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Isolation of ST398 methicillin-resistant *Staphylococcus aureus* from pigs at abattoirs in Tohoku region, Japan

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ABSTRACT. Here, we investigated the presence of ST398 livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in nasal swabs of 420 slaughtered pigs from 84 farms at three abattoirs in Tohoku, Japan. MRSA were isolated from 13 (3.1%) samples from 9 (10.7%) farms at two abattoirs. All isolates were classified as ST398 and were resistant to ampicillin and tetracycline. Ten and three isolates were classified as Staphylococcal Cassette Chromosome *mec* (SCC*mec*) types V and IVa, respectively. All type V isolates possessed *czrC*. The minimum inhibitory concentrations (MICs) of zinc chloride against types IVa and V were 1 and 4 mM, respectively. This study shows the presence of ST398 MRSA in pigs in this region. Antimicrobials and zinc compounds in feed and drugs might select SCC*mec* type V ST398 MRSA.

KEY WORDS: Methicillin-resistant *Staphylococcus aureus*, pig, ST398, zinc resistance

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen that causes healthcare and community-associated infections in humans. In recent decades, the emergence and worldwide spread of livestock-associated MRSA (LA-MRSA) in pigs have been reported [11, 30]. LA-MRSA, particularly sequence type (ST) 398, are of global concern in human and veterinary medicine. In a Dutch hospital, located in a pig-dense area, ST398 has led to a three-fold increase in MRSA incidence over a few years [29]. The report suggests that pig farming serves as a risk factor for MRSA carriage within the families of farmers and their neighbors in the area. Additionally, there are some reports on the transmission of LA-MRSA to workers and pig carcasses at abattoirs [21, 23, 28]. Slaughtering pigs infected with MRSA is a potential threat to public health as the infection can be transmitted to the general population *via* consumption of contaminated pork, as well as to workers at abattoirs. In Japan, although two previous studies concerning LA-MRSA in slaughtered pigs have been conducted in 2009 [4] and 2013 [25], respectively, ST398 was not reported. However, in other countries, including Asian countries, LA-MRSA isolation from pigs and pig carcasses has been reported frequently [6, 21]. In Japan, pigs are annually imported for breeding purposes. According to the Japanese Ministry of Agriculture, Forestry and Fisheries, 1,040 live pigs were imported in 2016 [20].

Recent overseas studies indicate that the use of zinc compounds, such as zinc sulfate and zinc oxide, leads to the selection and persistence of LA-MRSA in pig farms [2, 26]. This is because the zinc resistance is encoded in *czrC* and the gene is located within the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) type V in MRSA [5]. In Europe and North America, ST398 harboring SCC*mec* type V accounts for the majority of ST398 MRSA isolated from pigs [9, 15, 23, 24]. In Italy, Normanno *et al.* [23] reported that 16 and 3 ST398 MRSA strains were isolated from 215 pigs and 113 abattoir workers, respectively, and all the strains had SCC*mec* type V. In Germany, the zinc-resistance rate in the clonal complex CC398 MRSA from patients increased by 70% from 2009 to 2014 [27]. In Japan, zinc compounds have also been used as feed additives in pig farms. The Food Safety Commission of Japan conducted a risk assessment on antimicrobial-resistant bacteria arising from the use of colistin sulfate,

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which is used as a feed additive and veterinary medicinal products in the livestock. They concluded that the degree of possible reduction or loss of clinical effectiveness of human drugs was high in January 2017 [12]. After the release of this risk assessment, the government started discussing the restriction of using colistin. If the use of colistin sulfate is restricted, then the use of zinc compounds might increase to control post-weaning diarrhoea and improve growth performance, consequently increasing the risk of slaughtering pigs infected with zinc-resistant ST398 MRSA. Therefore, we conducted an investigation to confirm the presence of LA-MRSA in pigs at abattoirs in Tohoku region of Japan. This region is the third largest pork producing area in Japan, accounting for approximately 15% of the total pig production in the country [20].

Sampling was conducted at three abattoirs. Nasal swabs were collected from 420 pigs (5 per farm) shipped from 84 farms using cotton swabs (SEEDSWAB No. 2, Eiken Chemical, Japan), at 11 days (2, 1, 5 and 3 days in May, June, September and October 2017, respectively). All farms were located in Tohoku region. All pigs were diagnosed as healthy upon visual inspection by veterinarians. The collected samples were delivered under refrigeration to the Towada Meat Inspection Center (Aomori, Japan), and analyzed within no longer than 6 days after sampling. At the center, the tip of each swab was inoculated into 4 m*l* Tryptic soy broth (TSB) (Oxoid, Hampshire, UK) containing 6.5% NaCl and incubated for 18–24 hr at 37°C for enrichment. After the incubation, 0.1 m*l* enrichment culture was transferred to 4 m*l* TSB (Oxoid) containing 3.5 mg/*l* cefoxitin (Sigma-Aldrich, Tokyo, Japan) and 75 mg/*l* aztreonam (MP Biomedicals, Solon, OH, USA) and incubated for further 18–24 hr at 37°C. A loopful of the resulting TSB culture was plated onto a CHROMagar MRSA medium (CHROMagar, Paris, France) and oxacillin resistance screening agar base (Oxoid, Wesel, Germany) and incubated for 24–48 hr at 35°C. When suspected MRSA colonies were observed on this medium, up to two colonies per sample were isolated and identified by using a real-time PCR procedure (CycleavePCR *Staphylococcus aureus* (DnaJ gene) Detection Kit; Takara Bio Inc., Kusatsu, Japan). One MRSA isolate per sample was subjected to molecular typing, antimicrobial susceptibility testing, and zinc susceptibility testing. SCC*mec* typing was performed by multiplex PCR amplification of the *mec* and *ccr* regions using the primers described by Kondo *et al*. [17]. MRSA isolates were also characterized by *spa* [14] and multilocus sequence typing (MLST) [10]. The presence of *pvl*, *czrC* and hemolysin (α, β, γ and δ) genes was determined by PCR using previously described methods [5, 16, 19]. *S. aureus* BAA-1556 was used as a control. Minimal inhibitory concentrations (MICs) of antimicrobials were determined using the broth microdilution method in dried plates (Eiken Chemical, Tokyo, Japan), following the guidelines of the Clinical and Laboratory Standards Institute [7, 8]. *S. aureus* ATCC 29213 was used for quality control. MRSA isolates were tested for their susceptibility to ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, trimethoprim, gentamicin, teicoplanin, tetracycline, oxacillin and vancomycin. 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].

MRSA was isolated from 13 pigs (3.1% of 420 pigs) shipped from 9 farms (10.7% of 84 farms) at two abattoirs (a and b) (Table 1). At "Abattoir a" and "Abattoir b", MRSA were isolated from samples obtained on 5 of 8 and 4 of 11 sampling dates, respectively. From the samples from the one remaining abattoir, no MRSA was isolated, despite sampling being conducted across 10 dates. All the 13 isolates were positive for *mecA* and were classified as ST398. All the isolates possessed α-, β-, γ- and δ-hemolysin genes, but not *pvl*. Of the 13 ST398 MRSA isolates, 11 from 8 farms (fa, fb, fd, fe, ff, fg, fh, and fi) were classified as *spa* type t034. The remaining two isolates, from "Farm fc", were classified as *spa* type t011. Three isolates with *spa* type t034 from two farms (ff and fh) had SCC*mec* type IVa. The remaining 10 isolates from 7 farms (fa, fb, fc, fd, fe, fg and fi) had SCC*mec* type V as well as *czrC*. All isolates were resistant to both ampicillin and tetracycline. Against zinc chloride, *czrC*-positive (10) and –negative (3) isolates showed MIC of 4 and 1 mM, respectively.

The ST398 MRSA isolates in this study are closely similar to those reported in countries other than Japan [3, 21, 23, 26, 30],

Table 1. Genetic and phenotypic characteristics of the methicillin-resistant *Staphylococcus aureus* isolates obtained in this study

Isolate	Abottior code	Farm code	Date of sampling	ST	*spa* type	SCC*mec* type	Hemolysin genes	*pvl*	*czrC*	Antimicrobial resistance profile	MIC of $ZnCl_2$ (mM)
a-1	a	fa	May 8	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, TMP	4
b-1	a	fb	May 22	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, TMP	4
b-2	a	fb	May 22	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, TMP	4
c-1	a	fc	May 22	398	t011	V	α, β, γ, δ	—	+	ABPC, TC, EM, TMP	4
c-2	a	fc	May 22	398	t011	V	α, β, γ, δ	—	+	ABPC, TC, EM, TMP	4
d-1	a	fd	September 13	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, EM, TMP	4
e-1	a	fe	September 27	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, EM, TMP	4
f-1	b	ff	May 22	398	t034	IVa	α, β, γ, δ	—	—	ABPC, TC, CP, TMP	1
f-2	b	ff	May 22	398	t034	IVa	α, β, γ, δ	—	—	ABPC, TC	1
g-1	b	fg	September 14	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, TMP	4
g-2	b	fg	September 14	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, TMP	4
h-1	b	fh	September 29	398	t034	IVa	α, β, γ, δ	—	—	ABPC, TC, CP, TMP	1
i-1	b	fi	October 13	398	t034	V	α, β, γ, δ	—	+	ABPC, TC, EM, TMP	4

SCC*mec*: Staphylococcal Cassette Chromosome *mec,* AST: sequence type, ABPC: ampicillin, TC: tetracycline, TMP: trimethoprim, EM: erythromycin, CP: chloramphenicol, MIC: minimum inhibitory concentrations.

both genotypically and phenotypically. Furuno *et al.* [13] recently reported that 41 (32.8%) of 125 pigs imported from Europe and North America were positive for MRSA when tested during the quarantine period. Of 12 ST398 MRSA isolates obtained in that testing, 9 possessed both SCC*mec* type V and *czrC* and were resistant to both ampicillin and tetracycline. Because MRSA infection is not a targeted infectious disease according to the Domestic Animal Infectious Disease Control Law in Japan, the pigs tested in that study, including those carrying MRSA, would have been released to domestic pig farms after quarantine inspections. The results of the present study suggest that the ST398 MRSA lineages have been introduced into Japan from imported pigs and have spread in pig farms in Tohoku region. Moreover, because pig farmers can select the abattoirs where their pigs are slaughtered, according to the number of shipping pigs and the price of pork in the area where the abattoirs are located, pigs reared in Tohoku region are possibly shipped to abattoirs in regions other than Tohoku.

The government of Japan revoked the designation of colistin sulfate as a feed additive in July 2018. Although zinc resistance is not only factor contributing to the spread of ST398 MRSA, increased use of zinc compounds as an alternative of colistin sulfate in pig farms might lead to selection of zinc-resistant ST398 MRSA. The Ministry of Agriculture, Forestry and Fisheries of Japan reported that ST398 MRSA was isolated from 2 diseased pigs in FY 2017 (https://www.maff.go.jp/nval/yakuzai/pdf/h29_cyousa20190912.pdf) although the genotypic and phenotypic details of the strains were unclear. In addition, there are 2 case reports of *pvl*-positive CC398 MRSA isolation from patients in Japan [18, 22]. Monitoring the prevalence of LA-MRSA in pigs and the sales volume of zinc compounds, as well as the characterization of the LA-MRSA isolates, should be conducted to manage the risk of transmission of LA-MRSA to humans.

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