Epidemiological Studies on Methicillin-Resistant *Staphylococcus aureus* from Swine in Japan

(わが国の豚由来メチシリン耐性黄色ブドウ球菌の疫学的研究)

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ABBREVIATIONS

ABPC; ampicillin

ACME; arginine catabolic mobile element

AZM; azithromycin

CA-MRSA; community-acquired MRSA

CC; clonal complex

CEZ; cefazolin

CLSI; Clinical Laboratory Standards Institute

CP; chloramphenicol

CTF; ceftiofur

DSM; dihydrostreptomycin

EM; erythromycin

ERFX; enrofloxacin

FQ; fluoroquinolones

GM; gentamicin

HA-MRSA; hospital-acquired MRSA

IPM; imipenem

KM; kanamycin

LA-MRSA; livestock-associated MRSA

MIC; minimum inhibitory concentration

MLST; multi locus sequence typing

MPIPC; oxacillin

MRCNS; methicillin-resistant coaglase negative staphylococci

MRSA; methicillin-resistant *Staphylococcus aureus*

MSSA; methicillin-susceptible *Staphylococcus aureus*

OTC; oxytetracycline

PC; penicillin G

PCR; polymerase chain reaction

PVL gene; Panton-Valentine leucocidin gene

QRDR; quinolone-resistance determining regions

SCC*mec*; staphylococcal cassette chromosome *mec*

ST; sequence type

TMP; trimethoprim

TS; tylosin

VCM; vancomycin

CONTENTS

PREFACE

Staphylococcus aureus is a zoonotic bacteria and causes a variety of inflammatory diseases in humans and domestic animals; e.g. dermatitis, pneumonia, endocarditis, mastitis, pneumococcal otitis, arthritis in humans (Brandt et al., 1997, Røder et al., 1999, Hageman et al., 2006, Huijsdens et al., 2006), mastitis in dairy herds (Bakema et al., 2006), exudative epidermitis in swine (Straw et al., 2006), osteomyelitis and arthritis in poultry (Saif et al., 2003). Regarding *S. aureus* infection in humans, although approximately a third of the human population is colonized with *S. aureus* (Graham et al., 2006, Shorr 2007), asymptomatic infection is far more common than clinically symptomatic infection, similarly to animals (Chambers 2001, van Duijkeren et al., 2007, Chaberny et al., 2010). However, infection of *S. aureus* causes death in humans. Once onset of the symptoms occurs, it occasionally results in death. Lethal cases due to antimicrobial resistant, more specifically, methicillin-resistant *Staphylococcus aureus* (MRSA) infections in US Americans had eclipsed those from many other transmittable diseases, including human immunodeficiency virus /acquired immunodeficiency syndrome (Klevens et al., 2007). Similarly, in Japan, the bacterium is one of the most harmful pathogens in human medicine (Yokota et al., 1996).

In livestock production, although antimicrobial therapy is necessary for the treatment of the diseases for affected herds/flocks, use of antimicrobial agents selects resistant strains (Harada and Asai 2010). In commensal bacteria, enterococci and *Escherichia coli*, strains of swine

significantly exhibit higher resistance rates to antimicrobial agents and more multi-drug resistant phenotypes than those of cattle (Harada and Asai 2010, Kojima et al., 2010). In Japan, the amount of antimicrobial utilization is the largest in swine farming among livestock production (Harada and Asai 2010). According to the studies, the amount of tetracycline antibiotics is the key factor for high resistant rates and multi-resistant phenotypes, because of selection of cross-resistant and co-resistant strains. Use of antimicrobial agents in swine production, possibly, also facilitates emergence of novel antimicrobial-resistant pathogens including MRSA (Price et al., 2012).

MRSA, being resistant to β-lactam antibiotics, carries *mecA* gene, which encodes penicillin binding protein 2'; it is an exogenous cell wall enzyme and exhibits low binding-affinity to β-lactam antibiotics (Ito and Hiramatsu 1998, Leonard and Markey 2008). The MRSA strain was initially discovered in 1961 in the United Kingdom after methicillin was introduced to human medicine (Jevons 1961). Since then, MRSA has been drastically prevailing at hospitals in several countries of Europe and North America, and it has been called as hospital acquired (HA)-MRSA (Panlilio et al., 1992, Tiemersma et al., 2004). As HA-MRSA is typically multiple resistant, there is difficulty to treat patients infected with it in clinical practice (Polisena et al., 2011). Later, in 1981, another type of MRSA emerged in communities outside the hospital. It was primarily detected among drug users in the United States (American Academy of Orthopaedic Surgeons 2009), and it also infects healthy individuals without a

serious risk factor, such as hospitalization and immunocompromized state (Yamamoto et al., 2010). It was termed as community acquired (CA)-MRSA. In the late 90's, CA-MRSA became a major health concern throughout the world (Yamamoto et al., 2010). By contrast, in livestock production, MRSA strains had also been discovered, especially dairy cows developed mastitis in 1972, and another isolation in 1975 (Leonard and Markey 2008); the researchers suggest that the MRSA strains should be of human origins, and those isolates discovered in livestock had not been reported to be of a serious threat as an infection disease to human (Leonard and Markey 2008). Afterward, a new type of MRSA, named livestock associated (LA)-MRSA, emerged in Dutch swine production systems, and spread all over European live stock production systems including cattle and poultry immediately (de Neeling et al., 2007, European Food Safety Authority, 2009), and was transmitted to humans（Voss et al., 2005). Therefore, it has been notably in the attention on public health in the last decade (de Neeling et al., 2007, Smith et al.,2009). LA-MRSA was classified as sequence type (ST) 398 on multi locus sequence type (MLST), majorly with *spa* type of t011 or t034, and SCC*mec* type of VIa or V; however, some of them were not classified into the extant types (de Neeling et al., 2007, Lewis et al., 2008, Khana et al., 2008, Smith et al., 2009 , Li et al., 2011). These molecular types in MLST and *spa* typing are different from MRSA strains of human origin previously reported (Huijsdens et al., 2009).

The MRSA ST398 strains were instantly spread via colonized swine throughout the European swine production system. According to the studies, the prevalence rates in European and North American swine were between 24.9 and 85.7% and in swine producers were between 9.3% and 64% (de Neeling et al., 2007, Lewis et al., 2008, Khana et al., 2008, Smith et al., 2009, Morcillo et al., 2012). In areas where are farmed with high population of swine infected with MRSA ST398, the clone has influenced public health; after it emerged in the Netherland, it has led to a three-fold increase in MRSA incidence over a few years in a Dutch hospital located in a swine-dense area (van Rijen et al., 2008), and a similar incident is reported from a hospital in a region of Germany with intense livestock farming (Köck et al., 2009). At the moment, MRSA ST398 in livestock, primarily swine, has provided a reservoir of infection for humans, dairy cows and veal, and poultry, especially in European countries (European Food Safety Authority, 2009). Furthermore, other than European countries, the prevalence of LA-MRSA was confirmed at human hospitals, communities, and livestock production systems; patients in Hong Kong (Ip et al., 2005), a veterinarian in Thailand (Wulf et al., 2008), poultry of Iran (Nemati et al., 2008), and swine of Canada (Khanna et al., 2008), the United States (Smith et al., 2009), and South Korea (Lim et al., 2012).

The vast majority of LA-MRSA strains were resistant to tetracycline (Li et al., 2011), and it is the difference in LA-MRSA from HA- or CA-MRSA. Furthermore, LA-MRSA often exhibits

multi-drug resistance to fluoroquinolone, aminoglucoside, macrolide, in addition to β-lactam antibiotics and tetracycline (Witte et al., 2007, Lewis et al., 2008, Pantosti and Venditti 2009).

Epidemiological data on staphylococci in swine would be informative to estimate the risk of prevalence and spread of MRSA. Horizontal, interspecies transfer of staphylococcal cassette chromosome (SCC) *mec* could be an important factor in the emergence of MRSA (Bloemendaal et al., 2010). It is known in theory, that emergence of CA-MRSA might have acquired SCC*mec* from methicillin-resistant coaglase negative staphylococci (MRCNS) clone in a community (Nagao and Ota 2007). Thus, there is possible emergence of LA-MRSA in the swine production systems in Japan as methicillin-susceptible *S.aureus* (MSSA) evolves to MRSA upon the acquisition of SCC*mec* of MRSA or other methicillin-resistant staphylococci (Bioemendaal et al., 2010, www.staphylococcus.net).

The overall objective of the studies was to elucidate trends in antimicrobial resistant *S. aureus* and to evaluate the possible evolution of MRSA in swine in Japan. In the first chapter, the author determines an overview of the prevalence of antimicrobial resistance in *S. aureus* from diseased food-producing animals in Japan. In the second chapter, the author conducted a survey on the prevalence of MRSA in Japanese swine. Furthermore, in the last chapter, the author focused on molecular typing of MSSA in swine.

CHAPTER 1

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

1.1 Introduction

Staphylococcus aureus causes a variety of inflammatory diseases in domestic animals; e.g. mastitis in dairy herds (Barkema et al., 2006), exudative dermatitis in swine (Straw et al., 2006), and osteomyelitis and arthritis in poultry (Saif et al., 2003). 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 and β-lactams, are a serious threat to human health (Harada and Asai 2010). Fluoroquinolones (FQ) resistance was identified in *S. aureus* from food-producing animals (Lin and Davies 2007), but not in Japan (Yoshimura et al., 2002, Morioka et al., 2005). In addition, transmission of MRSA to humans through livestock products

is a worldwide concern on public health (Kojima et al., 2009, Aspiroz et al., 2010, Hata et al., 2010). Recently, MRSA ST398 of food animal origin was reported in several European countries, while in Japan, MRSA was detected in cattle (Hata et al., 2010). The objective of this study was to determine the prevalence of antimicrobial resistance *S. aureus* from diseased food-producing animals in Japan. Furthermore, we analyzed the characteristics of *S. aureus* isolates resistant to methicillin and FQ.

1.2 Materials and Methods

1.2.1 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.

1.2.2 Antimicrobial susceptibility testing

All the isolates were tested for penicillin G (PC), oxacillin (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 (CLSI, CLSI 2008a). *S. aureus* ATCC 29213, *Enterococcus.faecalis* ATCC29212, *E.coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. The MICs of each antimicrobial were interpreted using the CLSI criteria (CLSI 2008b). The resistant breakpoints of DSM, OTC, and ERFX were defined microbiologically when the MIC distribution of antimicrobials was bimodal.

1.2.3 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 (Kawano et al., 1996). MLST was conducted for the MRSA isolate: seven housekeeping genes identified in a study (Enright et al., 2000) 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 (Kondo et al., 2007). The *spa* type was identified according to the method previously published (Shopsin et al., 1999). Detection of the Panton-Valentine leucocidin gene (PVL) gene and the

arcA gene encoded by the (arginine catabolic mobile element) ACME was performed as previously described (Zhang et al., 2008, Ishihara et al., 2010).

ERFX-resistant *S. aureus* isolates were examined for mutations of the quinolone-resistance determining regions (QRDR) of *gyrA* and *parC* by PCR (Schmitz et al., 1998) and DNA sequencing of the PCR products. Genomic information of the *S. aureus* ATCC 12600 strain and COL strain (Gill et al., 2005) were utilized for *gyrA* and for *parC* as wild type strains, respectively.

1.2.4 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.

1.3 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 the 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 additional 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 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. 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 4μg/ml 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.

1.4 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 (Kojima et al., 2009, Kojima et al., 2010) and *E. coli* (Kojima et al., 2009). The amount of antimicrobial utilization is the largest in swine among food-producing animals, followed by broiler chickens (Harada and Asai 2010). Metaphylaxis is a common 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/ml, equivalent to 0.47 μg/ml) in 1997 and 1998 (Yoshimura et al., 2002). High resistance rates of staphylococci to PC were previously reported in porcine isolates (58.3%, Morioka et al., 2005) and pork isolates (more than 60%, Fujio et al., 2007), 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 antimicrobials in the Japanese veterinary field, especially the swine industry (Harada and Asai 2010). 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/ml) was observed in 2 isolates (4.8%) (Yoshimura et al., 2002). The high virulent MRSA clones (USA 300 clone; ST8-IVa with PVL and ACME) were spread among humans in communities and patients in hospitals in the United States (Otter et al., 2009, Yamamoto et al., 2010). This MRSA clone was also identified in some Japanese humans in 2008 (Higuchi et al., 2010). 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 swine were reported (Chapter 2, Hata et al.,

2010). 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 (Piao et al., 2005). 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 swine (Morioka et al., 2005), there are no FQ-resistant *S. aureus* obtained from food- producing animals in 2000 (Yoshimura et al., 2002, Morioka et al., 2005). 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 (Ferrero et al., 1995, Lin et al., 2007). 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 (4μg/ml). 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 (Li et al., 2009).

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.

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

		Isolation Year							
Animal	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	Others	2	4	7		9	3	3	28
	Total	33	20	29	18	47	51	92	290

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

	MIC[b] range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Break Point (μg/ml)	Number of resistant isolates (%)							
					Cattle[c] (n=246)		Swine (n=16)		Chicken (n=28)		Total (n=290)	
PC	≦0.125-256	≦0.125	4	0.25	54	(22.0)[d]	15	(93.8)[d,e]	3	(10.7)[e]	72	24.8
MPIPC	≦0.125-64	0.25	0.5	4	1	0.4	0		0		1	0.3
CEZ	≦0.125-128	0.5	0.5	32	1	0.4	0		0		1	0.3
OTC	≦0.125-256	0.25	8	16	2	(0.8)[f,g]	8	(50.0)[f]	19	(67.9)[g]	29	10
DSM	0.5-512<	4	8	32	10	4.1	2	12.5	0		12	4.1
KM	≦0.125-512	2	2	64	3	1.2	1	6.3	1	3.6	5	1.7
GM	≦0.0315-32	0.25	0.5	16	2	0.8	1	6.3	1	3.6	4	1.4
EM	≦0.125-512<	0.25	0.5	8	3	(1.2)[h,i]	3	(18.8)[h]	3	(10.7)[i]	9	3.1
ERFX	≦0.063-4	<0.125	0.25	4	1	(0.4)[j,k]	2	(12.5)[j]	3	(10.7)[k]	6	2.1

[a]PC; penicillin G, MPIPC; oxacillin, CEZ; cefazolin, OTC; oxytetracycline, EM; erythromycin, DSM; dihydrostreptomycin, KM; kanamycin, GM; gentamicin, ERFX; enrofloxacin

[b]Minimum inhibitory concentration

[c] Origin of strains

d,e,f,g,h,and j, a siginificant difference (p<0.01) was observed, respectively.

i and k, a significant difference (p<0.05) was observed, respectively.

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

No of antimicrobial	Antimicrobial resistance pattern[a]	Animal species			
		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]PC; penicillin G, MPIPC; oxacillin, CEZ; cefazolin, OTC; oxytetracycline, EM; erythromycin, DSM; dihydrostreptomycin, KM; kanamycin, GM; gentamicin, ERFX; enrofloxacin

[b]The number of multidrug resistant isolates is provided in parentheses.

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	Isolated site	MIC of ERFX (mg/L)	Amino acid substitution in GyrA	Amino acid substitution in ParC	Resistance pattern[a]
2003	Dairy cow	Milk	4	S84L	S80Y	PC-MPIPC-CEZ-KM-EM-ERFX
2005	Chicken	Liver	4	S84L	S80F	OTC-ERFX
2007	Chicken	Liver	4	S84L	S80F	OTC-ERFX
			4	S84L	S80F	ERFX
	Swine	Tonsil	4	S84L	S80F	PC-OTC-ERFX
2009	Swine	Skin	4	WT[b]	S80F	PC-ERFX

[a] PC; penicillin G, MPIPC; oxacillin, CEZ; cefazolin, OTC; oxytetracycline, EM; erythromycin, KM; kanamycin

[b]WT; wildtype

CHAPTER 2

Isolation of Methicillin-Resistant *Staphylococcus aureus* from Swine in Japan

2.1 Introduction

Since MRSA was isolated from humans working in close proximity to swine in the Netherlands, a number of studies have been conducted to determine the prevalence and characterisation of MRSA amongst swine, swine producers and their families (de Neeling et al., 2007, Lewis et al., 2008, Khanna et al., 2008, Smith et al., 2009). MRSA isolates transmitted between swine and swine farmers were classified as ST398 using MLST. MRSA ST398 is disseminated by colonised swine through swine production systems. According to data of the Animal Quarantine Service of the Ministry of Agriculture, Forestry and Fisheries in Japan (http://www.maff.go.jp/aqs/tokei/toukeinen.html), more than 100 heads of breeding swine are imported from the United States and Canada every year to Japan (http://www.maff.go.jp/aqs/tokei/toukeinen.html). In addition, during 2005, a total of 75 swine were imported from the Netherlands to Japan.

Some surveys on MRSA of swine origin have been conducted in Asian countries, for example in China, Malaysia and Singapore (Wagenaar et al., 2009, Neela et al., 2009, Sergio et al., 2007). Interestingly, MRSA CC 9 is predominantly isolated from swine in China and

Malaysia (Wagenaar et al., 2009, Neela et al, 2009). In Singapore, MRSA ST398 was isolated from swine (Sergio et al., 2007). In Japan, although MRSA (MLST analysis was not done) was isolated from retail ground pork in 2005 (Fujio et al., 2007), MRSA has not been isolated from swine. The objective of this study was to determine the prevalence of MRSA in swine in Japan.

2.2 Materials and methods

2.2.1 Sampling

A total of 115 nasal swab samples and 115 faecal samples from swine reared on 23 swine farms in seven prefectures of Eastern Japan were collected at a slaughterhouse between March and September 2009. Nasal and faecal samples were collected from five animals per farm. Nasal swabs were sampled from the nasal cavity using a sterile cotton swab. Faecal samples were collected using a medicine spoon and were placed in a sterilised sampling bag. One gram of each faecal sample was used for MRSA isolation.

2.2.2 Isolation of MRSA

Samples were inoculated into 9 mL of heart infusion broth (Difco Laboratories, Detroit, MI) containing 7.5% NaCl and were incubated for 18 h at 35°C. Following incubation, the enrichment culture was inoculated onto two commercial isolation agars, namely

CHROMagar™ MRSA Medium (Kanto Chemical Co. Inc., Tokyo, Japan) and MRSA Selective Agar (Becton Dickinson & Co., Franklin Lakes, NJ). Suspected colonies of staphylococci were tested with oxidase (Nissui Co. Ltd., Tokyo, Japan), catalase and gram stain. Subsequently, a tube coagulase test (Eiken Chemical Co. Ltd., Tochigi, Japan) and an N-ID test (Nissui Co. Ltd.) were conducted on isolates to identify *Staphylococcus* spp.

2.2.3 Molecular characterization of MRSA

SCC*mec* typing was performed by amplification of the *mec* regions (classes A, B and C) and the *ccr* regions (types 1, 2 and 3) by multiplex PCR using the primers described by Kondo et al., 2007. The genes of *mecI*, *ccrB2* and *ccrC* were additionally examined by PCR using the primers described by Milheirico et al. (2007). The ST of the isolate was determined from the MLST website (http://saureus.mlst.net/) and the *spa* type was identified using the Ridom database website (http://spaserver2.ridom.de/ index.shtml).

2.2.4 Antimicrobial susceptibility

MICs of antimicrobials were determined using a broth dilution method following the guidelines of the CLSI (CLSI 2008a, CLSI 2008b). *S.aureus* ATCC 29213 and *E faecalis* ATCC 29212 served as quality control isolates. Susceptibility testing was conducted against ampicillin

(ABPC), MPIPC, CEZ, ceftiofur (CTF), imipenem (IPM), DSM, GM, KM, chloramphenicol (CP), OTC, EM, azithromycin (AZM), tylosin (TS), vancomycin (VCM), ERFX and trimethoprim (TMP).

2.3 Results

MRSA was isolated from only 1 of 115 nasal samples (0.9%, 95% confidential interval 0.0–4.8%) (Table 5). MRSA isolates harboured the type 1 *ccr* complex. The sequence of the PCR product showed 87% homology with type 1 *ccr* complex of *S. aureus* PL72 strain (accession no. AB433542). However, no amplicon was detected by PCR for the *mec* complex or the *mecI*, *ccrB2* and *ccrC* genes.

The MRSA isolate was classified as ST221 (allelic profile 1-4-1-4-12-1-10) belonging to CC5, according to the MLST database. The *spa* type of the isolate was t002. The isolate was resistant to ABPC, MPIPC and DSM, but remained susceptible to the other 13 antimicrobials (Table 6).

2.4 Discussion

This study showed MRSA ST221 to be present in the nasal cavity of slaughtered swine in Japan. Since 2005, the high prevalence of MRSA ST398 in the nasal cavity of swine has been

reported in some parts of Europe and North America, with an isolation rate between 24.9% and 49% (de Neeling et al., 2007, Lewis et al., 2008, Khanna et al., 2008, Smith et al., 2009). Although numerous swine have been imported from these countries to Japan since 2005, ST398 does not as yet seem to have appeared in Japan. It should be noted that animals are not quarantined for carriage of MRSA when imported because it is not a targeted infectious disease according to the Domestic Animal Infectious Disease Control Law (http://www.cas.go.jp/jp/seisaku/hourei/data/adaidc.pdf). Although this study utilised an enrichment culture method using broth medium containing 7.5% NaCl, it seems likely that the isolation rate of MRSA from swine in Japan (0.9%) was lower than those in Europe and North America.

The MRSA isolate belonging to CC5 and t002 was related to the New York/Japan MRSA clone with SCC*mec* II. However, the SCC*mec* type of the MRSA ST221 in this study was untypeable since the class of *mec* complex was not determined. Moreover, DNA homology of *ccr* type 1 of this MRSA isolate with previously reported isolates was low. Zaraket et al. (2007) reported that the New York/Japan MRSA clone was predominant in Japan. Previously, a strain of MRSA ST221 with SCC*mec* II (*spa* type not determined) was isolated at a hospital between 2001 and 2003 in South Korea (Cho et al., 2006) and an isolate with SCC*mec* I and t149 was isolated at a hospital in 2005 in Paraguay (Mayor et al., 2007). The MLST database

(http://saureus.mlst.net/sql/burstspadvanced.asp) showed that *S. aureus* ST221 was isolated at French and Scottish hospitals in 2002 and 2003, respectively. To the best of our knowledge, there is no previous report detailing the isolation of MRSA ST221 from animals. In addition, the MRSA ST221 isolate in this study exhibited resistance only to β-lactam antibiotics and DSM (Table 6). The New York/Japan MRSA clone exhibited resistance not only to β-lactam antibiotics but also aminoglycosides, macrolides, tetracycline and FQ (Zaraket et al., 2007). MRSA from swine showed resistance to tetracycline antibiotics in several Western countries (de Neeling et al., 2007). MRSA isolated from ground pork in Japan in 2005, exhibited resistance to benzylpenicillin, ABPC, CEZ, MPIPC and OTC (Fujio et al., 2007). The resistance profile revealed that the isolate was considerably different from this MRSA clone. Originally, this MRSA ST221 clone might not emerge in the Japanese swine production system. Meanwhile, the origin of MRSA ST221 infection in swine remains unclear.

This study was the first survey on the prevalence of MRSA in Japanese swine. At present, the prevalence of MRSA amongst swine in Japan is low and MRSA ST398 was not isolated from swine in this study.

Table 5. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in swine

Sample	No. of farms positive/tested (%, 95% CI)[a]	No. of swine positive/tested (%, 95% CI)
Nasal swab	1/23 (4.3, 0.1-22.0)	1/115 (0.9, 0-4.8)
Faeces	0/23 (0, 0-12.2)	0/115 (0, 0-2.6)

[a]CI, confidential interval

Table 6. Minimum inhibitory concentration (μg/mL) for methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type ST221 strain in this study

Antimicrobial agent[a]	Breakpoint[b]	MRSA ST221	*S. aureus* ATCC 29213	*Enterococcus faecalis* ATCC29212
ABPC	0.5	>128	≤1	≤1
MPIPC	4	256	0.25	16
CEZ	32	8	≤1	N/D
CTF	N/D[c]	2	1	N/D
IPM	16	≤1	≤1	≤1
DSM	32	>128	8	64
GM	16	1	≤0.5	16
KM	64	8	≤4	64
CP	32	8	8	4
OTC	16	≤0.5	≤0.5	16
EM	8	0.5	0.5	2
AZM	8	2	2	8
TS	N/D	2	2	2
VCM	16	2	2	4
ERFX	N/D	0.063	0.063	0.25
TMP	16	≤1	2	≤0.5

[a]PC; penicillin G, MPIPC; oxacillin, CEZ; cefazolin, IPM; Imipenem, OTC; oxytetracycline, EM; erythromycin, AZM; Azithromycin, TS; Tylosin, DSM; dihydrostreptomycin, KM; kanamycin, CP; Chloramphenicol, GM; gentamicin, VCM; Vancomycin, ERFX; enrofloxacin, TMP; Trimethoprim

Table 6 (Continued)

[b]Breakpoints of dihydrostreptomycin and oxytetracycline were defined microbiologically using 116 isolates of *S. aureus* stored in the National Veterinary Assay Laboratory (Tokyo, Japan). Breakpoints of remaining antimicrobials were as recommended by the CLSI

[c]N/D, not defined

CHAPTER 3

Molecular Type of *Staphylococcus aureus* from Swine in Japan

3.1 Introduction

The emergence of LA-MRSA, belonging to ST 398, is recently recognized as a critical threat of human health (European Food Safety Authority, 2009, Vanderhaeghen et al., 2010). LA-MRSA ST398 isolates are found in domestic animals, mainly swine, humans contact with swine, and retail meats in Europe (European Food Safety Authority, 2009), North America (Khanna et al., 2008, Smith et al., 2009) and Asia (Sergio et al., 2007, Li et al., 2011). In Asian countries, different ST of MRSA, ST9, is identified in swine in China (Cui et al., 2009, Wagenaar et al., 2009) and Malaysia (Neela et al., 2009). Our study in Chapter 2 showed only MRSA ST221 was isolated from swine in Japan. *Spa* type in combination with ST provides additional epidemiological information on LA-MRSA isolates. In European surveillance (European Food Safety Authority, 2009), LA-MRSA ST398 isolates was belonged to four major *spa* types accounting for 90%: t011, t108, t034 and t899, although several *spa* types are found to be associated with ST398. Recent studies report prevalence of MSSA ST398 in swine (Hasman et al., 2009, Wagenaar et al., 2009). Based on the diverse *spa* types and SCC*mec* types (IVa or V) of LA-MRSA ST398, the strains may evolve from a variety of MSSA strains by

means of the horizontal transfer of *mecA* (Wielders et al., 2001). Additionally, MRCNS may play a role as a reservoir of the *mecA* gene (Li et al., 2011).

In Japan, as many swine are imported for breeding stock, the risk of invasion of LA-MRSA ST398 to swine industries is present. In addition, we cannot deny emergence of a new MRSA clone from MSSA in swine. In the present study, to evaluate the possibility of the evolution of MRSA in swine, we examined MLST and *spa* type of MSSA from swine. The SCC*mec* types were also determined in MRCNS from swine.

3.2 Materials and Methods

In total, 15 MSSA isolates from diseased swine between 2003 and 2009 were included in this study. In addition, 10 MRCNS isolates from different swine were also included: *S. warneri* (five isolates), *S. haemolyticus* (three isolates), *S. epidermidis* (one isolate) and *S. lentus* (one isolate). These MRCNS isolates used in this study were isolated from slaughter swine in the process of the MRSA isolation described in Chapter 2.

MLST was conducted for the MSSA isolates: seven housekeeping genes were amplified by PCR (Enright et al., 2000) and the sequence typing of the isolates was determined using the MLST website (www.mlst.net). The *spa* type of the isolates was identified using the

Ridom database website (http://spaserver2.ridom.de/index.shtml) after sequencing of PCR products according to the method previously published (Harmsen et al., 2003). As for MRCNS isolates, the SCC*mec* elements were typed by PCR (Kondo et al., 2007).

Susceptibilities of *S. aureus* against the following antimicrobials were examined in Chapter 1 using an agar dilution method of the CLSI (CLSI, 2008a): PC, MPIPC, CEZ, OTC, EM, DSM, KM, GM, and ERFX.

3.3 Results

Six porcine MSSA isolates belonged to ST398, six to ST9, and one each to ST5, ST97, and ST705 (Table 7). The *spa* types t034, t1298 and t3934 were identified among MSSA ST398 isolates, whereas the *spa* types t337, t526, t1430 and t6158 were identified among ST9 isolates (Table 7).

All the isolates of MSSA ST398 exhibited resistance to both PC and OTC w/wo others, whereas OTC resistance was not found in remaining isolates of STs, except for a isolates of ST9 (Table 8). Four isolates of *S. warneri* and two isolates of *S. haemolyticus* harbored SCC*mec* V. SCC*mec*IV was found in each isolate of *S. epidermidis* and *S. warneri.*

3.4 Discussion

In this study, porcine MSSA isolates were classified into five STs. Porcine MSSA isolates belonging to ST398 and ST9 were found in Denmark (Hasman et al., 2009) and China (Wagenaar et al., 2009), and were identified in swine in Japan during this study. The remaining three STs were reported in bovine isolates in Japan (Hata et al., 2010). MRSA ST9 has been reported recently to be the predominant ST in swine in China (Cui et al., 2009, Wagenaar et al., 2009) and Malaysia (Neela et al., 2009), whereas MRSA ST398 is predominant in European countries (European Food Safety Authority, 2009). These STs of MRSA may be originally adapted to swine. The information of *spa* types associated with MRSA ST398 has been limited in European surveillance (European Food Safety Authority, 2009, Hasman et al., 2009). The *spa* type t034 is the third predominant type of MRSA ST398 isolates from swine in the EU (European Food Safety Authority, 2009). The *spa* type t034 of ST398 is the most dominant among swine in Denmark (Hasman et al., 2009). Isolates of the *spa* type t1430 related to ST9 have been identified in MRSA from swine and MSSA from cattle in European countries (European Food Safety Authority, 2009, Hasman et al., 2009). Accordingly, this study showed that MSSA with ST9 and ST398, which were found in MRSA isolates of porcine origin, were prevalent among swine in Japan.

All the isolates of MSSA ST398 were resistant to both PC and OTC. Aarestrup et al. (2010) showed that tetracycline resistance was commonly found in not only MRSA ST398 isolates, but also MSSA ST398 isolates. Our study supports the suggestion by Aarestrup et al. that tetracycline resistance is a feature associated with ST 398 (Aarestrup et al., 2010).

LA-MRSA ST398 clones have been suspected to gain the *mecA* gene from MRCNS (Li et al., 2011) . The MRCNS strains harbored SCC*mec* IV and V in this study. In addition to presence of MSSA ST398 and ST9 in Japanese swine industry, MRCNS isolates were found among swine. The potential risk of emergence of a new MRSA clone is also realized in the Japanese swine production system.

Our study in Chapter 2 suggested that MRSA ST398 did not prevail among Japanese swine although Japan imported many swine for breeding stock. The additional risk for emerging MRSA in swine was described in this study. It is necessary to continue MRSA surveillance among livestock animals and retail meats because MRSA strains adapted to swine may intrude through live swine or animal products from the countries where MRSA strains are prevalent.

Table 7. Molecular type of MSSA and MRSA from swine in Japan

Species	MLST (allelic profile)	*spa* type (Repeat succession)	*mecA*	SCC*mec* type	*mec* complex	*ccr* complex	No. of isolates
S. aureus	ST398 (3-35-19-2-20-26-39)	t034 (08-16-02-25-02-25-34-24-25)	-[a]	NT[b]	NT	NT	4
		t1298 (15-16-02-16-02-25-17-24)	-	NT	NT	NT	1
		t3934 (07-02-25-34-24-25)	-	NT	NT	NT	1
	ST9 (3-3-1-1-1-1-10)	t337 (07-16-23-23-02-12-23-02-34)	-	NT	NT	NT	3
		t526 (07-16-23-23-02-12-23-34)	-	NT	NT	NT	1
		t1430 (07-16-23-02-12-23-02-34)	-	NT	NT	NT	1
		t6158 (07-16-23-02-12-23-02-02-34)	-	NT	NT	NT	1
	ST5 (1-4-1-4-12-1-10)	t179 (26-23-17-34-17-20-17-12-12-16)	-	NT	NT	NT	1
	ST97 (3-1-1-1-1-5-3)	t2112 (26-23-12-21-17-34-34-34-34-33-34)	-	NT	NT	NT	1
	ST705 (6-72-50-43-49-67-59)	t529 (04-34)	-	NT	NT	NT	1
S. warneri	NT	NT	+[a]	V	C	5	4
			+	IV	B	2	1
S. haemolyticus	NT	NT	+	V	C	5	2
			+	ND[c]	C	NA[d]	1
S. epidermidis	NT	NT	+	IV	B	2	1
S. lentus	NT	NT	+	ND	A	NA	1

[a] -: negative, +: positive, [b] NT: not tested, [c]ND: not determined, [d]NA: not amplified

Table 8. Antimicrobial resistance profile of MSSA by MLST type

MLST type	Antimicrobial resistance profile[a]	No. of isolates
ST398	PC-OTC-KM-GM	1
	PC-OTC-DSM	2
	PC-OTC-EM	1
	PC-OTC-ERFX	1
	PC-OTC	1
ST9	PC-OTC	1
	PC-EM	2
	PC	3
ST5	PC	1
ST97	PC-ERFX	1
ST705	Susceptible	1

[a]PC: penicillin G, OTC: oxytetracycline, EM: erythromycin, DSM: dihydrostreptomycin, KM: kanamycin, GM: gentamicin, ERFX: enrofloxacin

CONCLUSIONS

In livestock production, although antimicrobial therapy is essential for the treatment of the diseases in affected animals, use of antimicrobial agents can select antimicrobial-resistant bacteria. In Japan, the amount of antimicrobial utilization is the largest in swine farming among Japanese livestock production. Use of antimicrobial agents in swine production, possibly, also facilitates emergence of novel antimicrobial-resistant pathogens including MRSA. LA-MRSA ST398 was isolated from a four-year-old girl in a family working in a swine farm in the Netherlands in 2004. Each MRSA clone started spreading to the world around 2005, and it has been transmitted to humans; it became one of the major problems on public health. The purpose of the dissertation was to elucidate trends of antimicrobial resistance, conduct a survey on prevalence of LA-MRSA, and evaluate possibility of emergence of LA-MRSA among swine in Japan.

In Chapter 1, the author discussed and concluded an overview of antimicrobial resistance in *S. aureus* isolated from swine. This study clarified that porcine isolates of *S. aureus* exhibited resistance to the major antimicrobial agents using in the Japanese swine industry. High resistance rates and phenotype of multi-drug resistance in porcine isolates can be a problem on public health. Intensive large-scale farming is applied to reduce the production costs in Japan. However, it enables the diseases to spread more rapidly, causing significant

economic losses due to increased morbidity, mortality, and veterinary medicine. The amount of antimicrobial utilization is the largest in Japanese swine farming. Use of antimicrobial agents selects antimicrobial-resistant bacteria, and resistance rates are correlated with the amount of their antimicrobial utilization. The antimicrobial use in swine industry may be the key factor for the high prevalence of resistance in *S. aureus* in swine. Prudent use of antimicrobials is the best way to resolve the problem. In addition to the results of Chapter 1, any methicillin resistant strain was not detected from diseased swine between 2003 and 2009.

In Chapter 2, the study elucidated the prevalence of LA-MRSA in swine industries in Japan. The isolation rate of MRSA from swine in 2009 was 0.9% (1/115, 95% confidential interval 0.0-4.8%). It was lower than those in Europe and North America. One MRSA isolate was classified in ST211, which belonged to CC5, with *spa* type t002. It was not one of the LA-MRSA; the genotype and antimicrobial resistance profile suggested that the MRSA ST221 strain might have not originally emerged in swine industry. Meanwhile, Japanese swine farms still importing swine from western countries mainly from North America, and people intercommunicate from there to Japan where LA-MRSA is prevalent. Therefore, continuous survey on the prevalence of LA-MRSA is needed at the national level in the Japanese swine industry.

In the last Chapter, the study clearly identified high possibility for emergence of

LA-MRSA in Japan. In Japan, 40% of the MSSA isolates from swine (6/15) were classified as ST398 with *spa* type of t034. MRSA ST398 t034 was predominant among livestock-associated MRSA in Europe and North America. SCC *mec* types of the MRCNS strains from swine were IV or V. As a result, considering horizontal transfer of the *mecA* gene between staphylococci, a new LA-MRSA clone can emerge in an animal production system because of the presence both MSSA ST398 and MRCNS with SCC*mec* IV and V. If LA-MRSA emerges among swine once, it may rapidly spread to swine industries and human communities. Therefore, a continuous epidemiological study on staphylococci is needed.

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