FULL PAPER *Bacteriology*

Prevalence and Mechanism of Antimicrobial Resistance in *Staphylococcus aureus* Isolates from Diseased Cattle, Swine and Chickens in Japan

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ABSTRACT. Antimicrobial administration is essential for the control and treatment of diseases in animals, but the emergence and prevalence of antimicrobial-resistant *Staphylococcus aureus* is a significant concern during animal production. Here we investigated the antimicrobial susceptibility of *S. aureus* from diseased food-producing animals and molecularly characterized the methicillin-resistant and fluoroquinolone-resistant isolates. A total of 290 *S. aureus* isolates obtained from cattle (n=246), swine (n=16), and chickens (n=28) between 2003 and 2009 were examined for antimicrobial susceptibility against 9 antimicrobials using an agar dilution method. Resistance to penicillin (PC) was most frequently found (24.8%), followed by oxytetracycline (OTC, 10.0%), dihydrostreptomycin (4.1%), erythromycin (EM, 3.1%), enrofloxacin (ERFX, 2.1%), and kanamycin (1.7%). The PC resistance rate was significantly higher in swine than in cattle ($P<0.01$) and chickens ($P<0.01$). The resistance rates to OTC, EM and ERFX were significantly higher in swine and chickens than in cattle ($P<0.05$). Methicillin-resistant *S. aureus* (MRSA) was recovered from milk derived from a cow with mastitis in 2003; sequence type 8, SCC*mec* type IV and *spa* type t024. In the six ERFX-resistant strains isolated after 2003, amino acid substitutions in ParC with/without GyrA were detected. As the prevalence of MRSA and FQ-resistant *S. aureus* in the animals should be noticed, continuous monitoring is necessary to control resistance to clinically important antimicrobials in *S. aureus* from food-producing animals.

KEY WORDS: antimicrobial susceptibility, enrofloxacin resistance, food-producing animals, methicillin resistance, *Staphylococcus aureus*.

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Staphylococcus aureus causes a variety of inflammatory diseases in domestic animals; e.g. mastitis in dairy herds [3], exudative dermatitis in pigs [26], and osteomyelitis and arthritis in poultry [23]. The diseases caused by *S. aureus* are often connected to severe economic losses in animal industries. As antimicrobial administration is essential for the control and treatment of these diseases in animals, the emergence and prevalence of antimicrobial-resistant *S. aureus* is a significant concern in animal production. It is important to provide information on antimicrobial susceptibility of *S. aureus* isolates from food-producing animals for animal practitioners.

Bacteria of food animal origin showing resistance to clinically important antimicrobials, such as fluoroquinolones (FQs) and β-lactams, is a serious threat to human health [10]. FQ resistance was identified in *S. aureus* from food-producing animals [19], but not in Japan [20, 29]. In addition, transmission of methicillin-resistant *S. aureus* (MRSA) to humans through livestock products is a worldwide concern on public health [1, 2, 11, 27]. Recently, MRSA ST398 of food animal origin was reported in several European countries, while in Japan, MRSA was found in cattle [11] and swine [2].

The objective of this study was to determine the prevalence of antimicrobial resistance in *S. aureus* from diseased food-producing animals in Japan. Furthermore, we analyzed the characteristics of *S. aureus* isolates resistant to methicillin and FQ.

MATERIALS AND METHODS

Bacteria: A total of 290 *S. aureus* isolates from a variety of clinical specimens of cattle (n=246), swine (n=16), and chickens (n=28) were provided by the Livestock Hygiene Service Centers of 23 prefectures throughout Japan between 2003 and 2009 (Table 1). Of the 246 bovine isolates, 236 were isolated from clinical or subclinical mastitis milk of dairy cows. Of the 16 porcine isolates, 7 isolates were derived from dermatitis, one from arthritis, and the remaining from unknown sites. Twenty-eight avian isolates were isolated from the liver, meat, and spleen of diseased birds with growth insufficiency, debility, diarrhea, decreased egg-laying rate, and unknown death. The isolates were preserved in 10% skimmed milk at −80°C until use.

Antimicrobial susceptibility testing: All the isolates were tested for susceptibility to penicillin G (PC), oxa-

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Table 1. *Staphylococcus aureus* isolated from food-producing animals in Japan between 2003 and 2009

Origin		2003	2004	2005	2006	2007	2008	2009	Total
Cattle	Mastitis	28	16	19	16	33	42	82	236
	Others			1	1	2	3	3	10
Swine	Dermatitis			1			3	3	7
	Arthritis				1				1
	Unknown	3		1		3		1	8
Chicken		2	4	7		9	3	3	28
		33	20	29	18	47	51	92	290

cillin (MPIPC), cefazolin (CEZ), oxytetracycline (OTC), erythromycin (EM), dihydrostreptomycin (DSM), kanamycin (KM), gentamicin (GM), and enrofloxacin (ERFX) using an agar dilution method of the Clinical Laboratory Standards Institute [4]. *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. The minimum inhibitory concentrations (MICs) of each antimicrobial were interpreted using the CLSI criteria [5]. The resistant breakpoints of DSM, OTC, and ERFX were defined microbiologically when the MIC distribution of antimicrobials was bimodal.

Molecular characterization of MRSA and FQ-resistant isolates: For the identification of MRSA, the presence of the *mecA* gene was shown for an MPIPC-resistant isolate followed using a published method [14]. Multi-locus sequence typing (MLST) was conducted for the MRSA isolate: seven housekeeping genes identified in a study [6] were amplified by PCR, and the sequence typing of the isolate was determined using the MLST website (www.mlst.net). The SCC*mec* type was classified according to a previous study [17]. The *spa* type was identified according to the method previously published [25]. Detection of the Panton-Valentine leucocidin (PVL) gene and the *arcA* gene encoded by the arginine catabolic mobile element (ACME) was performed as previously described [13, 30].

ERFX-resistant *S. aureus* isolates were examined for mutations of the quinolone-resistance determining regions (QRDR) of *gyrA* and *parC* by PCR [24] and DNA sequencing of the PCR products. Genomic information of the *S. aureus* ATCC 12600 strain and COL strain [9] were utilized for *gyrA* and for *parC* as wild type strains, respectively.

Statistical analysis: The results of the antimicrobial susceptibility were statistically compared between each animal species using the chi-square test. For each comparison, a P value of<0.05 was considered to be a significant difference. When at least one expected frequency was less than five, Fisher's exact-test was used for comparison between two groups.

RESULTS

Overall, resistance to PC was most frequently found (24.8%), followed by OTC (10.0%), DSM (4.1%), EM (3.1%), ERFX (2.1%), and KM (1.7%) as shown in Table 2. Only one bovine isolate was resistant to MPIPC and CEZ. Regarding animal species, PC resistance was found in 22.0% (54/246) of the bovine isolates, 93.8% (15/16) of the porcine isolates, and 10.7% (3/28) of the chicken isolates. The PC resistance rate was significantly higher in swine than in cattle ($P<0.01$) and chickens ($P<0.01$). The OTC resistance rate was significantly higher in swine (50.0%, 8/16; $P<0.01$) and chickens (67.9%, 19/28; $P<0.01$) than in cattle (0.8%, 2/246). The EM resistance was frequently found in isolates from swine (18.8%, 3/16; $P<0.01$) and chickens (10.7%, 3/28; $P<0.05$), compared with cattle (1.2%, 3/246). The ERFX resistance rate was significantly higher in swine (12.5%, $P<0.01$) and chickens (10.7%, $P<0.05$) than in cattle (0.4%).

Antimicrobial resistance patterns are shown in Table 3. Multidrug resistance (resistance to two or more classes of antimicrobials) was observed in 68.8% (11/16) of the porcine isolates in this study. The percentage of multidrug-resistant isolates was greater in swine isolates than bovine isolates (4.5%) and chicken isolates (25.0%).

Of the PC-resistant isolates, one of dairy cow origin was resistant to MPIPC and CEZ, and harbored *mecA*. The sequence type (ST) of the MRSA isolate was 8 (3-3-1-1-4-4-3) as determined by MLST. The subtype of SCC*mec* IV for this MRSA isolate was neither a, b, c nor d and the *spa* type was classified as t024 (11-12-21-17-34-24-34-22-25). The MRSA isolate was negative for the PVL and *arcA* genes.

There were six ERFX-resistant isolates; one from cattle, two from swine and three from chickens (Table 4). The MIC of ERFX was 4 μg/m*l* in all the resistant isolates. One of the ERFX-resistant isolates was MRSA. Amongst ERFX-resistant isolates, the bovine isolate had mutations of Ser84Leu and Ser80Tyr in GyrA and ParC, respectively. The three avian isolates and a porcine isolate from 2007 had mutations of Ser84Leu and Ser80Phe in GyrA and ParC, respectively. The remaining porcine isolate had only a mutation of Ser80Phe in ParC.

DISCUSSION

The present study showed that most of bovine isolates exhibited susceptibility to the antimicrobials tested, whereas antimicrobial resistance, including multidrug resistance, was frequently found in porcine and chicken isolates. Similar results were obtained in several species of intestinal bacteria of animal origins, for example enterococci [15, 16] and *E. coli* [15]. In Japan, antimicrobial use is largest in swine farming among the domestic animal industry, followed by broiler chickens [10]. Metaphylaxis is a common

Table 2. Antimicrobial susceptibilities of *Staphylococcus aureus* isolates from food-producing animals in Japan between 2003 and 2009

Antimicrobial agent[a)]	Break Point (μg/m*l*)	MIC[b)] range (μg/m*l*)	MIC_{50} (μg/m*l*)	MIC_{90} (μg/m*l*)	No. of resistant isolates (%)							
					Cattle[c)] (n=246)		Swine (n=16)		Chicken (n=28)		Total (n=290)	
PC	0.25	≤0.125–256	≤0.125	4	54	(22.0)[d)]	15	(93.8)[d), e)]	3	(10.7)[e)]	72	(24.8)
MPIPC	4	≤0.125–64	0.25	0.5	1	(0.4)	0		0		1	(0.3)
CEZ	32	≤0.125–128	0.5	0.5	1	(0.4)	0		0		1	(0.3)
OTC	16	≤0.125–256	0.25	8	2	(0.8)[f), g)]	8	(50.0)[f)]	19	(67.9)[g)]	29	(10.0)
DSM	32	0.5–512<	4	8	10	(4.1)	2	(12.5)	0		12	(4.1)
KM	64	≤0.125–512	2	2	3	(1.2)	1	(6.3)	1	(3.6)	5	(1.7)
GM	16	≤0.0315–32	0.25	0.5	2	(0.8)	1	(6.3)	1	(3.6)	4	(1.4)
EM	8	≤0.125–512<	0.25	0.5	3	(1.2)[h), i)]	3	(18.8)[h)]	3	(10.7)[i)]	9	(3.1)
ERFX	4	≤0.063–4	≤0.125	0.25	1	(0.4)[j), k)]	2	(12.5)[j)]	3	(10.7)[k)]	6	(2.1)

a) Penicillin G, PC; oxacillin, MPIPC; cefazolin, CEZ; oxytetracycline, OTC; erythromycin, EM; dihydrostreptomycin, DSM; kanamycin, KM; gentamicin, GM; enrofloxacin, ERFX.
b) MIC: Minimum inhibitory concentration.
c) Origins of strains.
d), e), f), g), h) and j), the significant difference ($P<0.01$) was observed, respectively. i) and k), the significant difference ($P<0.05$) was observed, respectively.

Table 3. Resistance pattern of *Staphylococcus aureus* isolates from food-producing animals in Japan between 2003 and 2009

No. of antimicrobial	Antimicrobial resistance pattern [a)]	Origin			
		Cattle	Swine	Chicken	Total
0	Susceptible	186	1	6	193
1	PC	44	4	1	49
	DSM	3			3
	OTC	1		13	14
	EM	1			1
	ERFX			1	1
2	PC-DSM	6			6
	PC-GM	1			1
	KM-GM	1			1
	PC-OTC		3	1	4
	PC-EM	1	2		3
	PC-ERFX		1		1
	ERFX-OTC			2	2
	EM-OTC			3	3
3	PC-EM-OTC		1		1
	PC-GM-KM			1	1
	PC-DSM-OTC		2		2
	PC-ERFX-OTC		1		1
4	PC-DSM-KM-OTC	1			1
	PC-GM-KM-OTC		1		1
5	PC-CEZ-MPIPC-KM-EM-ERFX	1			1
Total		246 (11) [b)]	16 (11)	28 (7)	290 (29)

a) Penicillin G, PC; oxacillin, MPIPC; cefazolin, CEZ; oxytetracycline, OTC; erythromycin, EM; dihydrostreptomycin, DSM; kanamycin, KM; gentamicin, GM; enrofloxacin, ERFX.
b) The number of multidrug resistant isolates is provided in parentheses.

practice in these animal species to control bacterial disease. In this study, the levels of PC resistance were highest among the antimicrobials tested in bovine (22.0%) and porcine isolates (93.8%). PC and ampicillin or a PC combination drug with DSM is widely chosen for treatments of porcine dermatitis and bovine mastitis in Japan. A previous study showed that 13 (31.7%) of 41 *S. aureus* isolates from bovine mastitis exhibited resistance to PC (MIC: higher than 0.78 Units/m*l*, equivalent to 0.47 μg/m*l*) in 1997 and 1998 [29]. High resistance rates of staphylococci to PC were previously reported in porcine isolates (58.3%) [20] and pork isolates (more than 60%) [8] as well as this study. Almost half of the porcine isolates were resistant to OTC in this study. Tetracycline antibiotics are the most commonly used

Table 4. Origin of animals, isolation site, minimum inhibitory concentration (MIC) value, and amino acid substitution at GyrA and ParC in enrofloxacin-resistant strains

Isolation Year	Origin of Animal	Isolation site	Strain	MIC of ERFX (μg/m*l*)	Amino acid substitution in		Resistance pattern [a]
					GyrA	ParC	
2003	Dairy cow	Milk	15-10	4	S84L	S80Y	PC-MPIPC-CEZ-KM-EM-ERFX
2005	Chicken	Liver	17-33	4	S84L	S80F	OTC-ERFX
2007	Chicken	Liver	19-11	4	S84L	S80F	OTC-ERFX
			19-32	4	S84L	S80F	ERFX
	Swine	Tonsil	19-27	4	S84L	S80F	PC-OTC-ERFX
2009	Swine	Skin	21-1	4	WT [b]	S80F	PC-ERFX

a) Penicillin G, PC; oxacillin, MPIPC; cefazolin, CEZ; oxytetracycline, OTC; erythromycin, EM; kanamycin, KM.
b) WT: wild type.

antimicrobials in the Japanese veterinary field, especially the pig industry [10]. On the other hand, the rate of OTC resistance among bovine isolates remains low in this study. In bovine *S. aureus* isolates in the late of 1990s in Japan, OTC resistance (MIC: more than 25 μg/m*l*) was observed in 2 isolates (4.8%) [29].

The high virulent MRSA clone (USA 300 clone; ST8-IVa with PVL and ACME) were spread among humans in communities and patients in hospitals in the United States [21, 28]. This MRSA clone was also identified in some Japanese humans in 2008 [12]. However, the MRSA isolate (ST8-IV/t024) from dairy cow in this study did not harbor the PVL gene and ACME. As for food-producing animals in Japan, MRSA ST5-II/t002, ST5-II/t375 and ST509-IIIa/t5266 from mastitis milk of cattle and ST221-untypable (type 1 *ccr* and unidentified *mec* complex class)/t002 from the nasal swab of a slaughtered pig were reported [2, 11]. In Japan, MRSA ST8-IV (SCC*mec* subtype was not a, b and c; *spa* type was not determined) strains, which were negative for the PVL gene, had already emerged in outpatients in the early 2000s [22]. Thus, the MRSA isolate from a dairy cow may be derived from human MRSA carriers in the community.

The appearance of FQ resistance was found in bovine *S. aureus* isolates after 2003 and porcine and avian *S. aureus* isolates after 2007. Although FQ resistance was reported in *S. epidermidis* from a pig [20], there are no FQ-resistant *S. aureus* obtained from food-producing animals in 2000 [20, 29]. Most of the ERFX-resistant isolates were multidrug-resistant in this study. For Gram-positive bacteria including *S. aureus*, amino acid substitution at QRDR in ParC and GyrA is responsible for quinolone resistance [7, 19]. In this study, although five ERFX-resistant isolates had amino acid substitutions in QRDR of both GyrA and ParC, one ERFX-resistant isolate from swine only had an amino acid substitution in QRDR of ParC (Table 4). These six isolates showed identical MICs for ERFX (4 μg/m*l*). Thus, activation of efflux may be associated with ERFX resistance in the porcine isolate having only an amino acid substitution in QRDR of ParC, as suggested in a previous study [18].

Finally, the antimicrobial susceptibility of *S. aureus* isolates from food-producing animals obtained over 7 years was examined. Neither an increase nor decrease of antimicrobial resistance was observed over this study period. However, the emergence and prevalence of MRSA and FQ-resistant *S. aureus* in the animals should be noticed. It will be necessary to conduct continuous monitoring and epidemiological studies in food-producing animals to protect public health in Japan.

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