Studies on molecular epidemiology of methicillin-resistant *Staphylococcus aureus* originated from livestock animals, meat products, and humans

(家畜、食肉、およびヒト由来 MRSA の分子疫学に関する研究)

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ABBREVIATIONS

Antimicrobials:

AMP: ampicillin

OXA: oxacillin

KAN: kanamycin

GEN: gentamicin

ERY: erythromycin

CLI: clindamycin

VAN: vancomycin

CIP: ciprofloxacin

TET: tetracycline

MRSA: methicillin-resistant *Staphylococcus aureus*

HA-MRSA: healthcare-associated methicillin-resistant *Staphylococcus aureus*

CA-MRSA: community-associated methicillin-resistant *Staphylococcus aureus*

LA-MRSA: livestock-associated methicillin-resistant *Staphylococcus aureus*

MSSA: methicillin-susceptible *Staphylococcus aureus*

MRCNS: methicillin-resistant coagulase negative staphylococci

SCC*mec*: staphylococcal cassette chromosome *mec*

MLST: multilocus sequence type

CC: clonal complex

CLSI: Clinical and Laboratory Standards Institute

MIC: minimum inhibitory concentration

MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of –fright mass spectrometry

POT: phage open reading frame typing

PFGE: pulsed field gel electrophoresis

CONTENTS

PREFACE

Antimicrobial resistance is a looming public health crisis. While once believed to be the province of hospitals and other health-care facilities, a host of community factors are now known to promote antibiotic resistance, and community-associated resistant strains have now been implicated as the cause of many hospital-acquired infections [10, 66]. An inherent consequence of exposure to antibiotic compounds, antimicrobial resistance arises as a result of natural selection [2]. Due to normal genetic variation in bacterial populations, individual organisms may carry mutations that render antibiotics ineffective, conveying a survival advantage to the mutated strain. In the presence of antibiotics, advantageous mutations can also be transferred via plasmid exchange within the bacterial colony, resulting in proliferation of the resistance trait [14]. The emergence of antimicrobial resistance has been observed following the introduction of each new class of antibiotics, and the threat is compounded by a slow antimicrobial development pipeline and limited investment in the discovery and development of new antibiotic agents [60, 73, 76].

International, national, and local antibiotic stewardship campaigns have been developed to encourage prudent use of and limit unnecessary exposure to antibiotics, with the ultimate goal of preserving their effectiveness for serious and life-threatening infections [8] There is also considerable debate in veterinary medicine regarding use of antibiotics in animals raised for human consumption (livestock animals). The potential threat to human health resulting from inappropriate antibiotic use in livestock animals is significant, as pathogenic-resistant organisms propagated in these livestock are poised to enter the food supply and could be widely disseminated in food products [17, 28, 31, 42, 65]. Commensal

bacteria found in livestock are frequently present in fresh meat products and may serve as reservoirs for resistant genes that could potentially be transferred to pathogenic organisms in humans [20, 55]. I need coherent international action that spans antimiciobial regulation and antimicrobial use across humans and animals.

Until the 1990s, methicillin-resistant *Staphylococcus aureus* (MRSA) was traditionally considered a pathogen causing nosocomial infections, being the so-called HA-MRSA (healthcare-associated methicillin-resistant *Staphylococcus aureus*). However, over time, cases of MRSA-positive individuals were observed who never had contact with hospital services, and strains from these individuals were identified and named CA-MRSA (community-associated methicillin-resistant *Staphylococcus aureus*) [87]. In 2003 in the Netherlands, a new MRSA strain arose in patients that could not be typed through PFGE (pulsed field gel electrophoresis) with *Sma*I, with resistance to digestion by this enzyme [9], being called since then NT-MRSA (non typeable methicillin-resistant *Staphylococcus aureus*). Investigations of this NT-MRSA intensified, and it was observed that these patients carrying this strain had previous contact with pigs and the geographic distribution of cases showed clusters near pig farms [16]. With more advanced studies, it was possible to determine strains strictly related to animals, such as those found in pigs, which were named LA-MRSA (livestock-associated methicillin-resistant *Staphylococcus aureus*) in 2010 [80].

MRSA evolved from methicillin-susceptible *S. aureus* (MSSA) by acquisition of staphylococcal cassette chromosome *mec* (SCC*mec*) elements containing a *mec* gene (*mec*A, more rarely *mec*C), which codes for an additional penicillin binding protein that has low affinity for β-lactam antibiotics and therefore mediates resistance to nearly all compounds from this antibiotic class (besides ceftobiprole and ceftarolin) [63]. Methicillin-resistant coagulase negative staphylococci (MRCNS) are also commonly found in the nose of MRSA-

positive patient, and there is indirect evidence that MRSA clones arose from genetic transfer of SCC*mec* from MRCNS to MSSA [86]. Therefore, it is thought that the expansion of MRCNS in humans and animals is the threat for public health like MRSA.

LA-MRSA belonging to multilocus sequencing type (MLST) 398 (ST398) and related strains collectively grouped into clonal complex 398 (CC398) have been frequently found in pigs, chickens, veal calves, dairy cattle, horses, dogs, and milk in various countries. MSSA and MRSA have been associated with companion and livestock animals [4, 30, 52, 58, 85]. An examination of LA-MRSA in human case isolates in the Netherlands indicated an increase from 0% in 2002 to greater than 21% in mid-2006 [78] and 35% in 2009 [23]. In most European countries, CC398 remains the most commonly identified type of LA-MRSA [72]. However, the epidemiology of LA-MRSA differs in other geographic areas. A different strain of LA-MRSA, CC97 is also found associated with pig and cow carriage [26]. CC9 appears to be the prominent type in several Asian countries [48, 81]. Poultry may harbor CC398 strains [3] but CC5 [3] and other types unrelated to CC398 have also been reported [49]. Besides the importance of living animals as a source of MRSA, animal origin products also play a role in disseminating these strains to the humans. Previous studies reported that 3-32% of meat products were contaminated by MRSA [18], and characteristics of these isolates suggest that they can be of both animal and human origin, and although the presence of MRSA in food is low, it must be monitored, because it can contribute to its dissemination.

In this thesis, I aimed to detect MRSA among livestock animals in Japan and characteristics of these isolates, and to verify the potential role of meat in MRSA transmission. First, I derived MRSA from pigs at a slaughterhouse in Ibaraki, one of the top pig-producing prefectures in eastern Japan according to the Ministry of Agriculture, Forestry and Fisheries (MAFF) survey (http://www.maff.go.jp) (Chapter 1). Next, to clarify the actual

state about MRSA and the risk that MRSA emerge among livestock animals in Japan, I attempted to isolate MRSA, MRCNS, and MSSA from pigs and cows at a slaughterhouse in Hokkaido (Chapter 2). Finally, I compare the characteristics of MRSA isolates from pigs, cows, meat, and humans to elucidate the relationship among these origins and to assess transmission from animals to human in Japan (Chapter 3).

CHAPTER 1

Isolation of methicillin-resistant *Staphylococcus aureus* among pigs in Ibaraki, Japan

1.1.Introduction

MRSA is an important nosocomial pathogen in humans. Indeed, MRSA can cause purulent skin and soft-tissue infections (SSTIs) in healthy individuals. In the last decade, MRSA has also been detected in livestock animals, termed LA-MRSA. Most of the isolates from European livestock have been typed using MLST and are designated as ST398 [16]. In contrast to European countries, in Asia MRSA ST9 has been reported as the prevalent clonal type in swine [16]. LA-MRSA is the primary cause of community-acquired SSTIs in people exposed to livestock animals [12]. MRSA is an important concern in the hospital setting and in the community as well as for consumers of animal food products worldwide.

In Japan, there has only been a single report of the isolation of MRSA ST221 from a pig [7]. Since there have been few studies of MRSA in livestock animals in Japan, the present distribution of MRSA in these animals is unclear. The aim of this study was to determine the frequency and clonal types of MRSA among slaughter pigs in a top pig-producing area in Japan.

1.2.Materials and methods

From February to March 2013, nasal swabs were collected from 100 apparently healthy pigs from 21 different farms (3–5 pigs per farm) at a slaughterhouse in Ibaraki. This slaughterhouse processes a maximum of 1600 pigs and 100 cows per day. Immediately after sampling, nasal swabs were incubated in 3 mL of tryptic soy broth (TSB) (Becton Dickinson Japan, Tokyo, Japan) containing 6.5% NaCl at 37 °C for 24 h. A loopful of TSB was then plated on CHROMagar™ MRSA (Kanto Chemical, Tokyo, Japan) and was incubated at 35 °C for 24 h. Suspected MRSA colonies were transferred to trypticase soy agar (TSA) and were tested with catalase and Gram strain. Isolates were identified using API® Staph ID 32 (SYSMEX bioMérieux Co. Ltd., Tokyo, Japan), and PCR was used to amplify the MSSA-specific gene (*femA*) and methicillin resistance gene (*mecA*) [1]. Both *femA*- and *mecA*-positive isolates were identified as MRSA (one per sample) and were analysed in further experiments.

SCC*mec* typing [46], PCR for the Panton–Valentine leukocidin gene (*pvl*) [1], MLST [24] and *spa* typing [70] were performed for all MRSA isolates according to previously published methods. The sequence type and *spa* types were determined using the MLST website (http://www.mlst.net) and Ridom database website (http://*spa*.ridom.de/spatypes.shtml), respectively.

Antimicrobial susceptibility was determined by the agar dilution method following Clinical and Laboratory Standards Institute (CLSI) recommendations [82] for the following antibiotics: ampicillin (Sigma–Aldrich, St Louis, MO, USA), oxacillin, gentamicin, kanamycin, erythromycin, clindamycin, vancomycin, ciprofloxacin and tetracycline (Wako Pure Chemical Industries, Osaka, Japan). *S. aureus* ATCC 29213 and *Enterococcus faecalis*

ATCC 29212 served as quality control strains. The presence of *erm*(A), *lnu*(A) and *lnu*(B) was analysed by PCR as previously described [50, 74] and the amplification products were directly sequenced. Sequence data were analysed by NCBI BLASTn search (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

1.3.Results

Eight MRSA isolates (8%) were obtained from 100 nasal swabs of slaughter pigs; the percentage of MRSA-positive pig farms was 14% (3/21) (Table 1). Five isolates from the same farm belonged to ST97, identified as *spa* type t1236, and were typed as SCC*mec* V (*mec* class C and *ccr* type 5). Three isolates from two pig farms belonged to ST5 and were identified as *spa* type t002. However, the SCC*mec* type of these three isolates was not determined because although they harboured type *mec* class A, the *ccr* complex for *cc*r type 1, 2, 3, 4 and 5 was not amplified by PCR. No *pvl*-positive MRSA isolates were detected.

The MICs of MRSA isolates were then analyzed CLSI guidelines to categorize them as either susceptible, intermediate or resistant [82]. All MRSA isolates were resistant to tetracycline in addition to ampicillin and oxacillin. Seven isolates (88%) were resistant to clindamycin. The ST97 MRSA lineage was intermediate-resistant to ciprofloxacin and two of the five isolates were intermediate-resistant to gentamicin. The ST5 MRSA lineage was susceptible to ciprofloxacin and intermediate-resistant to kanamycin. All isolates were susceptible to vancomycin. The five ST97/*spa* t1236/SCC*mec* V MRSA isolates showed an unusual antimicrobial susceptibility profile (clindamycin-resistant/erythromycin-susceptible) and harboured the *lnu*(B) gene. No other *erm*(A)- and *lnu*(A)-positive isolates were detected.

Table 1. Characteristics of methicillin-resistant *Staphylococcus aureus* isolated from pigs

Strain No.	Farm	Molecular type			MIC (μg/mL)[a]								
		MLST	*spa* type	SCC*mec*	AMP (0.5)	OXA (0.5)	KAN (64)	GEN (16)	ERY (8)	CLI (4)	VAN (32)	CIP (4)	TET (16)
MR23	A	ST97	t1236	V	32	>32	2	8	≦0.125	8	1	2	>32
MR24	A	ST97	t1236	V	16	32	2	0.25	≦0.125	8	1	2	>32
MR25	A	ST97	t1236	V	16	32	2	0.25	≦0.125	8	1	2	>32
MR26	A	ST97	t1236	V	16	32	2	8	≦0.125	8	1	2	>32
MR27	A	ST97	t1236	V	16	32	2	0.25	≦0.125	8	1	2	>32
MR30	B	ST5	t002	Atypical	32	>32	32	0.5	32	>8	2	0.5	>32
MR31	B	ST5	t002	Atypical	16	32	32	0.5	32	>8	1	0.5	>32
MR33	C	ST5	t002	Atypical	32	32	32	0.5	0.25	0.5	1	0.5	>32

Abbreviations: MLST; multilocus sequence typing, SCC*mec*; staphylococcal cassette chromosome mec, MIC; minimum inhibitory concentration, AMP; ampicillin, OXA; oxacillin, KAN; kanamycin, GEN; gentamicin, ERY; erythromycin, CLI; clindamycin, VAN; vancomycin, CIP; ciprofloxacin, TET; tetracycline.

[a]Parenthesis is the break point recommended by the Clinical and Laboratory Standards Institute [82]. Black full, resistant; dark fill, intermediate resistant; white fill, susceptible.

1.4.Discussion

The observed prevalence of MRSA in pigs (8%) and pig farms (14%) was lower than the reported frequency among pigs in European countries (11–46%) and other Asian countries such as China (11.4%) [16]. Baba et al. reported a frequency of 0.9% MRSA among strains isolated from pigs on farms in East Japan [7]. The current study of pigs at a slaughterhouse in Ibaraki, which is a part of East Japan, showed a relatively higher frequency. Furthermore, the MRSA-positive farms exhibited a specific type of MRSA (ST97/*spa* t1236/SCC*mec* V or ST5/*spa* t002/atypical SCCmec). Indeed, the antimicrobial sensitivity pattern of the MRSA isolates from each farm was also the same. This fact indicates clonal spread within the pig population, similar to that reported in other parts of the world [16].

This study showed evidence for the existence of different lineage types of MRSA in pigs in Ibaraki: ST97/*spa* t1236/SCC*mec* V and ST5/*spa* t002/atypical SCC*mec*. SCC*mec* V is the type most frequently harboured by ST398 MRSA [79]. Although ST97 MRSA is a common pig-adapted clone in Europe [16] and ST97 MSSA has been isolated from bovine milk [36] and diseased pigs [5] in Japan, ST97 MRSA has never been isolated from animals in Japan. ST5/*spa* t002 is the specific genotype of the New York/Japan clone, mainly associated with nosocomial infection in human medicine [36]. ST5 of animal origin is commonly reported in poultry worldwide and in pigs in Europe. In Japan, ST5 MRSA from animals has been isolated from bovine milk, although it has never been reported to be isolated from pigs [5, 36, 64].

MRSA isolated from animals tends to exhibit unusual antimicrobial susceptibility profiles, such as clindamycin resistance/erythromycin susceptibility [50]. Erythromycin and clindamycin belong to the macrolide, lincosamide and streptogramin (MLS) family because

they share a binding site [50]. MLS resistance in *S. aureus* is largely mediated by the *erm* genes, and resistance to clindamycin confers cross-resistance to erythromycin. In this study, the five ST97 MRSA isolates showed an unusual susceptibility profile (clindamycin-resistant/erythromycin-susceptible) and all of them harboured the *lnu*(B) gene but not the *erm*(A) gene. Lnu(B) modifies a hydroxyl group of clindamycin and lincomycin at position 3 [50]. In pig veterinary practice in Japan, lincosamides are estimated to be used a great deal more than erythromycin according to the report of the MAFF (http://www.maff.go.jp/nval/iyakutou/hanbaidaka/). Use of lincosamides in pig production may play a role in the selection of lincosamide-resistant MRSA and it could be the reason for this unusual antimicrobial susceptibility profile.

1.5.Conclusion

This study revealed the existence of ST97 and ST5 MRSA among slaughter pigs and is the first report of the isolation of ST97 MRSA from an animal in Japan, although the origin of this MRSA was unclear. MRSA isolated from pigs, unlike those isolated from humans, are frequently highly resistant to clindamycin and harbour the *lnu*(B) gene, similar to ST398 LA-MRSA. The limitation of this study include small sample size and limited area, and further larger studies are required to confirm the prevalence of MRSA among livestock animals in Japan.

1.6.Summary of Chapter 1

The objective of this study was to determine the frequency and clonal types of MRSA among slaughter pigs in a top pig-producing area in Japan. In total, 100 nasal swabs were collected from slaughterhouse pigs originating from 21 different farms. MRSA isolates were analysed by SCC*mec* typing, *spa* typing and MLST and were examined for susceptibility to nine antimicrobial agents (ampicillin, oxacillin, gentamicin, kanamycin, erythromycin, clindamycin, vancomycin, ciprofloxacin and tetracycline). MRSA isolates were obtained from eight swabs (8%), representing three pig farms (14%). Five of the isolates were classified as ST97/*spa* t1236/SCC*mec* V and three were classified as ST5/*spa* t002/atypical SCC*mec* type. All of the isolates were resistant to ampicillin, oxacillin and tetracycline, and seven isolates (88%) were resistant to clindamycin. The five ST97 MRSA isolates displayed an unusual phenotype (clindamycin-resistant/erythromycin-susceptible). In conclusion, this is the first report of a ST97 MRSA isolate in Japan. The overall prevalence of MRSA is low in pigs, although it appears to be adapting among pigs in Japan owing to the new ST97 and ST5 MRSA strains.

CHAPTER 2

Distribution of methicillin-resistant *Staphylococcus aureus*, methicillin-resistant coagulase negative staphylococci, and methicillin susceptible *Staphylococcus aureus* among pigs and cows in Hokkaido, Japan

2.1. Introduction

Globally MRSA, an important pathogen in humans and animals, is responsible for considerable mortality, morbidity, and health care expenditure in both hospitals and the community [79]. Methicillin resistance is associated with the presence of the *mecA* gene, which encodes an additional penicillin-binding protein (PBP2a or PBP2'). This protein has a lower affinity for all beta-lactam antibiotics [33]. The *mecA* gene is located on a mobile genetic element called SCC*mec* [39]. SCC*mec* is considered that horizontal transfer between staphylococci, like MSSA or MSCNS, and contribute to emerge MRSA or MRCNS [32].

In chapter 1, I revealed that prevalence of MRSA among pig in Ibaraki (8%) was lower than foreign countries. However, the sample size, animal species (only pig) and investigation area were limited, and the presence of MRSA in the other area and among livestock animals except pigs are unknown. Worldwide MRCNS have been isolated from a number of animals, such as pigs, horses, cows, dogs, and cats [29, 83, 84], and MRCNS acts as a reservoir of SCC*mec*. Although the presence of MRCNS and MSSA among livestock animals in Japan are unclear. The object of this study was to investigate the population of MRSA, MRCNS, and MSSA, and characterize the isolates among pigs and cows in Hokkaido, where is prosperous animal industry.

2.2. Materials and methods

2.2.1. Sample collection and isolation of bacteria

From February 2014 to September 2015, a total of 436 nasal swabs were collected from 217 pigs and 219 cows at a slaughterhouse in Hokkaido. Nasal swabs were incubated in 3 mL TSB containing 6.5% NaCl immediately after sampling at 37°C for 48 h. A loopful of the TSB was then plated on Oxacillin Resistance Screening Agar Base (ORSAB; Oxoid Limited, Basingstoke, England) and Baird Parker agar (BP; Oxoid Limited, Basingstoke, England) for the isolation of methicillin resistant staphylococci and MSSA, respectively. Resulting blue colonies on ORSAB and black colonies with halos on BP agar (up to 3) were transferred to TSA and archived in TSB with 15% glycerol at –80°C.

2.2.2. Identification

All isolates were confirmed using Gram stain, the catalase test. Gram-positive cocci and catalase production isolates were identified by matrix-assisted laser desorption/ionization time-of –fright mass spectrometry (MALDI-TOF MS) analysis using the ethanol-formic acid extraction method. Species identification was considered valid when the matching score with reference spectra of the MALDI Biotyper system, version 3.1 database (Bruker Daltonique, Billerica, USA) was ≥2.0.

All staphylococci isolates were performed PCR for the *mecA* gene, which encodes for methicillin resistance. All *mecA*-positive CNS were regarded as MRCNS.

2.2.3. SCC*mec* typing and detection of the Panton-valentine leukocidin gene.

For MRCNS isolates, SCC*mec* typing was performed by amplification of the *mec* regions (classes A, B, and C) and the *ccr* regions (types 1, 2, 3, and 5) [46]. The carriage of lukF-PV and lukS-PV genes encoding PVL was examined by PCR as described previously [1]

2.2.4. Identification of the clonal complex (CC) of MSSA by Phage Open Reading Frame Typing (POT).

All MSSA isolates were investigation for the presence of 16 small genomic islets (SGIs) by PCR [75]. After the PCR, the 16 SGIs were scored in the order of islet numbers with a 1 (present) or 0 (absence). These scores were then converted to hexadecimal numbers with the internal bin2hex (number, places) function of Microsoft Excel and detected islet pattern (IP). The IPs were compared with already reported by Suzuki et al [75]., CC of MSSA isolates were identified.

2.3. Results

2.3.1. Prevalence of MRSA, MRCNS among pigs and cows, and SCC*mec* typing

In total, 17 MRCNS isolates from 6% of pigs (13/217) and 102 MRCNS isolates from 44% of cows (96/219) were identified. The most frequent MRCNS species were *S. fleurettii* (46%), *S. sciuri* (17%) and *S. lentus* (13%) in cows, and *S. epidermidis* (33%), *S. warneri* (28%) and *S. sciuri* (22%) in pigs. No MRSA were isolated from cows nor pigs. *S. fleurettii* (n=47) were excluded from SCC*mec* typing because of *mecA* of *S. fleurettii* has been reported

to be chromosomally encoded and only contains part of the class A *mec* gene complex; it is not associated with a SCC*mec* element. SCC*mec* type of MRCNS isolates except *S. fleurettii* (n=72) were identified on the basis of following combination *mec* gene complex (*mec* class A, B or C) and *ccr* gene complex (*ccr* type 1, 2, 3, or 5), a large proportion of MRCNS isolates were nontypable (53%, n=38), following SCC*mec* III (26%, n=19), IV (8%, n=6), II (7%, n=5), and V (6%, n=4).

Table 2. Distribution of MRCNS and SCC*mec* typing derived from pigs and cows

MRCNS isolates	**No. of isolates (%)**				**SCC*mec* type**					
	Pig		**Cow**		**II**	**III**	**IV**	**V**	**NT**	**Chromosome *mecA***
S. sciuri	4	24%	17	17%	3	10			8	
S. epidermidis	5	29%	6	6%	1		1	4	5	
S. fleurettii	0		47	46%						47
S. lentus	0		13	13%		9			4	
S. saprophyticus	3	18%	2	2%					5	
S. vitulinus	1	6%	2	2%	1				2	
S. xylosus	2	12%							2	
S. chonii	2	12%	9	9%					11	
S. haemolyticus			1	1%					1	
S. warneri			5	5%			5			
Total of isolates	17		102		5	19	6	4	38	47

NT: nontypable

2.3.2. Distribution and characteristics of MSSA among pigs and cows

MSSA were isolated from 21% of cows (45/219) and 70% of pigs (152/217). The CC for the MSSA isolates are shown in Table 3. 45 MSSA isolates from cows, 89% (n=40) were identified as IP 908C (CC97), 11% (n=5) were IP 0E12 and 0A0C that of CCs were unclear.

On the other hands, among 152 MSSA isolates from pigs, 36% (n=55) of *S. aureus* isolates from pigs were IP 9276 (CC9), 34% (n=51) were IP F90D (CC398), 13% (n=20) were IP9050 (CC5), 9% (n=14) were IP 7DD1 (CC30) and 8% (n=12) of CCs were unclear (IP 2A4C, 7FF7, FB7F and F90F). No same IP and CC *S. aureus* from cows and pigs was found.

Table 3. CC of MSSA isolates derived from pigs and cows

		No. of isolates	
CC[a]	**IP value**	**Pig (n=152)**	**Cow (n=45)**
5	9050	20 (13%)	
9	9276	55 (36%)	
30	7DD1	14 (9%)	
97	908C		40 (89%)
398	F90D	51 (34%)	
Unclassified	0E12		4 (9%)
	0A0C		1 (2%)
	2A4C	2 (1%)	
	7FF7	2 (1%)	
	FB7F	4 (3%)	
	F90F	4 (3%)	

[a]The clonal complex (CC) of all MRSA isolates were classified by phage ORF typing

2.4. Discussion

This study showed that porcine MSSA isolates majority belonged to CC9 or CC398, and bovine MSSA belonged to CC97, respectively. In general, the distribution of MSSA clones per production type was in agreement with results of previous surveys, which have shown the presence of MSSA CC1, CC9, CC30 and CC398 in pigs [34], CC97, CC133 and CC705/151 in bovines [38]. MSSA CC398 isolates also have been reported occasionally in pigs from Japan, as well as at high carriage rate (16.8%) in pigs from China [11], although the

study was targeted to diseased pigs and the distribution of MSSA in healthy animals was unclear. This study also found MRCNS were pervasive among healthy pigs and cows in Japan. It might be occurred that MSSA strains acquired SCC*mec* encoding *mecA* gene from MRCNS, and evolve MRSA. From these results, it is possible that the same type of LA-MRSA spreading all over the world may emerge on farms.

MRCNS like as derived from pigs and cows in this study has been reported in several species of animals, and a prevalence as high as around 60% [51, 77, 88, 89], and MRCNS gained great attention as being one of the leading bacterial group associated with mastitis in cows and sheep and nosocomial infections in humans [47]. In this study, the diversity of MRCNS species and the SCC*mec* elements were found in isolates from healthy pigs and cows. It is suggested that these bacterial species to cause suppurative disease in these animals render them a potential threat to humans and constitutes a potential risk from the consumption of foods of animal origin.

MRSA were not present among pigs and cows in Hokkaido despite our present study revealed that 8% of pigs in Ibaraki carried MRSA. It is reported that the prevalence of MRSA in human healthcare setting were dependent upon the various regions within countries [27, 62]. Limited other investigations have been performed to elucidate factors possibly influencing LA-MRSA prevalence in pigs. In one study, different farm management systems showed significant differences in LA-MRSA prevalence; fattening farms were higher than farrow-to-finish farms [79]. In another study, LA-MRSA prevalence seemed to differ greatly when comparing among two closed farm systems; one production system was highly MRSA positive and the other system appeared MRSA negative [71]. Part of the sows of MRSA positive system had been imported from Canada, where pigs have been found to be affected by LA-MRSA [45]. Although the authors could not give epidemiological evidence, LA-

MRSA was thus possibly brought into the positive farm via import of affected live swine or pork products [71]. At present, we didn't have information about introduction of sows from foreign countries and farm management system, and we do not know why MRSA were recovered from only pigs in Ibaraki. More studies are required to reliably assess the influence of farm management and related aspects on LA-MRSA prevalence, these studies suggest an important role for national and international pig trading in the dissemination of LA-MRSA in livestock animals.

2.5. Conclusion

The present study warned of possibility of the emergence of LA-MRSA in Japan, although the prevalence of MRSA among livestock animals is low at the present time. Further monitoring of MRSA, MRCNS, and MSSA in livestock animals is strongly required due to possible impact on public health.

2.6. Summary of Chapter 2

To investigate the prevalence of MRSA, MRCNS, and MSSA, and characterize the isolates among pigs and cows, we collected nasal swabs from 217 pigs and 219 cows at a slaughterhouse in Hokkaido. MRCNS were derived from 6% of pigs and 44% of cows, although no MRSA were isolated. Species of bacteria of MRCNS and SCC*mec* type had variety and accorded with previous reports in foreign countries. MSSA were derived from 70% of pigs and 21% from cows, and isolates were classified into CC9 and CC398, and CC97, respectively; it is same genotypes as LA-MRSA spreading around the world. In conclusion, this study reported that presence of MRCNS and CC9, CC97, and CC398 MSSA in healthy pigs and cows, and warned of possibility of the emergence of LA-MRSA in Japan. Consequently, it is required monitoring MRSA in livestock animals to controlling infection.

CHAPTER 3

Closely related methicillin-resistant *Staphylococcus aureus* isolates from retail meat, cows with mastitis, and humans in Japan

3.1. Introduction

In Japan, MRSA has been detected in various food products, including chicken meat [43, 61], duck meat [61], meat products (the details regarding the type of meat product are unclear) [57], and bovine milk [36]. Furthermore, MRSA have been detected in livestock animals, including bovine mastitis [35] and nasal swabs of pigs [7, 68]. Although some MRSA related articles were reported in several origins (animals, meats, and humans), these MRSA isolates were not compared. Therefore, it has remained unsolved the relationship among these MRSA isolated from different origins. To elucidate the relationship among animal, meat, and human isolates, and to assess transmission from animals to humans in Japan, we investigated the characteristics of MRSA from retail meat, cows with mastitis, a common animal disease caused by MSSA, nasals of pigs (MRSA isolates recovered in Chapter 1) and humans.

3.2. Materials and Methods

3.2.1. Sample collection

A total of 5,435 food samples were collected from 2008 to 2009, and eight MRSA were isolated from eight meat samples used in this study. Various types of food (e.g. fish, rice balls) were included in addition to meat in the 5,435 samples, although the number of meat samples was uncertain. Eight MRSA were isolated from retail meat which was purchased in Osaka (n = 5: two ground beef samples and one sample each of pork ribs, ground pork, and Taiwanese frozen duck loin) and Tokyo (n = 3: one sample each of pork ribs, ground beef, and chicken) from 2008 to 2009. All meat, with the exception of the Taiwanese frozen duck loin, was produced domestically. MRSA from meat was isolated using a 1:10 dilution

emulsion of the meat sample in sterile phosphate buffer saline. A total of 0.5 ml of the emulsion was added to 4.5 ml of TSB with 7.5% NaCl and incubated for 18 to 20 h at 37 °C. A loopful of enrichment broth was spread on Mannitol Salt Agar with Egg Yolk (MSEY; Eiken Chemical, Tokyo, Japan) and incubated for 48 h at 37 °C. The presumptive colonies of *S. aureus* (yellow colonies with halo) were streaked and purified onto TSA. Isolates from meat were confirmed to be *S. aureus* by using PS LATEX (Eiken Chemical). The PCR was performed to confirm of the presence of the *mecA* gene [1].

Seven MRSA were isolated from seven cows with mastitis in 2011, all bred at the same private farm in Hokkaido. We isolated bacteria from the milk, which were taken from the breast, and identified MRSA to detect the pathogen of the mastitis by request from the owner. The owner of the farm consented to use of the isolates in this study anonymously, including non-disclosure of the city of the farm. We did not perform any animal experiments or field studies in this study. This study also did not involve endangered or protected species. Therefore, the special permission in the authorities for this investigation was not necessary. Milk samples were streaked onto MRSA screening agar (cefoxitin containing Mannitol Salt Agar with Egg Yolk (MS-CFX); Nissui Pharmaceutical, Tokyo, Japan) and overnight at 37 °C. The presumptive colonies were further cultured onto TSA and repeatedly sub-cultured to get pure culture. Methicillin resistance was confirmed by testing for the presence of penicillin binding protein 2 (PBP2') (MRSA-LA; Denka-Seiken, Tokyo, Japan). Eight MRSA recovered from pigs were isolated as described in Chapter 1.

A total of 100 human MRSA isolates collected in Kitasato University Hospital from 2014 to 2016 (46 HA-MRSA isolates and 54 CA-MRSA isolates) were obtained from the Infection Control Research Center, Kitasato University, Tokyo, Japan. All HA- and CA-MRSA isolates were recovered from blood samples. Infections were classified as either HA-

or CA-MRSA according to origin of MRSA isolates and standard epidemiological definitions established by the U.S. Centers for Disease Control and Prevention [44]. MRSA isolates were classified as HA-MRSA if (i) they were isolated from a culture obtained 48 hours or more after a patient was hospitalized, (ii) the patient had a history of hospitalization, surgery, dialysis, or residence in a long-term care facility within 1 year before the MRSA culture date, (iii) the patient had an indwelling device at the time of culture, or (iv) the patient had a history of MRSA infection or colonization. All other MRSA isolates were considered CA-MRSA. We could not obtain information about the patients (age, symptoms, sex, and places of residence or infection) because of ethical constraints imposed by Kitasato University.

All MRSA isolates were confirmed to be *S. aureus* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using the Bruker MALDI Biotyper system with the ethanol-formic acid extraction method. All were subsequently confirmed as *mecA*-positive by PCR [1].

3.2.2. Molecular typing

For all MRSA isolates, SCC*mec* typing, phage open reading frame (ORF) typing and *spa* typing were performed as described in Chapter 1. For MRSA isolates identified as SCC*mec*IV, further PCR for detection of the CWASP/J gene (*spj*) was performed to identify subtype SCC*mec* IVl [41]. The Clonal complex (CC) of all MRSA isolates were classified by phage ORF typing according to the methods described in Chapter 1.

Pulsed-field gel electrophoresis (PFGE) was performed for CC8 MRSA isolates with genetic DNA fragments generated using 30 U *Sma*I (TaKaRa, Otsu, Japan) as previously described [56]. Cluster analysis was performed with the software program BioNumerics v6

(Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient and the unweighted pair group method. MLST for retail meat, cows with mastitis, and CC8 human MRSA isolates was performed as described in Chapter 1. The founder and CC of each ST were determined using the enhanced version of Based Upon Related Sequence Types (eBURST) [25].

3.2.3. Virulence gene analysis

The presence of genes encoding six staphylococcal enterotoxins, SEA to SEE, which main source of food poisoning [54], in addition to SEL, which CA-MRSA/J carries in Japan frequently [41] (SEs: *sea, seb, sec, sed, see*, and *sel*), toxic shock syndrome toxin-1 (TSST-1: *tst*) [54], which cause of TSS, exfoliative toxin A (ETA: *eta*) and B (ETB: *etb*), which are implicated in the cause of staphylococcal scalded-skin syndrome [54], PVL (*pvl*), which is associated with increased disease severity and found in a high proportion of CA-MRSA strains [1], and ACME (*acr*) which is a striking feature of USA300 and plays an important role in its growth and survival [21] was determined by PCR using previously reported primers [1, 15, 21, 54].

3.2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by the agar dilution method following Clinical and Laboratory Standards Institute (CLSI) recommendations [82] for the following antibiotics: ampicillin (AMP; Sigma-Aldrich, St. Louis, MO, USA), oxacillin (OXA; Sigma-Aldrich), kanamycin (KAN; Sigma-Aldrich), gentamicin (GEN; Sigma-Aldrich), erythromycin (ERY; Sigma-Aldrich), clindamycin (CLI; Sigma-Aldrich), vancomycin (VAN;

Sigma-Aldrich), ciprofloxacin (CIP; Sigma-Aldrich), and tetracycline (TET; Wako Pure Chemical Industries, Osaka, Japan). *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 served as quality control strains. The breakpoints of these antimicrobial agents were determined according to CLSI interpretation criteria [82] using Muller-Hinton Agar (Oxoid).

3.3. Results

3.3.1 Molecular characterization of MRSA isolates from meat, cows with mastitis, and humans

Characteristics of MRSA isolates in this study are summarized in Table 4. Among eight MRSA isolates from meat, two (one from ground pork and one from ground beef) were classified as ST8 (CC8)/t1767/SCC*mec* IVl, two (one from pork ribs and one from chicken) were ST8 (CC8)/t1767/SCC*mec* untypable (harbored *ccr* type 2, but multiplex PCR for *mec* class was not amplified), one from ground beef was ST8 (CC8)/t4133/SCC*mec* IVl, one from pork rib was ST88 (CC88)/t1028/SCC*mec* IV, one from ground beef was ST59 (CC59)/t3385/SCC*mec* V, and one from Taiwanese frozen duck loin was ST573/t3525/SCC*mec* IV (Table 4). Eight MRSA isolates from nasal swabs of pigs were classified as CC5/SCC*mec* untypable (harbored *mec* class A, but multiplex PCR for *ccr* type was not amplified) and CC97/SCC*mec* V as described in Chapter 1. All seven MRSA isolates from cows with mastitis were classified as ST8 (CC8)/t1767/SCC*mec* IVl. Among MRSA isolates from humans, all 46 HA-MRSA isolates were classified as CC5/SCC*mec* II, and were divided into *spa* type t002 (n=30), t045 (=12), and t7455 (n=4). Fifty-four CA-MRSA isolates yielded 16 different *spa* types. These 16 *spa* types belonged to 6 different CCs: 14 of CC1 (t1784: n=13, t2207: n=1); 7 of CC5 (t002: n=5, t045: n=1, and newly identified t17193:

n=1), 29 of CC8 (t008: n=2, t986: n=1, t1476: n=1, t1767: n=16, t1852: n=3, t 4133: n=1, t12760: n=1, and newly identified t17177: n=3 and t17194: n=1); one of each CC45 (t065), CC89 (t375), and CC509 (t375). The majority of CA-MRSA was CC8/t1767/SCCmec IVl (n=15), following CC1/t1784/SCC*mec* IV (n=12). SCC*mec* type of CC45 and CC89 were untypable (harbored *mec* class A, but multiplex PCR for *ccr* type was not amplified). CC of one CA-MRSA isolate, *spa* type t1767, was not able to be classified by phage ORF typing because its IP (04C6) was not reported previously. Accordingly, MLST was performed; however, ST was not able to identified because two of seven genes (*aroE* and *glpF*) could not amplify using primers described previously [24].

Table 4. Molecular characterization of MRSA isolates from meat, pigs, cow mastitis, and humans (HA-MRSA and CA-MRSA)

			Origin					Resistant isolates									Pattern of virulence genes
CC[a]	**SCC**[b] ***mec***	***spa***	**Meat (n=8)**	**Pigs (n=8)**	**Cow with mastitis (n=7)**	**CA-MRSA (n=54)**	**HA-MRSA (n=46)**	**AMP**[f]	**OXA**	**KAN**	**GEN**	**ERY**	**CLI**	**VAN**	**CIP**	**TET**	
1	IV	t1784				13		13	13	1	1	13	0	0	13	0	*sea* (n=9), *sea* + *see* (n=3), negative (n=1)
		t2207				1		1	1	0	0	1	0	0	1	0	negative
5	II	t002				4	30	34	34	32	26	34	33	0	34	27	*seb* (n=13), *sec* + *sel* + *tst* (n=11), *sea* + *sec* + *sel* + *tst* (n=2), *sel* + *tst* (n=1), *sel* (n=1), negative (n=6)
		t045				1	12	13	13	13	7	13	13	0	10	13	*sec* + *sel* + *tst* (n=10), *seb* + *sec* + *sel* + *tst* (n=2), *sea* + *sec* + *see* + *sel* + *tst* (n=1)
		t7455					4	4	4	4	3	4	4	0	4	4	*sec* + *sel* + *tst* (n=3), *seb* + *sec* + *sel* + *tst* (n=1)
	IV	t002				1		1	1	0	0	0	0	0	0	0	*tst* (n=1)
		t17193				1		1	1	0	0	1	0	0	0	0	negative
	UT[d]	t002		3				3	3	0	0	2	2	0	0	3	negative
8	IV	t008				2		2	2	2	0	2	0	0	1	0	*pvl* + *acr* (n=2)
		t986				1		1	1	1	1	1	0	0	1	0	negative
		t1476				1		1	1	0	0	0	0	0	0	0	*tst*
		t1767				1		1	1	0	0	0	0	0	0	0	*sel* + *tst*
		t1852				3		3	3	3	3	0	0	0	3	0	negative (n=3)
		t17194				1		1	1	0	0	1	0	0	1	0	negative
	IVl	t1767	2 (1 GB, 1 GP)[e]		7	15		24	24	24	19	9	0	0	0	0	*sec* + *sel* + *tst* (n=23), negative (n=1)
		t4133	1 (GB)			1		2	2	2	2	0	0	0	0	0	*sec* + *sel* + *tst* (n=1), negative (n=1)
		t17177				3		3	3	3	3	0	0	0	0	0	*sec* + *sel* + *tst* (n=3)
	V	t12760				1		1	1	1	1	1	0	0	1	0	negative (n=1)
	UT[c]	t1767	2 (1 PR, 1 C)					2	2	2	2	0	0	0	0	0	*sec* + *sel* + *tst* (n=2)
45	UT[d]	t065				1		1	1	0	0	0	0	0	0	0	negative
59	V	t3385	1 (GB)					1	1	0	0	0	0	0	0	0	*sea* + *seb* + *sel*
88	IV	t1028	1 (PR)					1	1	1	0	0	0	0	0	0	negative
89	UT[d]	t375				1		1	1	1	1	1	1	0	0	0	*etb*
97	V	t1236		5				5	5	0	0	0	5	0	0	5	negative
509	II	t375				1		1	1	1	1	1	1	0	0	0	negative
573	IV	t3525	1 (TD)					1	1	1	1	0	0	0	0	1	*sec*
Unclassfied	IV	t1767				1		1	1	0	0	0	0	0	0	0	*sed*

[a]; The Clonal complex (CC) of all MRSA isolates were classified by phage ORF typing

[b]; SCC*mec* types I to V were determined based on the *mec* complex class (*mec* classes A, B, and C) and the type of *ccr* (*ccr* 1, 2, 3, and 5)

[c]; Untypable (*ccr* type 2 + *mec*-untypable)

[d]; Untypable (*ccr*-untyable + *mec* class A)

[e]; GB: ground beef, GP: ground pork, PR: pork ribs, C: chicken, TD: Taiwanese frozen duck loin

[f]; AMP: ampicillin, OXA: oxacillin, KAN: kanamycin, GEN: gentamicin, ERY: erythromycin, CLI: clindamycin, VAN: vancomycin, CIP: ciprofloxacin, TET: tetracycline

3.3.2 Toxin genes of MRSA isolates from meat, cows with mastitis, and humans

The toxin genes detected in each MRSA isolate are summarized in Table 4. In total, 88% (7/8) from meat, 100% (7/7) from cows with mastitis, 93% (43/46) HA-MRSA isolates, and 70% (38/54) of CA-MRSA isolates carried at least one toxin gene. None of the isolates from nasal of pigs harboured toxin genes. Among the fourteen CC1/SCC*mec* IV MRSA isolates, *sea* (n = 9) was most common, followed by *sea* + *see* (n = 3). Among the 51 CC5/SCC*mec* II MRSA isolates, including 46 HA-MRSA and 5 CA-MRSA isolate, *sec* + *sel* + *tst* (n = 24) was most common, followed by *seb* (n = 13), *seb* + *sec* + *sel* + *tst* (n = 3), *sea* + *sec* + *sel* + *tst* (n = 2), *sea* + *sec* + *see* + *sel* + *tst* (n = 1), *sel* + *tst* (n = 1), and *sel* (n = 1). One CC5/SCC*mec* IV carried *tst*. Among the nine CC8/SCC*mec* IV MRSA isolates, *pvl* + *acr* (n = 2), *sel* + *tst* (n = 1), and *tst* (n = 1) were observed. Among the twenty-nine CC8/SCC*mec* IVl MRSA isolates, including three from meat, seven cows with mastitis, and nineteen from

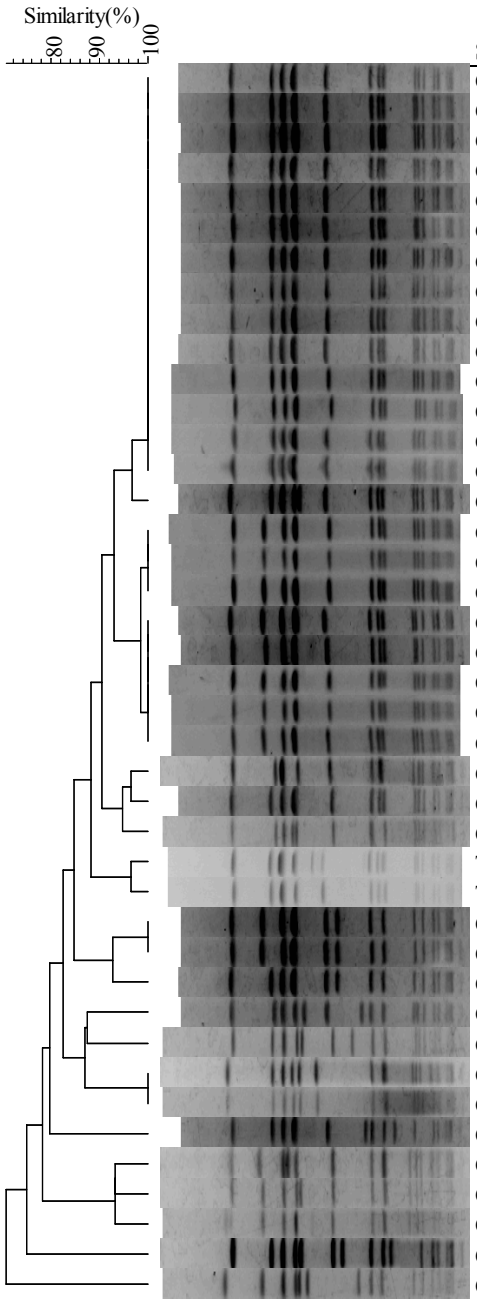
humans, 97% (27/29) carried *sec* + *sel* + *tst*. Two CC8 SCC*mec* untypable isolates from pork ribs and chicken carried *sec* + *sel* + *tst*. One CC59/SCC*mec* V (isolated from ground beef) carried *sea* + *seb* + *sel*, one CC573/SCC*mec* IV (isolated from Taiwanese frozen duck) carried *sec*, and one CC unclassified/SCC*mec* IV (belonging to CA-MRSA) carried *sed*.

3.3.3 Antimicrobial susceptibility

All MRSA isolates in this study were resistant to β-lactams (AMP and OXA); however, they were susceptible to VAN. In addition, CC8/SCC*mec* IVl was resistant to KAN (29/29: 100%) and GEN (24/29: 83%). CC5/SCC*mec* II was resistant to all tested antimicrobial agents except for VAN.

3.3.4 PFGE analysis and MLST of CC8 MRSA isolates

PFGE analysis and MLST were performed to classify MRSA isolates according to CC8 (n=41, determined by phage ORF typing), which was the common clone among retail meat (n=5), cows with mastitis (n=7), and CA-MRSA from humans (n=29) (Fig 1). CC8 isolates were classified into a total of three STs: ST8 (allelic profile 3-3-1-1-4-4-3), ST380 (3-3-61-42-4-4-3), and ST1516 (3-3-1-42-4-4-3). Three CC8/SCC*mec* IVl MRSA isolates from meat (two from beef and one from pork), one from a cow with mastitis, and ten human CA-MRSA isolates showed 100% PFGE similarity. Similarly, two human CA-MRSA isolates and three from cow with mastitis showed 100% similarity with different origin. These were ST8/SCC*mec* IVl containing three *spa* types with similar repeat profiles (t1767: 11-19-12-21-17-34-24-24-34-22-25, t4133: 11-12-21-17-34-24-24-34-22-25, and t17177: 11-19-12-21-17-34-24-24-24-24-34-22-25).



Strain	CC	ST	*spa*	SCC*mec*	Origin
CA1	8	8	t1767	IVl	Human
CA12	8	8	t1767	IVl	Human
CA15	8	8	t1767	IVl	Human
CA2	8	8	t1767	IVl	Human
CA21	8	8	t1767	IVl	Human
CA23	8	8	t1767	IVl	Human
CA34	8	8	t17177	IVl	Human
CA40	8	8	t17177	IVl	Human
CA43	8	8	t1767	IVl	Human
CA50	8	8	t17177	IVl	Human
OL6	8	8	t1767	IVl	Cow mastitis
OM3	8	8	t4133	IVl	Ground beef
OM4	8	8	t1767	IVl	Ground beef
OM5	8	8	t1767	IVl	Ground pork
CA9	8	8	t1767	IVl	Human
OL2	8	8	t1767	IVl	Cow mastitis
OL5	8	8	t1767	IVl	Cow mastitis
OL3	8	8	t1767	IVl	Cow mastitis
CA11	8	8	t1767	IVl	Human
CA17	8	8	t4133	IVl	Human
OL1	8	8	t1767	IVl	Cow mastitis
OL4	8	8	t1767	IVl	Cow mastitis
OL7	8	8	t1767	IVl	Cow mastitis
CA24	8	8	t1476	IV	Human
CA48	8	8	t1767	IVl	Human
CA25	8	8	t17194	IV	Human
TM1	8	8	t1767	UT	Chicken
TM2	8	8	t1767	UT	Pork ribs
CA14	8	8	t1767	IVl	Human
CA26	8	8	t1767	IVl	Human
CA6	8	8	t1767	IVl	Human
CA45	8	8	t1767	IVl	Human
CA49	8	8	t1767	IV	Human
CA4	8	8	t008	IV	Human
CA46	8	8	t008	IV	Human
CA39	8	8	t1767	IVl	Human
CA13	8	8	t986	IV	Human
CA16	8	380	t1852	IV	Human
CA31	8	380	t1852	IV	Human
CA19	8	1516	t1852	IV	Human
CA32	8	8	t12760	V	Human

Fig 1. Genetic relationships among CC8 MRSA isolates.

UPGMA dendrogram showing genetic relatedness among representative CC8 MRSA isolates as determined by PFGE with *Sma*I.

UT; *ccr* type 2 + *mec*-untypable

3.4. Discussion

This study showed that three MRSA isolates from retail meat, one MRSA from a cow with mastitis, and ten CA-MRSA isolates were closely related according to *spa* type, and identical according to PFGE pattern, ST, and SCC*mec*, they all had the characteristics of ST8/SCC*mec* IVl. Our study is the first to detect the closel molecular epidemiological relationship of MRSA among retail meat, cows with mastitis, and CA-MRSA from humans in Japan.

ST8 MRSA isolated in this study showed a similar genotype and antimicrobial susceptibility pattern to ST8 CA-MRSA/J in Japan, which has some different characteristics from foreign countries. In Japan, a ST8 CA-MRSA/J strain was described; ST8 CA-MRSA/J which can be characterized as carrying SCC*mec* IVl, *spa* type t1767, negative for PVL and ACME, positive for *sec, sel,* and *tst,* and resistant to gentamicin [41]. It is reported that 37.5% (18/48) of CA-MRSA from human were typed as ST8 CA-MRSA/J in Japan [41]. Gentamicin is used in an outpatient for the treatment of skin infections in Japan [40]. Therefore, antibiotic therapy based on antimicrobial susceptibility test is needed for skin infections caused by MRSA. ST8/SCC*mec* IVa, positive for PVL and ACME MRSA (USA300), is a predominant CA-MRSA genotype in the US and worldwide [19]. USA300 clones show resistance to many non-β-lactams (macrolides, fluoroquinolones, and tetracycline) in addition to β-lactams [19]. ST8 MRSA isolates used in this study showed the same genotype and antimicrobial profile, aminoglycosides resistance, as those of CA-MRSA/J. Although the geographical area where human MRSA was derived, as well as the sample size of meat and animals were all limited, this study revealed that ST8 CA-MRSA/J spreads not only to the human community setting, but also among meat and living livestock (cow), but not yet to the healthcare setting.

Four STs (ST8, ST59, ST88, and ST573) were identified in isolates from retail meat in this study. All of these STs are human-associated types: ST8 in the United States and worldwide [19], ST59 in Taiwan [19], ST88 in Africa and Asia [53], ST573 which is a rare clone found previously in Taiwan [33] and Australia [34]. Three of four STs (ST8, ST59, and ST88) were found primarily in human CA-MRSA in Japan [87]. MRSA isolates from retail chicken and duck meat in Japan were ST8/SCC*mec* IV, and regarded as CA-MRSA [61]. These observations suggest a relationship of MRSA between retail meats and humans. However, these four STs have not been reported in Japanese livestock animals, and the prevalence of MRSA is low (0.9%–8% [7, 68]) in Japanese livestock. Considering these reports, there is a strong possibility that MRSA isolates from meat in this study are contaminated from humans, although we cannot draw any definitive conclusions regarding the source of contaminated retail meat with MRSA.

Among STs from retail meat, ST573 MRSA from Taiwanese duck loin was first isolated in Japan. ST573 is a rare clone, found previously in Taiwanese children (SCC*mec* V, 0.3% (1/294)) [13], healthcare settings (SCC*mec* IV, 2.4% (5/206)) in Taiwan [69], and in community settings (SCC*mec* V, 0.05% (2/4,099)) in Australia [59]. There are several reports of pathogens associated with the import of food; an oxacillin-susceptible *mecA*-positive *S. aureus* (OS-MRSA), has never been isolated in Europe, was isolated from imported cheese. It might indicate that OS-MRSA may enter the EU via the import of food [67]. Although the source of ST573/SCC*mec* IV MRSA from Taiwanese frozen duck in this study is unclear, it might have been brought to Japan via imported meat from Taiwan, the only region where ST573 carrying SCC*mec* IV MRSA has been detected [69].

Since *S. aureus* can produce enterotoxins, it also poses a threat to humans who ingest food contaminated with these toxins [37]. Staphylococcal food poisoning, characterized by

vomiting and diarrhea, is a leading cause of food-borne illness in Japan [6]. Food sources of *S. aureus* have expanded to include livestock animal products and low-fat milk [6]. Toxic shock syndrome (TSS), which can be life-threatening, is defined by clinical and laboratory evidence of fever, rash, desquamation, hypotension, and multiple organ failure caused not only by toxic shock syndrome toxin-1 (TSST-1), but also by enterotoxins [22]. In this study, 88% (7/8) of MRSA isolates from retail meat were positive for enterotoxin genes and *tst*, higher than previous study (28.6% [61]). In Japan, the contamination rate of MRSA in meat is low (0.45% to 1.5% [43, 61]), but MRSA isolates from retail meat frequently carry virulence genes, and the spread of MRSA can cause human disease via the handling of contaminated retail meat.

3.5. Conclusion

This study showed that ST8 CA-MRSA/J is detected in the community setting, including retail meat and cows, and suggested that there is the transmission route of ST8 CA-MRSA/J among these sources. However, the direction of transfer of MRSA could not be established, and the results might not be reflective of Japan overall because the number of MRSA isolates from meat and animals was very low. Additional studies are needed to determine the origin of MRSA from retail meat, confirm the distribution of ST8 CA-MRSA/J in living animals, and assess the risk of the spread of MRSA to consumers and others who handle meat.

3.6. Summary of Chapter 3

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pervasive healthcare-acquired (HA) pathogen with recent emergence as a community-acquired (CA) pathogen. To elucidate whether meat mediates MRSA transmission between animals and humans in Japan, this study examined MRSA isolates from retail meat (n = 8), cows with mastitis (n = 7), and humans (HA-MRSA = 50 and CA-MRSA = 50) by molecular typing, virulence gene analyses, and antimicrobial susceptibility testing. MRSA isolates from retail meat were classified into sequence type (ST) 8/*spa* type t1767 (n=4), ST8/t4133 (n=1), ST59/t3385 (n=1), ST88/t375 (n=1), and ST509/t375 (n=1). All seven MRSA isolates from cow with mastitis were ST8/t1767. 50 HA-MRSA were clonal complex (CC) 5, divided into t002 (n=33), t045 (n=13), and t7455 (n=4). 50 CA-MRSA were classified into 6 different CCs: CC1 (n = 14), CC5 (n = 3), CC8 (n = 29), CC45 (n = 1), CC89 (n = 1), CC509 (n = 1), and into 15 different *spa* types including newly identified t17177, t17193, and t17194. The majority were CC8/t1767 (n=16). CC of one CA-MRSA isolate (*spa* type t1767) was not classified. Among 41 CC8 MRSA (five from meat, seven from cow with mastitis, and 29 CA-MRSA), 14 ST8/SCC*mec* IVl isolates (three from meat, one from a cow with mastitis, and 10 CA-MRSA) had identical pulsed-field gel electrophoresis patterns and similar *spa* type (t1767, t4133, and t17177), and were typed as CA-MRSA/J (ST8/SCC*mec* IVl, positive for *sec* + *sel* + *tst* but negative for Panton–Valentine leukocidin and the arginine catabolic mobile element). These results suggest that there is transmission cycle of CA-MRSA/J among meat, cows, and humans in Japan, although it is unclear whether the origin is cow.

CONCLUSION

First, I derived MRSA from pigs at a slaughterhouse in Ibaraki, as described in Chapter 1. Eight of 100 pigs (8%) carried MRSA, and molecular epidemiology analysis revealed that two MRSA strains presented in Ibaraki: ST97/*spa* t1236/SCC*mec* V and ST5/*spa* t002/atypical SCC*mec*, and the antimicrobial susceptibility patterns and carrying antimicrobial resistance genes were close to LA-MRSA. Although it appears to be adapting among pigs in Japan owing to the new ST97 and ST5 MRSA strains, the overall prevalence of MRSA is low in pigs (8%).

Next, I attempted to isolate MRSA, MRCNS, and MSSA from pigs and cows to clarify the actual state about MRSA and the risk that MRSA emerge among livestock animals in Japan, as described in Chapter 2. I derived 6 - 44% of MRCNS from pigs and cows, although nor MRSA. MSSA were isolated from 70% of pigs and 21% of cows, and phage open reading flame typing revealed that the majority CC of MSSA were CC9, CC398 in pigs and CC97 in cows, same genotype as LA-MRSA. This study provided the population genetic structure of MRCNS and MSSA in livestock animals in Japan, which reflect the natural habitation of bacterial clones in the host species, and warned of possibility of the emergence of LA-MRSA.

Finally, I compare the characteristics of MRSA isolates from pigs, cows, meat, and humans to estimate the transmission route, as described in Chapter 3. Molecular characteristics revealed that closely ST8 CA-MRSA/J is detected in the community setting, including retail meat and cows, and suggested that there is the transmission cycle of ST8 CA-MRSA/J among these sources, although it is unclear whether the origin is cow.

In conclusion, I successfully provided new findings about the epidemiological information of MRSA, MRCNS and MSSA in Japan, and elucidated that related MRSA isolates from cows, meat, and humans. The public health relevance of MRSA in retail meat is entirely unclear. Although it is plausible that nasal MRSA colonization could occur if humans contaminate their hands by touching meat (or contaminated surfaces) then touching their nose before handwashing. I could present an essential requirement for efficient antibiotic resistant bacteria control measure to be implemented.

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ABSTRACT IN JAPANESE

先進国における新規抗菌薬の開発が停滞している一方、ヒトに対する抗菌薬の不適切な使用を背景として新たな薬剤耐性菌が増加している。そのため薬剤耐性菌の出現と拡散は世界的脅威となっており対策が必要とされている。さらに医療現場だけでなく、家畜に対しても多くの抗菌薬が治療および成長促進目的として使用され、それに伴う薬剤耐性菌が出現している。動物で出現した薬剤耐性菌は獣医療分野の治療効果を減弱させるほか、畜産物等を介してヒトに伝播することが危惧されている。世界保健機関（WHO）は 2011 年、世界保健デーで薬剤耐性菌の問題を取り上げ、薬剤耐性菌の抑制と減少のためにヒト、動物という垣根を超えた一体的な取り組み（ワンヘルス・アプローチ）の必要性を訴えた。この考えは国際的に受け入れられ、薬剤耐性に関するワンヘルス・アプローチの取組が強化されている。

メチシリン耐性黄色ブドウ球菌（MRSA）はわが国において院内感染の主要な原因である。MRSA は黄色ブドウ球菌 *Staphylococcus aureus* が可動性遺伝子カセット(SCC*mec*)により運ばれる *mecA* 遺伝子を外部から獲得することでメチシリンに対して耐性を獲得する。MRSA はヒトへの病原性が非常に強く、感染した場合に死亡する例も多い。そのため医療現場では MRSA に対する様々な対策が取られているが、2014 年の厚生労働省の調査によると入院患者からの MRSA の分離率は 49.1%と諸外国に比べ非常に高く、MRSA の制御は院内感染対策の最優先課題の一つである。

海外では家畜が高率（11-46%）に MRSA を保有している事実が報告されている。家畜から分離された MRSA は Multi Locus Sequence Typing で主に ST398 に型別

され、獣医療で多く使用されるテトラサイクリン系抗菌薬へ高度耐性を示すなど、医療現場由来株と性状が異なることから「家畜関連型 Livestock-associated(LA-)MRSA」と名付けられ、新型の MRSA として注目された。LA-MRSA は、家畜にとどまらず医療現場へ侵入し、院内感染を起こした報告もあることから、家畜―ヒト間の MRSA の伝播防止対策のために、家畜現場における MRSA の保菌状況に関する調査が海外で活発に実施されている。わが国でも毎年多くの抗菌薬が家畜へ使用され、その量は医療現場の使用量のおよそ 2 倍とされる。家畜由来薬剤耐性菌の出現への懸念から、その動向は農林水産省の家畜由来細菌の薬剤耐性モニタリング事業（JVARM）により監視されている。しかし JVARM によるモニタリングは大腸菌やカンピロバクターなどを対象菌種としており、MRSA の動向は明らかにされていない。

本研究では、ワンヘルス・アプローチに基づき、わが国の家畜における MRSA の現状の解明とヒトへの伝播防止対策への応用を目的とした。第 1 章では、家畜における MRSA の分布状況を明らかにするため、国内における畜産地域の 1 つである茨城県の豚 100 頭から MRSA を分離した。続いて第 2 章では、より詳細に国内における MRSA の分布状況を調査するため調査対象家畜および対象地域を拡大し、北海道の牛と豚の合計 436 頭から MRSA の分離を試みた。また同時に、MRSA 同様に SCC*mec* を保有し、SCC*mec* のリザーバーとされるメチシリン耐性コアグラーゼ陰性ブドウ球菌（MRCNS）、およびメチシリン感受性黄色ブドウ球菌（MSSA）を分離し性状解析を実施した。最後に、第 3 章では家畜由来 MRSA とヒト由来

MRSA との関連、および食肉が MRSA の伝播媒体となる可能性について検討するため、家畜、食肉、ヒト由来 MRSA の性状を比較した。

第 1 章における研究の結果、茨城県のと畜場搬入豚 100 頭中 8 頭（8%）から MRSA が分離された。分子疫学解析の結果、海外の LA-MRSA の 1 つである ST97/SCC*mec* V が分離され、テトラサイクリン耐性、マクロライド系感受性、リンコサミド系耐性と LA-MRSA に特徴的な薬剤感受性パターンを示した。日本では多くの家畜を輸入しており、海外から持ち込まれた可能性が考えられた。本研究では、海外で流行する LA-MRSA に類似する MRSA が、わが国の豚にも分布することを初めて明らかにした。

第 2 章では、北海道のと畜場に搬入された豚 217 頭、牛 219 頭から MRSA、MRCNS、および MSSA の分離を行った。MRSA は豚および牛から分離されなかったが、6－44%の MRCNS が、21－70%の MSSA が分離された。さらに、豚由来 MSSA は CC9 及び CC398、牛由来 MSSA は CC97 が優勢なタイプで、海外の LA-MRSA と遺伝子型が同じ MSSA が分布していることが明らかとなった。今回得られた結果より、日本の家畜における MRSA の保菌割合は海外に比べ低いものの、今後海外と同じ LA-MRSA が発生するおそれがあることが示唆された。

第 3 章では、第 1 章で分離された豚由来 8 株、分与を受けた食肉由来 8 株、牛乳房炎由来 7 株、ヒト由来 100 株の MRSA を対象に分子疫学解析を実施し、その性状を比較した。結果、牛乳房炎由来、食肉由来、ヒト由来市中感染型 MRSA（CA-MRSA）が非常に近縁であったことを明らかとした。これら近縁な MRSA は日本の市中で近年急激に広まっているタイプの MRSA（CA-MRSA/J）で、牛および

食肉からの分離報告は本研究が初めてとなった。これまでの調査より、日本の家畜における MRSA 分布状況は非常に低く、CA-MRSA/J の分離報告は無い。また、食肉から分離される MRSA は多くがヒト由来で、ハンドリングなどによる汚染であることが疑われている。こうした背景より、本研究において食肉由来 MRSA の汚染源を特定することはできないが、ヒト由来であった可能性が高いことが考えられた。本研究において牛―食肉―ヒト間において、何らかの伝播経路が存在しうることが示唆された。

以上の成績から、わが国の豚および牛の MRSA、MRCNS、および MSSA に関する新たな分子疫学情報を提供することができた。さらに、牛、食肉、ヒト由来 MRSA に遺伝学的関連があり、これらの間で伝播経路が存在する可能性を示した。本研究により、伝播経路を遮断するという、新たな薬剤耐性菌対策を構築するための有用な知見を提供することができた。