection and persistence of MRSA *SCCmec* type V in pig farms [3, 24]. This is because zinc resistance is encoded by *czrC*, which is expressed by MRSA *SCCmec* type V [5]. The ST398 MRSA of *SCCmec* type IVd obtained in the present study expressed *czrC* and can be selected by using zinc compounds similar to the approach used for MRSA of *SCCmec* type V.

The core-genome phylogenetic analysis showed relatively small numbers of single nucleotide polymorphisms (SNPs) (range, 13–26) in the seven ST398 MRSA isolates (a-1, a-2, b-1, c-1, d-1, e-1, and f-1) carrying *SCCmec* type IVd (Fig. 1). Conversely, the core-genome phylogenetic analysis showed relatively large numbers of SNPs (range, 478–486) between the ST398 MRSA isolate (g-1) carrying *SCCmec* type IVd isolate and the seven ST398 MRSA isolates carrying *SCCmec* type V. Duchêne *et al.* [9] reported that

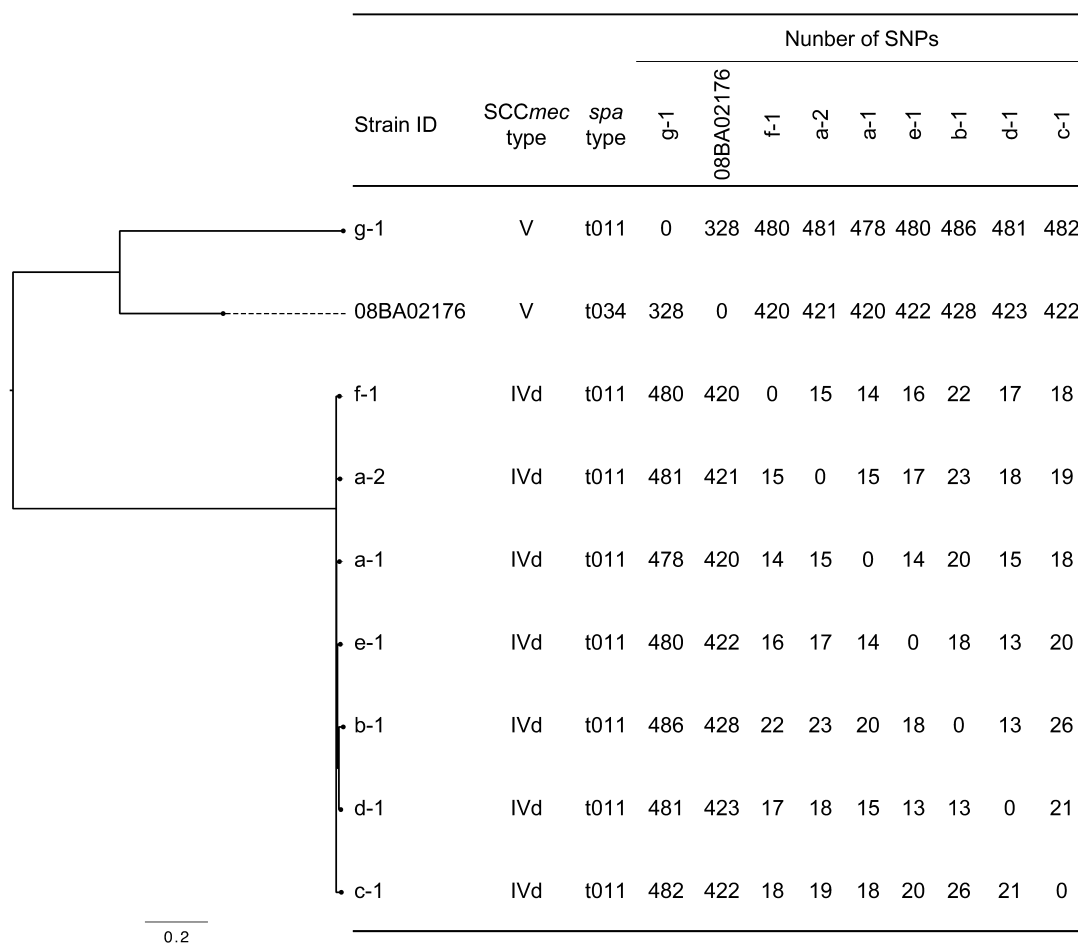


Fig. 1. Phylogenetic tree of ST398 methicillin-resistant *Staphylococcus aureus* isolates constructed with maximum-likelihood phylogenetic analysis based on single nucleotide polymorphisms (SNPs) in the core-genome and excluding homologous recombination sequences. The core-genome region was 92.5% (2,574,746/ 2,782,313 bp) of the genome of the reference strain, *S. aureus* 08BA02176 (accession number: CP003808.1). The scale distance corresponds to the number of SNPs per site.

the mean substitution rate in the clonal complex (CC) 398 *S. aureus* (genome size, 2.8 megabase pairs) was 2.43×10^{-6} substitutions per site per year. The mean substitution rate can be interpreted as an average of 6.8 SNPs occurring in the CC398 genome per year. Therefore, the small numbers of SNPs among the seven ST398 MRSA of *SCCmec* type IVd indicate that they originated from a common ancestor a few years ago. In Japan, pigs are annually imported for breeding purposes. Furuno *et al.* [12] reported that 12 of 125 pigs imported from Europe and North America tested positive for MRSA ST398 during the quarantine period. A herd of pigs infected with ST398 MRSA of *SCCmec* type IVd may have been imported a few years ago, and the infected animals delivered to pig farms in the Tohoku and Kanto regions after the quarantine period.

Ears contaminated with ST398 MRSA originated from pigs in the Tohoku and Kanto regions. In contrast to the characteristics of ST398 MRSA reported in the two regions [20–22], the characteristics of the isolates in the present study were different. Fifty-eight ST398 MRSA isolates were obtained in the Kanto region between 2012 and 2014, and the most frequent *spa* type was t16450 (33 isolates), followed by t034 (14 isolates), and t3934 (11 isolates) [20]. The *SCCmec* types of t16450 and t034 were atypical and V, respectively. In the Tohoku region, 13 ST398 MRSA isolates were obtained in 2017, and the most frequent *spa* type was t034 (11 isolates) followed by t011 (two isolates) [21]. The *SCCmec* types of t034 were IVa (three isolates) and V (eight isolates).

The eight ST398 isolates were all positive for *blaI*, *blaZ*, *ermC*, *tet(38)*, and *tet(M)*. Of the seven *SCCmec* type IVd isolates, three had the chloramphenicol/florfenicol resistance gene (*fexA*) and four had the disinfectant resistance gene (*qacG*). One *SCCmec* type V isolate was multidrug-resistant and had *ant(6)-Ia* (aminoglycoside resistance), *dfrG* (trimethoprim resistance), *fexA*, *lnu(B)* and *lsa(E)* (both lincomycin resistance), and *tet(K)* (Table 1). A similar profile of resistance genes in ST398 MRSA *SCCmec* type V was reported in Korea [16]. However, the existence of the florfenicol resistance gene (*fexA*) and lincomycin resistance genes *lnu(B)* and *lsa(E)*, which had not been reported in Japan, was verified. In addition, the resistance gene profiles of *SCCmec* type IVd isolates were obtained in this study.

In the present study, the prevalence of ST398 MRSA in pig ears was 7.8% (8/102, 95% confidence interval 3.8–14.9). The prevalence of MRSA in 40 domestic pork products was investigated in 2017 [23] but no ST398 MRSA was isolated from any of the pork products tested. However, other STs (ST97 and ST8) were isolated from two domestic pork products. Moreover, the prevalence of MRSA in swabs of the skin behind the ear and surface of the carcass collected from 276 pigs was investigated in 2019 and the prevalence of ST398 MRSA was statistically higher in skin swabs taken from behind the ear (14.5%, 40/276) than those from the carcasses (0.4%, 1/276) [22]. The risk of ST398 MRSA transmission to humans could be higher from ears than from pork.

CONFLICTS OF INTEREST. The authors have no conflicts of interest to disclose.

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