คุณลักษณะและการจำแนกชนิดเชิงโมเลกุลของเชื้อ METHICILLIN-RESISTANT COAGULASE-POSITIVE STAPHYLOCOCCI ในสุนัข ผู้เลี้ยงสุนัข และสัตวแพทย์

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CHARACTERISTICS AND MOLECULAR TYPING OF METHICILLIN-RESISTANT COAGULASE-POSITIVE STAPHYLOCOCCI IN DOGS, DOG'S OWNERS AND VETERINARIANS

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ภัทรรัฐ จันทร์ฉายทอง : คุณลักษณะและการจำแนกชนิดเชิงโมเลกุลของเชื้อ METHICILLIN-RESISTANT COAGULASE-POSITIVE STAPHYLOCOCCI ในสุนัข ผู้เลี้ยงสุนัข และสัตวแพทย์. (CHARACTERISTICS AND MOLECULAR TYPING OF METHICILLIN-RESISTANT COAGULASE-POSITIVE STAPHYLOCOCCI IN DOGS, DOG'S OWNERS AND VETERINARIANS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.น.สพ.ดร. ณุวีร์ ประภัสระกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.นพ.ดร. ชาญวิทย์ ตรีพุทธรัตน์, ผศ. นพ. อนันต์ จงเถลิง, 125 หน้า.

การศึกษานี้เสนอการทดสอบคุณสมบัติทางชีวเคมีเพื่อใช้ในการระบุสปีชีส์ของเชื้อ coagulase-positive staphylococci (CoPS) ในสุนัข ได้แก่ *Staphylococcus pseudintermedius*, *S. schleiferi* subsp. *coagulans* และ *S. aureus* ประกอบด้วย การสร้างอะเซโทอิน, การใช้น้ำตาลมอลโทส, กาแลกโทส, ทรีฮาโลส และแลกโทสในอาหารเลี้ยงเชื้อเหลว และการหมักน้ำตาลแมนนิทอล วิธีนี้ให้ผลการระบุสปีชีส์สอดคล้องกับวิธีการทางพันธุกรรมและการวิเคราะห์รูปแบบโปรตีน ในแง่ของการส่งผ่านเชื้อระหว่างคนและสุนัข เชื้อ *S. pseudintermedius* และ *S. schleiferi* subsp. *coagulans* สามารถแยกได้จากผู้ที่สัมผัสใกล้ชิดสุนัข ได้แก่ ผู้เลี้ยงสุนัขและสัตวแพทย์ แต่ไม่พบในผู้ที่ไม่สัมผัสใกล้ชิดสุนัข อีกทั้งสามารถแยกเชื้อ methicillin-resistant CoPS (MRCoPS) ได้จากสุนัขและผู้ที่สัมผัสใกล้ชิดสุนัขด้วย โดยเชื้อ methicillin-resistant *S. pseudintermedius* (MRSP) แยกได้มากที่สุด ตามด้วย methicillin-resistant *S. schleiferi* subsp. *coagulans* และ methicillin-resistant *S. aureus* ตามลำดับ จากการทดสอบความไวรับต่อยาต้านจุลชีพและการตรวจหายีนดื้อยาด้วยเทคนิคไมโครแอเรย์ พบว่าเชื้อมากกว่า 80% ดื้อต่อยาต้านจุลชีพหลายกลุ่ม ได้แก่ เตตร้าซัยคลีน อะมิโนกลัยโคไซด์ อีริโทรมัยซิน คลินดามัยซิน คลอแรมเฟนิคอล ไตรเมตโทรปริม ไซโปรฟลอกซาซิน และซัลฟาเมโทซาโซล เมื่อทำการจำแนกคุณลักษณะเชิงโมเลกุลด้วยเทคนิค multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) และการจำแนกชนิด staphylococcal cassette chromosome *mec* (SCC*mec*) พบเชื้อที่แยกได้จากสุนัขและผู้สัมผัสสุนัขมีทั้งที่มีลักษณะเป็นโคลนร่วมกันและหลากหลาย โดยเฉพาะเชื้อ MRSP ทั้งนี้ผลการศึกษาสนับสนุนหลักฐานการส่งผ่านเชื้อข้ามโฮสต์ระหว่างสุนัขและคน จากผลการทดลองได้นำเสนอวิธีการวินิจฉัยเชื้อ CoPS ที่เป็นมาตรฐาน ทำให้พบการกระจายของเชื้อ MRCoPS ที่มีการดื้อต่อยาหลายชนิดใน สุนัข สัตวแพทย์ และเจ้าของสุนัข ดังนั้นการรับมือกับปัญหาเชื้อแบคทีเรียดื้อยาและลดโอกาสการแพร่กระจายเชื้อสู่คน จึงเป็นพันธกิจที่สำคัญของสัตวแพทย์

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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PATTRARAT CHANCHAITHONG : CHARACTERISTICS AND MOLECULAR TYPING OF METHICILLIN-RESISTANT COAGULASE-POSITIVE STAPHYLOCOCCI IN DOGS, DOG'S OWNERS AND VETERINARIANS. ADVISOR : ASST. PROF. NUVEE PRAPASARAKUL, D.V.M., Ph.D., CO-ADVISOR : ASST. PROF. CHANWIT TRIBUDDHARAT, M.D., Ph.D., ASST. PROF. ANAN CHONGTHALEONG, M.D., 125 pp.

This study propose the conventional biochemical scheme for species identification of canine coagulase-positive staphylococci (CoPS), *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus*, consisting of acetoin production, assimilation of maltose, galactose, trehalose and lactose in broth base and fermentation of mannitol. The scheme could generate reliable results with genotypic identification and protein pattern analysis. Regarding interspecies transmission, *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* could be isolated from dog-associated people including veterinarians and dog's owners but not from people without dog association. With respect to methicillin-resistant trait, MRCoPS could be only recovered from dogs and dog-associated people. The methicillin-resistant *S. pseudintermedius* (MRSP) was mostly isolated that followed by methicillin-resistant *S. schleiferi* subsp. *coagulans* (MRSSc) and methicillin-resistant *S. aureus* (MRSA), respectively. Using antimicrobial susceptibility test and genetic resistance detection by microarray, over 80% of MRCoPS isolates exhibited multidrug resistance to approved antimicrobials for veterinary use including tetracycline, aminoglycosides, erythromycin, clindamycin, chloramphenicol, trimethoprim, ciprofloxacin and sulfamethoxazole. By multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing, the molecular characteristics revealed that Thai dogs and dog-associated people shared common and various clones of MRCoPS, especially MRSP. These supported the evidence of interspecies transmission of canine MRCoPS between dogs and humans. The standard diagnostic schemes were proposed in this study leading to discovery of multidrug resistance MRCoPS distributing among dogs, veterinarians and dog owners. Thus, to deal with the challenge condition of antimicrobial-resistant bacteria and to diminish the spread to human beings, veterinarians should seriously take action.

Department : Veterinary Pathology Student's Signature .....

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ATCC	=	American type culture collection
APS	=	Ammonium persulfate
BLAST	=	Basic local alignment search tool
bp	=	base pair
BSA	=	Bovine serum albumin
BURST	=	Based upon related sequence types
CA-MRSA	=	Community-acquired methicillin-resistant *Staphylococcus aureus*
CHL	=	Chloramphenicol
CIP	=	Ciprofloxacin
CLI	=	Clindamycin
CoPS	=	Coagulase-positive staphylococci
CFU	=	Colony-forming unit
CLSI	=	Clinical Laboratory Standard Institute
CU-ACUC	=	Chulalongkorn University Animal Care and Use Committee
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
EDTA	=	Ethylenediaminetetraacetic acid
ERY	=	Erythromycin
EUCAST	=	European Committee of Antimicrobial Susceptibility Testing
FUS	=	Fusidic acid
GEN	=	Gentamicin

HA-MRSA	=	Hospital-acquired methicillin-resistant *Staphylococcus aureus*
h	=	hour
HCl	=	Hydrochloric acid
IS	=	Insertion sequence
KAN	=	Kanamycin
kDa	=	kiloDalton
KOH	=	Potassium hydroxide
l	=	liter
LA-MRSA	=	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LZD	=	Linezolid
mA	=	milliampere
MIC	=	Minimal inhibitory concentration
min	=	minute
MLST	=	Multilocus sequence typing
MLVA	=	Multiple-locus variable-number tandem repeat
mg	=	milligram
ml	=	milliliter
mM	=	Millimolar
MRCoPS	=	Methicillin-resistant coagulase-positive staphylococci
MRS	=	Methicillin-resistant staphylococci
MRSA	=	Methicillin-resistant *Staphylococcus aureus*

MRSP	=	Methicillin-resistant *Staphylococcus pseudintermedius*
MRSSc	=	Methicillin-resistant *Staphylococcus schleiferi* subsp. *coagulans*
MUP	=	Mupirocin
µl	=	microliter
µM	=	micromolar
NaCl	=	Sodium chloride
OXA	=	Oxacillin
PA-MRS	=	Pet-associated methicillin-resistant staphylococci
PCR	=	Polymerase chain reaction
PBP	=	Penicillin-binding protein
PCR-RFLP	=	Polymerase chain reaction-restricted fragment length polymorphism
PEN	=	Penicillin
PFGE	=	Pulsed-field gel electrophoresis
pH	=	Positive potential of hydrogen ions
ψSCC*mec*	=	Pseudo staphylococcal cassette chromosome *mec*
RAPD	=	Random-amplified polymorphic DNA analysis
RIF	=	Rifampicin
s	=	second
SCC*mec*	=	Staphylococcal cassette chromosome *mec*

SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIG	=	*Staphylococcus intermedius* group
ST	=	Sequence type
ST_4	=	Sequence type by multilocus sequence typing using 4 genes
ST_7	=	Sequence type by multilocus sequence typing using 7 genes
STR	=	Streptomycin
SMX	=	Sulfamethoxazole
SYN	=	Synecid
TEMED	=	N, N, N',N'-tetramethylethylenediamine
TET	=	Tetracycline
TIA	=	Tiamulin
TMP	=	Trimethoprim
TSA	=	Tryptic soy agar
U	=	Unit
UK	=	United Kingdom
UPGMA	=	Unweighted pair group method with arithmetic mean
USA	=	United State of America
UV	=	Ultraviolet
VAN	=	Vancomycin
VNTR	=	Variable-number tandem repeat
VP	=	Voges-Prausker
^{o}C	=	Degree Celsius

CHAPTER I

INTRODUCTION

Staphylococcus spp. are commensal bacteria exhibiting on skin of animal as a part of normal flora. These are facultative anaerobic gram-positive cocci with grape-like arrangement under microscopic inspection. Coagulase-positive staphylococci (CoPS) are members of a group in this genus that frequently cause diseases as opportunistic pathogens because of their pathogenic trait. To date, CoPS include *Staphylococcus aureus*, *S. intermedius*, *S. pseudintermedius*, *S. delphini*, *S. schleiferi* subsp. *coagulans* and *S. lutrae*, and *S. hyicus* is categorized as coagulase-variable species (Baird-Parker, 1965; Devriese et al., 1978; Devriese et al., 2005; Foster et al., 1997; Hajek, 1976; Igimi et al., 1990; Varaldo et al., 1988). In human, *S. aureus* is the major species colonizing on skin and can associate with skin infection with underlying conditions (Lin et al., 2007). *S. pseudintermedius* is the predominant species colonizing on canine skin especially at mucosa and occlusion area such as anus, perineum, axilla and groin. Also, *S. pseudinteremdius* are frequently isolated from canine skin infection such as pyoderma, otitis externa and wound infection (Mason et al., 1996). This species was formerly classified as *S. intermedius* and now is a member of *S. intermedius* group (SIG) (Bannoehr et al., 2007). Otherwise, *S. schleiferi* subsp. *coagulans* is also a member of commensal CoPS on healthy canine skin and can be isolated from skin lesions of dog in lesser frequencies by comparison with *S. pseudintermedius*. The *S. schleiferi* subsp. *coagulans* exhibits phenotypes similar to *S. aureus* and *S. pseudintermedius* that might cause in misidentification and underestimation (Zdovc et al., 2004). By commercial test kits, CoPS species isolated from animal cannot be accurately differentiated

(Jousson et al., 2007). Accordingly, the lack of simple biochemical scheme for CoPS derived from canine origin is an obstacle for routine diagnosis and epidemiological study.

Methicillin-resistant staphylococci (MRS) are considered as multidrug-resistant bacteria that can resist to β-lactams. With higher pathogenicity, methicillin-resistant coagulase-positive staphylococci (MRCoPS) including methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *S. pseudintermedius* (MRSP) and methicillin-resistant *S. schleiferi* subsp. *coagulans* (MRSSc) are concerned as important species in both human and veterinary medicine (Weese and van Duijkeren, 2010). MRSA was firstly recognized frequently causing nosocomial infection in patient with long-term hospitalization including wound infection, pneumonia and bacteremia, known as hospital-acquired MRSA (HA-MRSA). The MRSA strains associating skin infection in community is known as community-acquired MRSA (CA-MRSA) (Tong et al., 2011). Dog can be infected or colonized with MRSA which are possibly acquired from human by reverse zoonosis. For canine-specific species, MRSP was firstly recognized in 2007 that was carried by dogs and veterinary staffs in a veterinary teaching hospital (Sasaki et al., 2007). The mechanism conferring the methicillin-resistant trait of MRSP and MRSA is mediated by *mecA* gene.

The *mecA* gene is located on staphylococcal cassette chromosome *mec* (SCC*mec*), a specific mobile genetic element of this genus (Katayama et al., 2000). This gene mediates resistance to β-lactams by production of penicillin binding protein 2a (PBP2a). Therefore, staphylococcal strains carrying SCC*mec* can survive with continuing peptidoglycan synthesis in the presence of β-lactams. By antimicrobial susceptibility testing, determination of oxacillin resistance was used for phenotypic identification of MRS with different criteria for interpretation between MRSA and MRSP (Bemis et al., 2009; Brown et al., 2005; Papich, 2010; Schissler et al., 2009). Also,

cefoxitin susceptibility testing is recommended for MRSA screening, yet this is not acceptable for MRSP identification (Papich, 2010). By serological technique, detection of PBP2a can be used to confirm the phenotype in MRSA, but this technique gave false-positive result for SIG (Pottumarthy et al., 2004). Thus, phenotypic techniques can cause misidentification of MRS derived from animals. Using genotypic detection, the presence of *mecA* is recognized as a standard for identification of MRS which derived from both human and animals (Bemis et al., 2009; Brown, 2001). Therefore, the staphylococci carrying *mecA* can be definitively interpreted as MRS. These not only resist to β-lactams but also mediate resistance to other antimicrobial classes by accumulation of particular genetic determinants.

In veterinary practice, various antimicrobial classes are used for treatment and control of bacterial infection in animals (Morley et al., 2005). Uses of antimicrobial drugs are empirically based on recommendations or experiences of practitioner (White, 1996). Cephalexin monohydrate and amoxicillin-clavulanic acid are recommended for treatment of canine staphylococcal skin infection as empirical treatment (Dowling, 1996; Mason and Kietzmann, 1999). In veterinary clinical practice, bacterial identification and antimicrobial susceptibility testing are commonly performed in case of non-responsive patients. (Umber and Bender, 2009). Other groups of broad-spectrum antimicrobial are also required in single or combination to enhance the efficacy of treatment. Nevertheless, intensive and prolonged uses of antimicrobials are factors of positive selection of MRS to proliferate on dog skin (Guardabassi et al., 2004; Lloyd, 2007; Schwarz and Chaslus-Dancla, 2001). This is concerned as a source of antimicrobial-resistant bacteria that can spread to human in term of pet-associated MRS (PA-MRS) (Epstein et al., 2009).

Transmission of CoPS between dog and human have been increasingly reported (Cohn and Middleton, 2010). To date, dog become an integral part of family and share

the same environment in households that might be an opportunity of transmission. In case of dog's owner infected with MRSA, dog can be colonized with the strain from human and serve as a reservoir causing recurrent infection in human pateint (Manian, 2003). Regarding a zoonotic problem, *S. pseudintermedius and S. schleiferi* susp. *coagulans* were isolated from dog's owner with invasive local and systemic infection (Chuang et al., 2010; Kumar et al., 2007; Stegmann et al., 2010). Dog-associated people, which include veterinary staffs and dog's owners, can only be colonized with MRSP also. The MRSP-colonized veterinary surgeons are suspected as a cause of post-operative infection in canine patients (van Duijkeren et al., 2008). For MRSSc, information of MRSSc transmission between two hosts is scarcely available. Therefore, knowledge about species identification of CoPS, molecular and antimicrobial-resistant characteristics can provide the epidemiological information to manipulate and management of possible MRCoPS transmission.

CHAPTER II

LITERATURE REVIEW

2.1 Coagulase-positive staphylococci and species identification

The genus *Staphylococcus* is a member in Family *Staphylococcaceae* that are gram-positive cocci because of peptidoglycan components of cell wall. These commonly colonize on skin and mucous membrane of animals as a commensal organism and can associate with disease in host with predisposing causes. Concerning pathogenic potential, CoPS frequently associate with diseases by coagulase production as a strategy for immune evasion. Furthermore, other virulence factors were identified in this group. Nevertheless, coagulase-negative staphylococci can cause infection, especially in immunocompromised patients with medical intervention. Also, these are concerned as a reservoir of antimicrobial resistance genes (Kloos and Bannerman, 1994). *Staphylococcus* sp. was firstly proposed in 1882, which obtained from human abscess, and *S. aureus* was classified as a type species in 1917. CoPS isolated from animal were formerly identified as *S. aureus* biotype E and F according to biochemical variants from human strains (Baird-Parker, 1965). In 1976, S. *intermedius* was proposed as a new species of CoPS isolated from animals including dog, cat, pigeon, horse and mink (Hajek, 1976). These play role as a part of normal flora and opportunistic pathogen in animals. In 1990, *S. schleiferi* subsp. *coagulans* was firstly reported as a member of CoPS, which associated canine otitis externa (Igimi et al., 1990). Phenotypes of this species are similar to *S. aureus* and *S. intermedius*, but the genotype resemble to coagulase-negative *S. schleiferi* subsp. *schleiferi*. Consequently, *S. schleiferi* subsp. *coagulans* is undistinguishable from other staphylococci using commercial biochemical kits (Jousson et al., 2007). In 2005, *S. intermedius* isolated from dogs was reclassified

as *S. pseudintermedius* by DNA-DNA hybridization that resulted only 38-46% homology to *S. intermedius* type strain from pigeon (Devriese et al., 2005). With close genetic relationship, SIG is a group of CoPS including *S. intermedius*, *S. pseudintermedius* and *S. delphini* (Bannoehr et al., 2007). The results from commercial biochemical kits are obvious for species identification in this group; therefore, the sequencing of particular gene comprising *cpn60*, *nuc* and *sodA* is suggested for definitive identification (Sasaki et al., 2007). Many molecular techniques were created for species identification of CoPS such as polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) for *S. pseudintermedius* and multiplex-polymerase chain reaction (PCR) for CoPS identification (Bannoehr et al., 2007; Bannoehr et al., 2008; Sasaki et al., 2010). Up to date, 7 species of staphylococci with ability of coagulase production were identified including *S. aureus*, *S. intermedius*, *S. delphini*, *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus* and *S. lutrae* (Bannoehr et al., 2007; Devriese et al., 1978; Foster et al., 1997; Varaldo et al., 1988). Nevertheless, *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus* are commonly isolated from canine origins. To date, *S. pseudintermedius* was recognized as major CoPS species colonizing on canine skin and associating skin infection. Likewise, *S. schleiferi* subsp. *coagulans* can be isolated from healthy dog and dog with skin diseases, and *S. aureus* can be recovered from canine origin at lower frequency.

2.2 Epidemiology of CoPS and MRCoPS in human and dog and (reverse) zoonotic aspects

By longitudunal study, *S. aureus* can be recovered from 20% of healthy human as persistent carrier, and 60% of human can be intermittent carriers (Peacock et al., 2001). Nasal carriage of *S. aureus* can be a marker to determine a risk for developing infection (Toshkova et al., 2001). Health-care workers are a risk group for MRSA colonization in

prevalence at 1.6-15.5% worldwide (Albrich and Harbarth, 2008). In veterinary medicine, dogs and veterinary staffs can also be carriers of MRSA in prevalence at 0-9% and 4.4-32%, respectively (Weese and van Duijkeren, 2010). Furthermore, transmissible evidences of MRSA between dog and human have been reported, and dog can act as a reservoir for MRSA reversely infecting in humans (Manian, 2003; van Duijkeren et al., 2004; van Duijkeren et al., 2005). In dog, *S. pseudintermedius* can be isolated up to 90% from healthy canine carriers at various sites such as nare, mouth, groin, perineum and anus (Bannoehr and Guardabassi, 2012). Also, dog can be colonized with MRSP in wide range from 0 to 66.5% that might be varied by populations, samples and geographical regions (van Duijkeren et al., 2011). The highest frequency was found in a study by sampling from canine pyoderma (Kawakami et al., 2010). In human, the colonization of MRSP has been increasingly reported in small animal veterinarians and dog's owners (Boost et al., 2009; Morris et al., 2010; van Duijkeren et al., 2008). Unfortunately, this can cause localized and systemic infection in human (Campanile et al., 2007; Chuang et al., 2010; Kempker et al., 2009; Stegmann et al., 2010). *S. schleiferi* subsp. *coagulans* and MRSSc can be isolated from dogs, but frequencies are lower than that of *S. pseudintermedius* and MRSP (Griffeth et al., 2008; Hanselman et al., 2008; May et al., 2005; Yamashita et al., 2005). The pathogenicity of *S. schleiferi* subsp. *coagulans* in human was also reported in case of endocarditis and metastatic infection (Kumar et al., 2007).

2.3 Mechanism of methicillin resistance in staphylococci

Methicillin resistance is a specific resistant trait of staphylococci that was firstly identified in methicillin-resistant *S. aureus* (MRSA). As a β-lactamase-resistant penicillin, methicillin was introduced for treatment of β-lactamase-producing *S. aureus* in 1959. Unfortunately, MRSA was emerged in one year after launching of this drug and

spread in hospitals in Europe as a nosocomial pathogen in 1961. The mechanism of resistance is the production of PBP 2a or 2' which is an additional PBP with low affinity to β-lactams. This protein functions as a transpeptidase with catalytic activity in process of peptidoglycan synthesis. Therefore, MRS can continue synthesizing the cell wall during the presence of β-lactams (Chambers, 1988). Methicillin-resistant trait is not only presented in MRSA but also found in various staphylococcal species. In 2007, MRSP was firstly described as in 2007 (Sasaki et al., 2007). The mechanism of resistance is mediated by *mecA* gene which encodes PBP2a and locates on SCC*mec*, a specific mobile genetic element for *Staphylococcus* spp.

2.4 Staphylococcal cassette chromosome *mec* (SCC*mec*)

SCC*mec* is a gene cassette that was firstly discovered in MRSA strain N315 in 2000 (Katayama et al., 2000). The exact origin of SCC*mec* in MRSA has not been clarified, but *S. sciuri* is suspected as the origin of this (Wu et al., 1996). The *mec* gene complex, which consists of *mecA* and its operating system, and the chromosomal cassette recombinase (*ccr*) genes are responsible for resistance mechanism and site-specific recombination into chromosome, respectively (Katayama et al., 2000). The specific –integrated site is called *attBSCC* which is located at open reading frame of unknown function or *orfX*. Not only *mecA*-mediating resistance to β-lactams, transposons and plasmids containing other resistance genes can be incorporated in SCC*mec* (Hiramatsu et al., 2001). However, *ccr* complex and *mec* complex are the key roles for classification of SCC*mec*. The three allotypes of *ccr* gene including A, B and C were differentiated by ≤50% homology of DNA sequences, and each allotype can be classified into subtype by 50-80% homology. About *mec* complex, the complete components comprise *mecA*, *mecI*, *mecR1* and insertion sequences (IS). The class of *mec* complex can be categorized by the truncated operon of *mec*, type of IS elements

and their orientation (IWG-SCC, 2009). To date, 11 types of SCC*mec* were classified in MRSA that are illustrated in Table 2.1.

Table 2.1 The 11 types of SCC*mec* classified MRSA and their components (IWG-SCC, 2009; Li et al., 2011; Shore et al., 2011)

SCC*mec* type	*ccr* complex	*mec* complex
I	1 (A1/B1)	B (IS*431*-*mecA*-Δ*mecR1*-ΨIS*1272*)
II	2 (A2/B2)	A (IS*431*-*mecA*-*mecR1*-*mecI*)
III	3 (A3/B3)	A (IS*431*-*mecA*-*mecR1*-*mecI*)
IV	2 (A2/B2)	B (IS*431*-*mecA*-Δ*mecR1*-ΨIS*1272*)
V	5 (C)	C2 (IS*431*-*mecA*-Δ*mecR1*-IS*431*)
VI	4 (A4/B4)	B (IS*431*-*mecA*-Δ*mecR1*-ΨIS*1272*)
VII	5(C)	C1 (IS*431*-*mecA*-Δ*mecR1*-IS*431*)
VIII	4 (A4/B4)	A (IS*431*-*mecA*-*mecR1*-*mecI*)
IX	1 (A1/B1)	C2 (IS*431*-*mecA*-Δ*mecR1*-IS*431*)
X	7 (A1/B6)	C1 (IS*431*-*mecA*-Δ*mecR1*-IS*431*)
XI	8 (A1/B3)	E (*blaZ*-*mecA*-*mecR1*-*mecI*)

SCC*mec* type IV and V, which have smaller size than SCC*mec* I, II and III, were proposed to be a cassette associating in CA-MRSA (Ito et al., 2004; Ma et al., 2002). Moreover, the variant type of SCC*mec* type V, named type V_T, was identified with truncated IS*431* presenting premature stop codon (Boyle-Vavra et al., 2005). The difference between SCC*mec* type V and VII is only the orientation of IS*431* that differentiate the class C *mec* complex into 2 subclasses (C1 and C2) (Figure 2.2) (Berglund et al., 2008). Various combinations of *ccr* and *mec* complexes were identified and were reported as novel SCC*mec* types in MRSA (Figure 2.1-2.4) (Li et al., 2011; Zhang et al., 2009). Recently, SCC*mec* type XI incorporated with novel class E *mec*

complex containing *blaZ* was identified (Figure 2.4) (Shore et al., 2011). Many SCC*mec* identified in MRSA can be found in MRSP including type III, IV, V and V_T (Black et al., 2009; Perreten et al., 2010). Otherwise, variant SCC*mec* are specifically carried by MRSP. For instance, SCC*mec* type II-III exhibiting hybrid character of SCC*mec* type II and III, and SCC*mec* VII-241 are commonly found in European lineage of MRSP (Figure 2.5) (Descloux et al., 2008; Ruscher et al., 2010). Concerning advantages, typing of SCC*mec* has been used to characterize the MRS for epidemiological purposes.

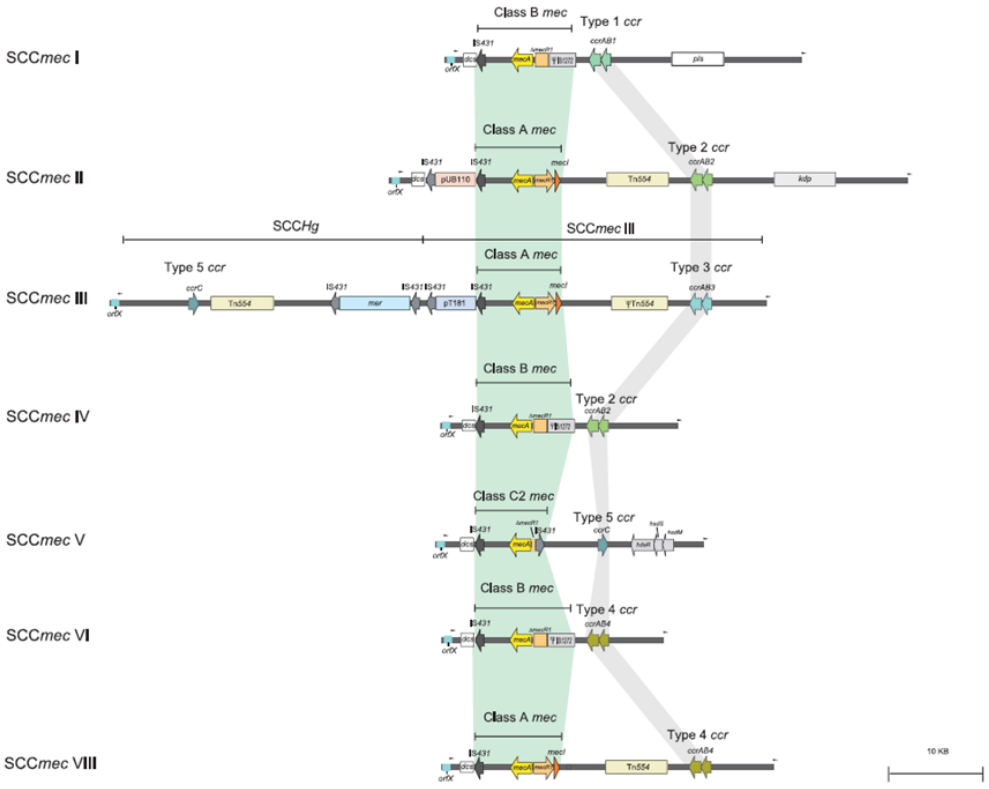


Figure 2.1 Schematic diagram of SCC*mec* I to VI and VIII described in MRSA prototypes (MRSA NCTC10442, SCC*mec* type I; MRSA N315, SCC*mec* type II; MRSA 85/2082, SCC*mec* type III; MRSA CA05, SCC*mec* type IV; MRSA WIS; SCC*mec* type V; MRSA HDE 288; SCC*mec* type VI; MRSA10682, SCC*mec* type VI) (Chen et al., 2009)

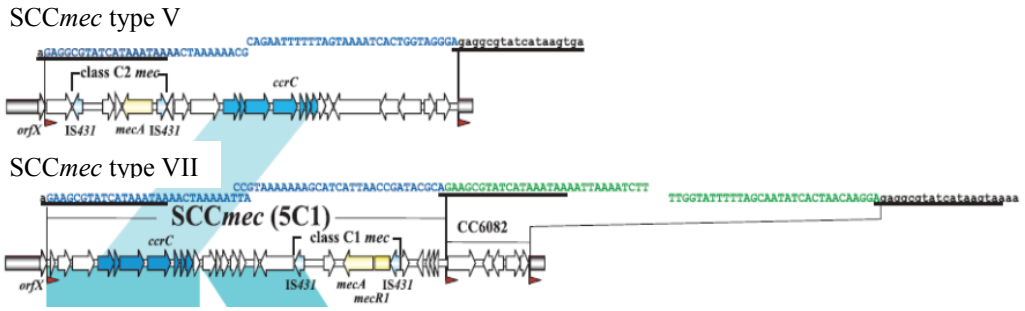


Figure 2.2 Structural comparison of SCC*mec* type V (MRSA WIS) and SCC*mec* type VII (MRSA JCSC6082) (Berglund et al., 2008)

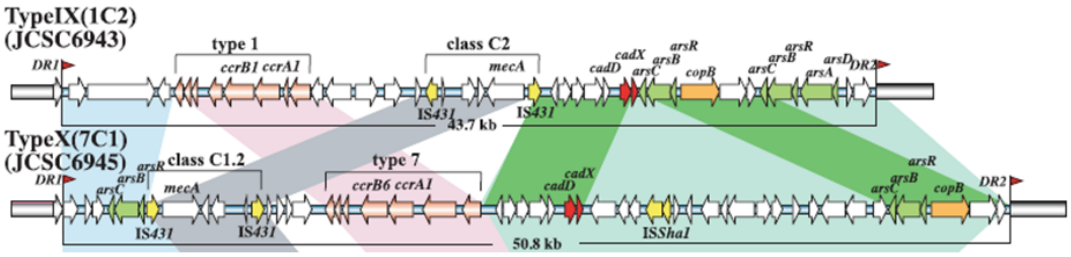


Figure 2.3 Structure of SCC*mec* type IX (MRSA JCSC6943) and X (MRSA JCSC6945) (Li et al., 2011)

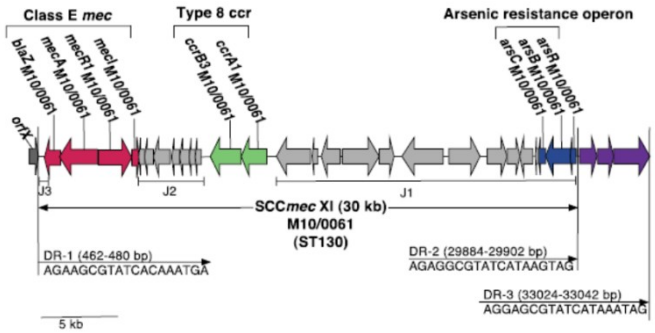


Figure 2.4 Structure of SCC*mec* type XI of MRSA M10/0061 (Shore et al., 2011)

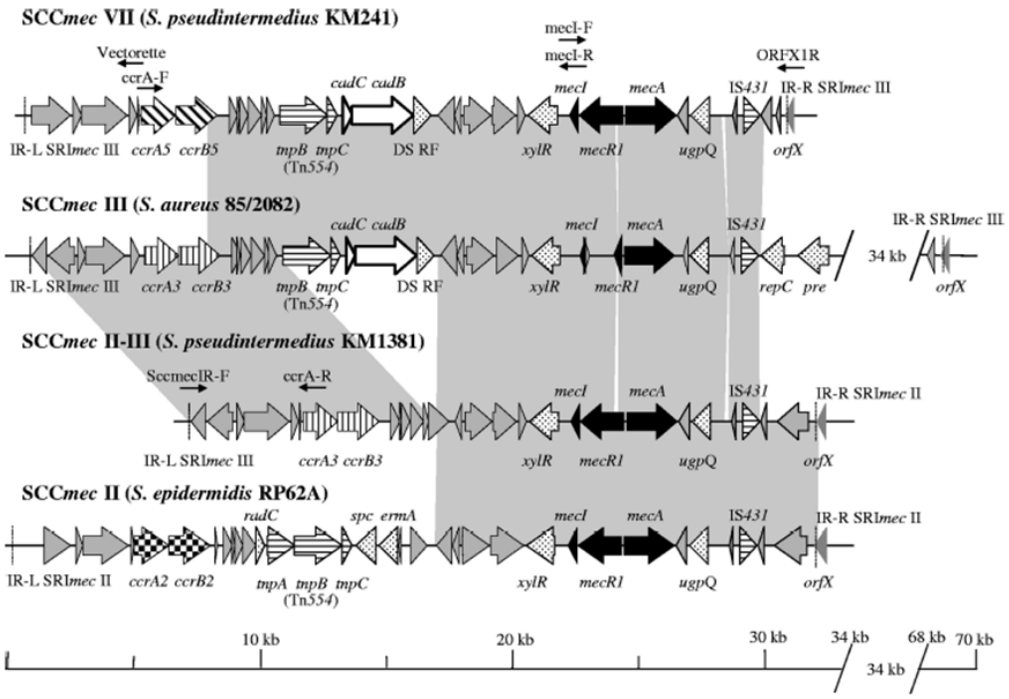


Figure 2.5 Structure of SCC*mec* type VII-241 (MRSP KM241) and II-III (MRSP KM 1381) by comparison with SCC*mec* type II (*S. epidemidis* RP62A) and SCC*mec* type III (MRSA 85/2082) (Descloux et al., 2008)

2.5 Molecular typing of staphylococci

Genetic characterization can provide information about taxonomy, evolution and population genetics of bacteria (van Belkum et al., 2001). Many techniques were created to clarify clonal relatedness of bacteria for epidemiological study. To investigate about molecular epidemiology of MRSA, pulsed-field gel electrophoresis (PFGE), random-amplified polymorphic DNA (RAPD) analysis and PCR-RFLP are developed as the tools to discriminate among strains (Fl-Adhami et al., 1991; Melles et al., 2007; Murchan et al., 2003). Generally, PFGE is used for typing of outbreak strains of MRSA because of the high discriminatory power for analysis and comparison. Based on the concept of gel-based technique, chromosomal DNA is digested at specific sites by restriction enzyme, and megabase-sized DNA are migrated in the electric field with

different directions (Goering, 2010). *Sma*I has been commonly used for PFGE typing of staphylococci with harmonizing to increase reproducibility (Bannerman et al., 1995; Murchan et al., 2003). Incapability of *Sma*I digestion in MRSA producing C5-cytosine methyltransferase was identified, and *Cfr*9I, a neoschizomer of *Sma*I, can be used for PFGE (Argudín et al., 2010). To determine evolution and clonal spread of MRSA, multilocus sequence typing (MLST) is a useful sequence-based technique by analysis of polymorphism of 7 housekeeping genes with establishment of international database. For *S. aureus*, genes encoding metabolic enzymes are used for typing including carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*) (Enright et al., 2000). For *S. pseudintermedius*, only 4 genes was designed for MLST consisting of elongation factor (*tuf*), chaperonin 60 (*cpn60*), phosphate acetyltransferase (*pta*) and autoinducing peptide (*agrD*) (Bannoehr et al., 2007). Recently, novel MLST scheme by analysis of 7 housekeeping genes (MLST-7) was developed for *S. pseudintermedius*. This comprises 3 former genes, *tuf*, *cpn60* and *pta*, and 4 new genes including acetate kinase (*ack*), formate dehydrogenase (*fdh*), adenylsuccinate synthetase (*purA*) and sodium sulfate symporter (*sar*) (Solyman et al., 2013). To date, MLST and PFGE are accepted to characterize MRSA and MRSP derived from human and animal sources worldwide (Weese and van Duijkeren, 2010). Furthermore, techniques based on variable-number tandem repeat (VNTR) were also created such as multiple-locus variable-number tandem repeat (MLVA) for *S. aureus* (Sabat et al., 2003) and *spa* typing for both *S. aureus* and *S. pseudintermedius* (Moodley et al., 2009; Strommenger et al., 2008). By comparison with PFGE, *spa* typing is not 100% typeability and provides lower discriminatory power than PFGE for *S. pseudintermedius* (Feng et al., 2012; Perreten et al., 2010). For methicillin-resistant strain, SCC*mec* typing is included to identify

characteristics of the resistance element. Indeed, MLST, PFGE and SCC*mec* typing are sufficient to study the spread of MRSA and MRSP which provide information in many reports. For example, MRSP ST71(MLST)-J(PFGE)-II-III(SCC*mec*) is the major clone distributing in European countries and infecting in human (Stegmann et al., 2010) and MRSA ST398 is globally recognized as livestock-associated MRSA (Wulf and Voss, 2008). Despite unavailability of MLST for *S. schleiferi* subsp. *coagulans*, PFGE and SCC*mec* typing have been used to identify relationships among strains (Roberts et al., 2005).

2.6 Antimicrobial resistance in staphylococci

In addition to β-lactams, staphyloccci are also resistance to other antimicrobial groups. Various mechanisms and resistance genes have been frequently identified in MRSA and MRSP. With multidrug resistance properties, these generally resist to β-lactams, tetracyclines, aminoglycosides, macrolides, lincosamides, chloramphenicol, trimethorprim and fluoroquinolones. Genes mediating the resistance mechanisms commonly founded in staphylococci such as β-latamase gene cluster (*blaZ-blaI-blaR*), *tet*(M) and *tet*(K) for tetracycline resistance, *erm*(A), *erm*(B) and *erm*(C) for macrolide-lincosamide-streptogramin B resistance, *aac(6')-Ie-aph(2')-Ia* for gentamicin and kanamycin resistance, cat_{pC221} and cat_{pC223} for non-fluorinated phenicol resistance and *dfrA* and *dfrG* for trimethoprim resistance (Kadlec et al., 2012; Kadlec and Schwarz, 2012). Interestingly, MRSA resisting to conserved antimicrobials for treatment of nosocomial infection has been emerged. MRSA reducing susceptibility to vancomycin was firstly reported in 1997 (Hiramatsu, 1998). The *vanA* mediating vancomycin resistance in MRSA was possibly gained from vancomycin-resistant *Enterococcus faecalis* (Chang et al., 2003). Many other antimicrobials are last-resort options for treatment of MRSA infection or decolonization such as linezolid (oxazolidone), rifampicin

(rifamycin), quinupristin/dalfopristin (streptogramin A and B), fusidic acid and mupirocin, but emergence of MRSA resisting to these drugs have been increasingly concerned. Linezolid resistance in *S. aureus* can be caused by mutation of *23SrRNA* target or acquisition of 23S methyl transferase (*cfr*) gene (Morales et al., 2010; Pillai et al., 2002). The *cfr* also mediated resistance to lincosamides, streptogramin A, and phenicols (Roberts, 2008). MRSA ST398 and ST9 carrying *cfr* from swine origin was reported (Kehrenberg et al., 2009) but not in MRSP and MRSSc. For streptogramin A resistance, *vga* and *lsa* genes encoding efflux pump were identified in MRSA (Kadlec and Schwarz, 2009; Schwendener and Perreten, 2011; Wendlandt et al., 2013). Both MRSA and MRSP mediating rifampicin resistance by mutation of *rpoB* were characterized (Aubry-Damon et al., 1998; Kadlec et al., 2011). Furthermore, various genes mediating multidrug resistance have been emergently found in MRSA derived from animals such as *fexA*, *dfrK*, *tet*(L) and *lnu*(B) which are rarely found in human strains (Kadlec et al., 2012).

CHAPTER III

HYPOTHESES, OBJECTIVES AND CONCEPTUAL FRAMEWORK

3.1 Hypotheses

The hypotheses of this study are:

Dogs and dog-associated people including dog's owners and veterinarians are colonized with various species of CoPS that can be identified by biochemical scheme, and the clones of canine CoPS can be found and shared between Thai dogs and dog-associated people.

The research questions of this study which were relied on the hypotheses include:

1. Can canine CoPS be identified phenotypically by using biochemical properties and protein pattern analysis?
2. How much prevalence are Thai dogs, dog's owners and veterinarians colonized with CoPS and MRCoPS?
3. What are characteristics of CoPS isolated from Thai dogs and dog-associated people?

3.2 Objectives

The objectives of this study are:

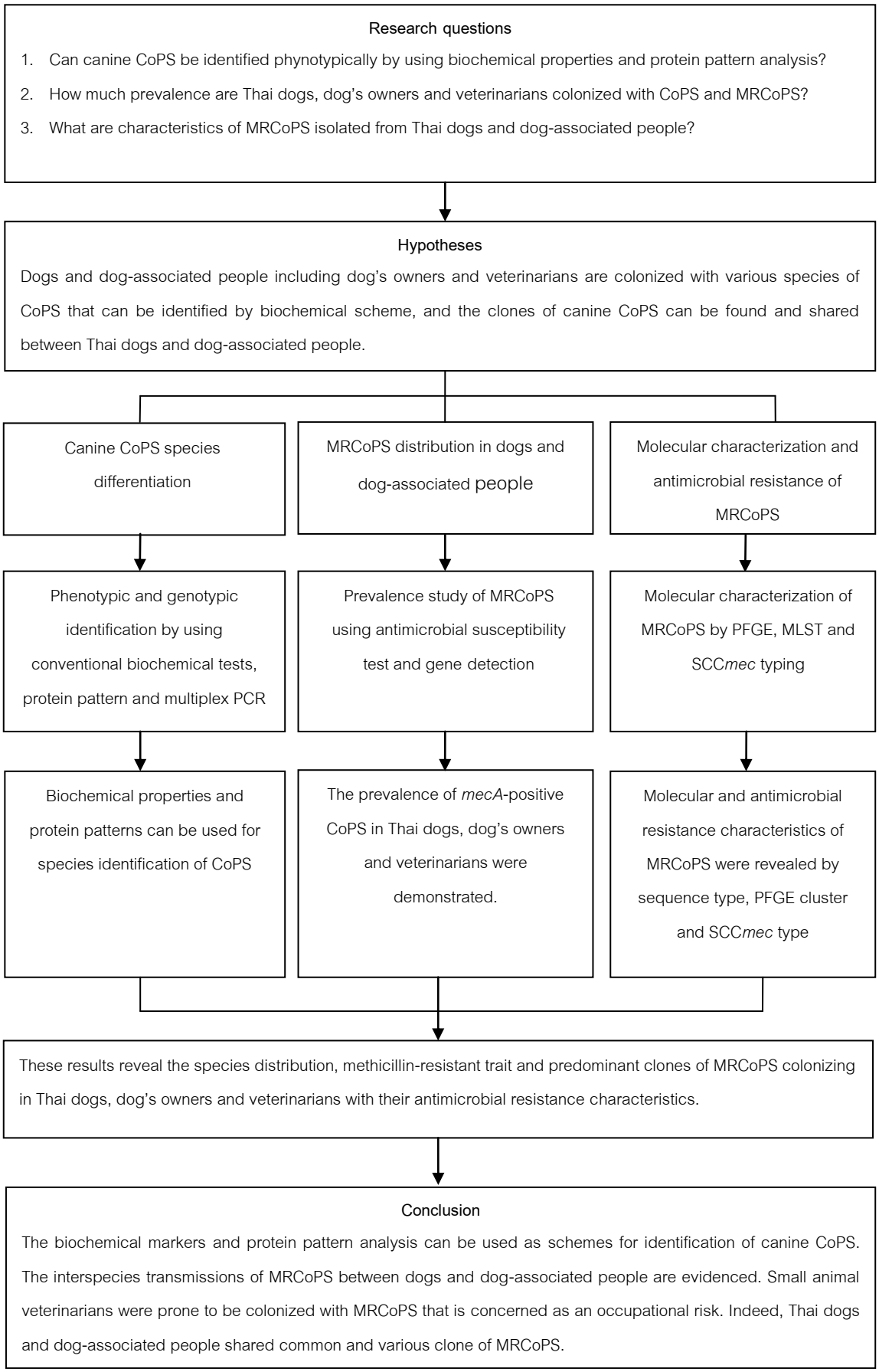
1. To determine the biochemical markers and protein pattern of CoPS isolated from canine origins
2. To investigate the prevalence and antimicrobial resistance of CoPS and MRCoPS colonization in Thai dogs, dog's owners and veterinarians

3. To characterize phenotypes and genotypes of MRCoPS isolated from Thai dogs and compare to those of dog-associated people

3.3 Conceptual framework

This study was conducted according to the conceptual framework as follows:

1. Species identification of CoPS isolated from dogs and dog-associated people using conventional biochemical tests and protein pattern analysis by comparison with genotypic identification.
2. Investigation of prevalence of CoPS and MRCoPS in Thai dogs and dog-associated people by detection of methiciliin-resistant phenotypes and *mecA* with determination of their antimicrobial resistance characteristics.
3. Characterization of genotypic features of Thai MRCoPS isolated from Thai dogs and dog-associated people by molecular typing including MLST, PFGE and SCC*mec* typing


Research questions
1. Can canine CoPS be identified phynotypically by using biochemical properties and protein pattern analysis?
2. How much prevalence are Thai dogs, dog's owners and veterinarians colonized with CoPS and MRCoPS?
3. What are characteristics of MRCoPS isolated from Thai dogs and dog-associated people?
Hypotheses
Dogs and dog-associated people including dog's owners and veterinarians are colonized with various species of CoPS that can be identified by biochemical scheme, and the clones of canine CoPS can be found and shared between Thai dogs and dog-associated people.
Canine CoPS species differentiation
MRCoPS distribution in dogs and dog-associated people
Molecular characterization and antimicrobial resistance of MRCoPS
Phenotypic and genotypic identification by using conventional biochemical tests, protein pattern and multiplex PCR
Prevalence study of MRCoPS using antimicrobial susceptibility test and gene detection
Molecular characterization of MRCoPS by PFGE, MLST and SCCmec typing
Biochemical properties and protein patterns can be used for species identification of CoPS
The prevalence of mecA-positive CoPS in Thai dogs, dog's owners and veterinarians were demonstrated.
Molecular and antimicrobial resistance characteristics of MRCoPS were revealed by sequence type, PFGE cluster and SCCmec type
These results reveal the species distribution, methicillin-resistant trait and predominant clones of MRCoPS colonizing in Thai dogs, dog's owners and veterinarians with their antimicrobial resistance characteristics.
Conclusion
The biochemical markers and protein pattern analysis can be used as schemes for identification of canine CoPS. The interspecies transmissions of MRCoPS between dogs and dog-associated people are evidenced. Small animal veterinarians were prone to be colonized with MRCoPS that is concerned as an occupational risk. Indeed, Thai dogs and dog-associated people shared common and various clone of MRCoPS.

CHAPTER IV

MATERIALS AND METHODS

4.1 Species identification of CoPS

4.1.1 Sample collection

Bacteria were collected from 66 healthy dogs entering small animal hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand for vaccination. All dogs did not present clinical sign of systemic illness. These were enrolled with owner's consent. The bacterial samplings were performed at 137 carriage sites including nasal mucosa of 66 dogs, perineum of 56 dogs and the groin of 15 dogs by sterile cotton swabs. The samples were collected in Stuart's transport medium (Difco, France) and were cultured within 24 h (Eriksen et al., 1994).

4.1.2 Bacterial culture and isolation

Bacteria were grown on nutrient agar (Merck, UK) with 5% sheep blood and Baird-Parker agar (Oxoid, England) for staphylococcal selection. After incubation at 37^{o}C for 24 h, 3-5 distinct patterns of CoPS-suspected colony, exhibiting 1-3 mm of diameter sizes, colony color (white to yellow) and hemolytic pattern on blood agar and gray to black colonies with or without a lecithovitelline zone on Baird-Parker agar, were selected (Cowan and Steel, 1961).

4.1.3 Determination of biochemical properties

Primary biochemical tests including Gram's staining, motility test, oxidation-fermentation test, catalase and oxidase tests were conducted for identification of the genus *Staphylococcus*. Coagulase production was determined by tube coagulase test

using Bactident® rabbit plasma (Merck, Germany). To evaluate biochemical properties for species identification, secondary tests were selected according to the ability of discrimination of CoPS from previous studies consisting of acetoin production, assimilation of maltose, galactose, trehalose and lactose and fermentation of mannitol (Devriese et al., 2005; Igimi et al., 1990; Roberson et al., 1992; Sasaki et al., 2007; Schissler et al., 2009; Zdovc et al., 2004). All tests were performed according to conventional schemes. The acetoin production was detected by Voges-Prausker (VP) test (Barritt, 1936). The assimilation of carbohydrates was determined by using purple agar and broth media containing 1% of sugar and bromcresol purple as a pH indicator (Freney et al., 1999; Igimi et al., 1989). For mannitol fermentation test, the bacteria were inoculated by stabbing into the butt of tube containing purple agar base with 1% of mannitol, covered by paraffin overlay (Roberson et al., 1992). After inoculation, all tests were incubated at 37^{o}C for 24 h. The acetoin production was interpreted as positive by developing red color after adding 40% KOH and α-naphthol. The positive results for carbohydrate assimilation tests and mannitol fermentation were shown by changing the color of media to yellow because of acid production.

4.1.4 multiplex PCR for CoPS identification

For genotypic identification, multiplex PCR was developed as a tool for species differentiation of CoPS (Sasaki et al., 2010). This is relied on specific amplification of *nuc* gene by using species-specific primers for 7 species (Table 4.3). Bacterial DNA was extracted by 2 methods including DNA extraction kit and conventional boiling technique. A total of 50 µl of PCR reaction consisted of 25 µl of GoTaq Green master mix (Promega, USA), 1 µl of 10 µM of each primer, 1 µl of extracted DNA template and 11 µl of nuclease free water. The specific condition included an initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 35 s, and

extension at 72°C for 1 min; and a final extension at 72°C for 2 min. PCR products were run in 1.0% agarose gel electrophoresis and were inspected under UV illumination. The standard strains validated by gene sequencing were used as control strains.

Table 4.1 Primers for multiplex PCR for species identification of CoPS (Sasaki et al., 2010)

Species	Primer name	Oligonucleotide sequence (5'→ 3')	Product size (bp)
S. aureus	au-F3	TCGCTTGCTATGATTGTGG	359
	au-nucR	GCCAATGTTCTACCATAGC	
S. intermedius	in-F	CATGTCATATTATTGCGAATGA	430
	in-R3	AGGACCATCACCATTGACATATTGAAACC	
S. schleiferi	sch-F	AATGGCTACAATGATAATCACTAA	526
	sch-R	CATATCTGTCTTTCGGCGCG	
S. delphini group A	dea-F	TGAAGGCATATTGTAGAACAA	661
	dea-R	CGRTACTTTTCGTTAGGTCG	
S. hyicus	hy-F1	CATTATATGATTTGAACGTG	793
	hy-R1	GAATCAATATCGTAAAGTTGC	
S. pseudintermedius	pse-F3	TRGGCAGTAGGATTCGTTAA	926
	pse-R5	CTTTTGTGCTYCMTTTTGG	
S. delphini group B	deb-F	GGAAGRTTCGTTTTTCCTAGAC	1,135
	deb-R4	TATGCGATTCAAGAACTGA	

4.1.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein pattern analysis

Protein patterns of CoPS were demonstrated by using SDS-PAGE. The procedure was conducted according to the previous study (McDougal et al., 1986). Overnight-grown bacteria were harvested and were twice washed with 0.5% physiological saline. Approximately, 50 mg of bacterial cell was mixed with glass beads (Sigma, USA) and denaturing buffer [0.06 M Tris–HCl, 2.5% glycerol, 0.5% sodium dodecyl sulfate (SDS) 1.5% β-mercaptanol and 2% bromphenol blue (Fisher Scientific, UK)] and were rigorously shake for 30 min. Then, the proteins were denatured by boiling at 100 °C for 10 min, and the pellets were excluded by centrifugation at 9,000Xg for 5 min for supernatant collection. To view the protein pattern, the extracted proteins were run in discontinuous SDS-PAGE, including a 4% stacking gel [0.625 M Tris–HCl, acrylamide-bis acrylamide 30:0.8, distilled water,10% ammonium persulfate (APS) and N, N, N',N'-tetramethylethylenediamine (TEMED) (Fisher Scientific, UK)] and a 10% separating gel [1.125 M Tris–HCl, distilled water, 10% SDS, 10% APS and TEMED (Fisher Scientific, UK)] in electrophoresis buffer [196 mM glycine, 0.1% SDS and 2.5 mM Tris base (Fisher Scientific, UK)]. The system was performed at a constant of 10 mA (ATTO, Japan). The separating gel was stained with 0.25% Coomassie Brillant Blue R-250 (Sigma, USA). The molecular weight of protein bands was calculated using PhotoCaptMW software (Vilber Lourmat, France). For dendrogram construction, the protein patterns were compared for clustering by unweighted pair group method with arithmetic mean (UPGMA) by online dendroUPGMA program (http://genomes.urv.cat/UPGMA) (Thomson-Carter and Pennington, 1989). The protein patterns of all isolates in this study were characterized, and the identical pattern was compared with the standard strains.

4.1.6 Standard strains and gene sequencing

S. aureus ATCC (American Type Culture Collection) 25923 and *S. aureus* ATCC 29213 were used as control strains for species identification and antimicrobial susceptibility tests. In addition, *S. aureus*, *S. pseudintermedius*, *S. intermedius*, *S. delphini* and *S. schleiferi* subsp. *coagulans*, derived from collection of Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, were also used as standard strains of CoPS from animal origins.

The species of these was confirmed by *sodA* and/or *cpn60* (*hsp60*) sequence analysis using specific primers (Table 4.1). The specific condition for *cpn60* amplification included an initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; and a final extension at 72°C for 7 min (Bannoehr et al., 2007). For *sodA* amplification, the specific condition included an initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 37°C for 45 s and extension at 72°C for 30 s; and a final extension at 72°C for 7 min. Bacterial DNA were extracted to use as template by using Wizard® Genomic DNA purification kit (Promega, USA). In total 50 µl of reaction, polymerase chain reactions (PCR) consisted of 25 µl of GoTaq Green master mix (Promega, USA), 1 µl of 10 µM of each of *cpn60* primer or 25 µM of each of *sodA* primer, 1 µl of extracted DNA template and 22 µl of nuclease free water and then performed in PCR thermal cycler (ThermoHybaid, USA). The PCR products were inspected under UV illuminator (Viber Loutmat, France) after running in 1.5% agarose gel electrophoresis. The PCR products were purified by using nucleic acid purification kits (Nucleospin® Extract II) (Macherey-Nagel, Germany). The validated sequences were submitted to online database providing GenBank accession numbers (Table 4.2). DNA sequencing was performed using the BigDye Terminator (Qiagen, USA). All DNA sequences of each gene were analyzed using nucleotide BLAST for comparison the

nucleotide homology with type strains (Tatusova and Madden, 1999). To illustrate relationship among species, the phylogenetic trees were constructed using the Neighbor-Joining method by MEGA4 program (Saitou and Nei, 1987; Takezaki et al., 1995; Tamura et al., 2007).

Table 4.2 Primers for *cpn60* and *sodA* amplification and sequencing (Bannoehr et al., 2007; Poyart et al., 2001)

Primer for PCR	Oligonucleotide primer (5'→3')	Gene detected by primer pair	Size of product (bp)
cpn60-F	GCGACTGTACTTGCACAAGCA	*cpn60*	550
cpn60-R	AACTGCAACCGCTGTAAATG		
sodA-d1	CCITAYICITAYGAYGCIYTIGARCC	*sodA*	480
sodA-d2	ARRTARTAIGCRTGYTCCCAIACRTC		

Table 4.3 Standard strains of CoPS derived from collection of Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University and their GenBank accession numbers.

Strains/isolates	Species	Source	Gene	GenBank accession No.
CVMC 0509	*Staphylococcus aureus*	dog	*sodA*	GU930723
CVMC 0609	*S. aureus*	dog	*sodA*	GU930724
CVMC 0108	*S. pseudintermedius*	dog	*sodA*	GU930731
CVMC 0108	*S. pseudintermedius*	dog	*cpn60*	GU930715
CVMC 0608	*S. pseudintermedius*	dog	*sodA*	GU930732
CVMC 0608	*S. pseudintermedius*	dog	*cpn60*	GU930716
CVMC 0708	*S. pseudintermedius*	dog	*sodA*	GU930733
CVMC 0708	*S. pseudintermedius*	dog	*cpn60*	GU930717
CVMH 0808	*S. pseudintermedius*	human	*cpn60*	GU930718
CVMP 0309	*S. intermedius*	pigeon	*sodA*	GU930734
CVMP 0309	*S. intermedius*	pigeon	*cpn60*	GU930719
CVMP 0109	*S. delphini*	pigeon	*sodA*	GU930735
CVMP 0109	*S. delphini*	pigeon	*cpn60*	GU930720
CVMP 0209	*S. delphini*	pigeon	*sodA*	GU930736
CVMP0409	*S. delphini*	pigeon	*cpn60*	GU930722
CVMC 0208	*S. schleiferi* subsp. *coagulans*	dog	*sodA*	GU930725
CVMC 0308	*S. schleiferi* subsp. *coagulans*	dog	*sodA*	GU930726
CVMC 0408	*S. schleiferi* subsp. *coagulans*	dog	*sodA*	GU930727
CVMC 0508	*S. schleiferi* subsp. *coagulans*	dog	*sodA*	GU930728
CVMP 0609	*S. schleiferi* subsp. *coagulans*	pigeon	*sodA*	GU930729
CVMP 0709	*S. schleiferi* subsp. *coagulans*	pigeon	*sodA*	GU930730

4.2 Prevalence of MRCoPS and antimicrobial resistance

4.2.1 Populations and sample collection

A. Dog population

The sample size of dog was calculated from formula of sample estimation for prevalence study (Naing et al., 2006): $n = z^2 \frac{P(1-P)}{d^2}$; where

n = sample size,

z = statistic for a level of confidence,

p = expected prevalence in proportion of one and

d = desired precision in proportion of one.

The 95% z value is 1.96 by confidence of interval and the estimated prevalence of MRCoPS in dog in the previous study is 32% (Sasaki et al., 2007) with the desired precision at 10%. As a result, the sample size of this study is 84 dogs at least, $n = 1.96^2 \frac{0.32(1-0.32)}{(0.1)^2} = 83.5$.

Skin swabbing was randomly performed to obtain the bacteria at nasal mucosa, perineum and/or groin of 100 owned-dogs without systemic clinical illness entering for vaccination unit and dermatological unit in veterinary teaching school, Chulalongkorn University, Bangkok, Thailand during 2009-2012. The history about age, breed, sex and antimicrobial exposure during 1 year were recorded. The method for sample collection from dogs was approved by Chulalongkorn University Animal Care and Use Committee (CU-ACUC) and consented by dog's owners. All samples were stored in Stuart's transport medium (Difco, France) at 4°C. Bacterial culture was performed within 24 h after the sample collection.

B. Human populations

Human populations were divided into 3 groups including veterinarians, dog's owners and people without dog and cat association. The sample sizes of each group

were calculated by the formula as above when the prevalence of MRCoPS in dog-associated people is 15% (Sasaki et al., 2007). As a result, the sample size must be 49 persons at least in each group, $n = 1.96^2 \frac{0.15(1-0.15)}{(0.1)^2} = 48.9$.

The 100 people without dog and cat association were persuaded to be control people. For dog's owners, 100 people who had dog(s) and lived with their dog(s) in their households were included in this group. The history including age, sex, sharing area with dog in their households and pattern of dog contact was recorded. The 200 Thai small animal veterinary practitioners who worked in animal hospitals and clinics were persuaded in a group of veterinarian. The history including age, sex, job, and hygiene was recorded.

Bacteria from human populations were obtained by nasal swabbing. The swabs were stored in Stuart's transport medium (Difco, France), and bacterial culture was performed within 24 h as well. The procedure of sample collection in human was approved by The Ethical Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University (081/54).

4.2.2 Bacterial culture, isolation and identification

Bacteria were primarily grown on nutrient agar (Merck, UK) or tryptic soy agar (TSA) (Difco, France) with 5% sheep blood at 37°C for 24 h. For selection of methicillin-resistant strain, swabs were streaked on mannitol salt agar (Difco, France) containing 0.5 mg/l of oxacillin (Oxoid, England). Then, the selective plates were incubated at 32°C for 48 h (Brown et al., 2005; Sasaki et al., 2007). Colony morphology of CoPS, exhibiting white to yellow, convex, round with smooth surface in diameter 0.2-1 mm in size and presenting hemolytic activity on blood agar, were inspected for selection. At least three CoPS-suspected colonies were selected to grow as pure colonies on TSA for species

identification. As above, species of CoPS was identified by biochemical properties and multiplex PCR.

4.2.3 Screening of methicillin resistance

Methicillin-resistant phenotype of CoPS was screened by oxacillin disk diffusion test according to protocol of Clinical Laboratory Standard Institute (CLSI, 2009). Overnight-grown CoPS colonies were diluted in 0.85% physiological saline and were adjusted to be 1.5 X 10^8 CFU/ml or 0.5 McFarland standard. The diluted bacteria were streaked on Müller-Hinton agar (Difco, France), and 1 µg-oxacillin disk (Oxoid, England) was placed on the agar. All plates were incubated at 32°C for 24 h. Then, zone of inhibition was measured and interpreted according to the criteria (Table 4.4). *S. aureus* ATCC 25923 was used as a control strain for this prodedure.

Table 4.4 Interpretative criteria for MRCoPS screening by oxacillin disk diffusion test (May et al., 2005; Papich, 2010)

Species	Zone diameter breakpoints (mm)		
	Susceptible (S)	Intermediate (I)	Resistant (R)
S. aureus	≥13 mm	11-12 mm	≤10 mm
S. pseudintermedius	≥ 18 mm	not applicable	≤17 mm
S. schleiferi subsp. *coagulans*	≥18 mm	not applicable	≤ 18 mm

4.2.4 *mecA* detection

All CoPS isolates were confirmed the presence of *mecA* by PCR (Strommenger et al., 2003). Specific primers used for this PCR were shown in Table 4.5. Total 25 µl of PCR mixture consisted of 12.5 µl of 2X GoTaq Green master mix (Promega, USA), 0.5 µl of 10 µM of each primer, 1 µl of extracted DNA template and 10.5 µl of nuclease free water. The reaction was performed in condition as follows, an initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; and a final extension at 72°C for 4 min. The amplified fragment was detected under UV illuminator after 1.5% agarose gel electrophoresis. For control of procedure, MRSA NCTC 10442 and *S. aureus* ATCC 25923 were used as positive and negative control strains, respectively. The *mecA*-positive isolates were definitively identified as methicillin-resistant strain in this study.

Table 4.5 Primers for *mecA* detection (Strommenger et al., 2003)

Primer names	Oligonucleotide sequences (5'→ 3')	Product size (bp)
mecA 1	AAAATCGATGGTAAAGGTTGGC	532
mecA 2	AGTTCTGCAGTACCGGATTTGC	

4.2.5 Antimicrobial susceptibility testing

To determine antimicrobial resistance of MRCoPS, minimal inhibitory concentration (MIC) of 18 antimicrobial drugs was determined including ciprofloxacin (CIP), chloramphenicol (CHL), clindamycin (CLI), erythromycin (ERY), fusidic acid (FUS), gentamicin (GEN), kanamycin (KAN), linazolid (LZD), mupirocin (MUP), oxacillin (OXA), penicillin (PEN), rifampicin (RIF), streptomycin (STR), sulfamethoxazole (SMX), tetracycline (TET), tiamulin (TIA), trimethoprim (TMP), vancomycin (VAN) and one combined quinupristin/dalfopristin (synecid or SYN). The broth microdilution technique

was performed for MIC determination according to CLSI recommendation (CLSI, 2009). The 24 h-grown bacterial colonies were suspended in 0.85% physiological saline (BioMérieux, France) in equivalent to 0.5 McFarland, measured by densitometer (Densimat®) (BioMérieux, France). The 10 µl of 0.5 McFarland bacterial suspensions were diluted in 11 µl of cation-adjusted Müller-Hinton broth (Trek Diagnostic System, The Netherlands). Suspended bacteria were inoculated in a custom Sensititre® 96-well plate model NLEUST containing these antimicrobial agents using Sensititre® Auto Inoculator (Trek Diagnostic System, The Netherlands). For oxacillin susceptibility test, all dilutions were supplemented with sodium chloride to be 2% in final concentration. The inoculated plates were incubated at 35°C for 24 h. The results of MIC were interpreted as susceptible or resistant according to breakpoint criteria recommended by European Committee of Antimicrobial Susceptibility Testing (EUCAST) version 2.0 (www.eucast.org). Criteria for kanamycin, streptomycin and sulfamethoxazole were used according to French Society for Microbiology in 2010 (www.sfm.asso.fr). Range of tested concentrations and breakpoint of each antimicrobial agent are shown in Table 4.6. To detect inducible clindamycin resistance phenotype, D-test was performed using disk diffusion test according to recommendation of CLSI (CLSI, 2009). All bacterial isolates were examined by placing 15 µg erythromycin disk (Oxoid, England) and 2 µg clindamycin disk (Oxoid, England) at 1.5 cm apart on Müller-Hinton agar. The positive result was evaluated by the presence of D zone (Figure 4.1) after incubation at 35°C for 24 h.

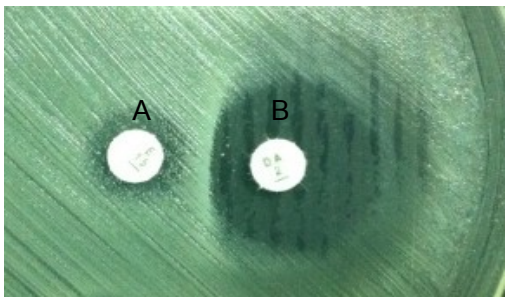


Figure 4.1 The presence of D zone of inducible clindamycin resistance phenotype using 15 µg erythromycin disk (A) and 2 µg clindamycin disk (B)

MRCoPS isolates presenting quinupristin/dalfopristin resistance were confirmed the resistance to streptogramin A by virginiamycin M1 susceptibility test. The MIC of virginiamycin M1 was manually performed by broth microdilution technique. Resistance to streptogramin A was judged when MIC was ≥128 mg/L. MRSA IMD49-10 and *S. aureus* RN4220 were used as positive and negative control strains, respectively (Schwendener and Perreten, 2011).

Table 4.6 Ranges of concentration and breakpoint criteria for interpretation of antimicrobial resistance in MRCoPS

Antimicrobial	Range (mg/l)	Breakpoint (mg/l)*	
		Susceptible (S)	Resistant (R)
Oxacillin (OXA) (for MRSA)	0.25-8	≤2	>2
Oxacillin (OXA) (for MRSP and MRSSc)	0.25-8	≤0.25	>0.25
Penicillin (PEN)	0.12-2	≤0.12	>0.12
Ciprofloxacin (CIP)	0.25-8	≤1	>1
Gentamicin (GEN)	1-16	≤1	>1
Kanamycin (KAN)	4-64	≤8	>16
Streptomycin (STR)	4-32	≤8	>16
Erythromycin (ERY)	0.25-8	≤1	>2
Clindamycin (CLI)	0.12-4	≤0.25	>0.5
Tiamulin (TIA)	0.5-4	NA	NA
Chloramphenicol (CHL)	4-64	≤8	>8
Tetracycline (TET)	0.5-16	≤1	>2
Trimethoprim (TMP)	2-32	≤2	>4
Sulfamethoxazole (SMX)	64-512	≤64	>256
Vancomycin (VAN)	1-16	≤2	>2
Mupirocin (MUP)	0.5-2 and 256	≤1	>256
Rifampicin (RIF)	0.015-0.5	≤0.06	>0.5
Linezolid (LZD)	1-8	≤1	>1
Fusidic acid (FUS)	0.5-4	≤4	>4
Quinupristin/dalfopristin (SYN)	0.5-4	≤1	>2

*Breakpoints were recommended by EUCAST version 2.0. With exception of kanamycin, streptomycin and sulfamethoxazole, the breakpoinits were recommended by French Society for Microbiology in 2010.

NA, not applicable

4.2.6 DNA microarray

DNA microarray was used for detection of antimicrobial resistance genes founded in gram-positive bacteria (Perreten et al., 2005). To prepare genetic materials as template, bacterial cells were digested by 2 µl of 1 mg/ml of lysostaphin solution in 100 µl of Tris-EDTA (TE) buffer incubated at 37°C for 30 min. Then, the suspension was mixed with 450 µl of lysis buffer [0.01 M Tris-HCl (pH 8.5), 0.05% Tween20, 240 µg/ml Proteinase K)], incubated at 60°C for 45 min and denatured at 95 °C for 15 min. The supernatant was collected by centrifugation at 4,000xg and used as template for DNA amplification and hybridization in array tube.

To optimize the DNA for amplification, concentration of nucleic acids were measured by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and were diluted to 100 ng/µl with sterile ultrapure water. DNA templates were randomly amplified in round A reaction. The total 25 µl of round A reaction mixture included 5 µl of diluted lysate containing at least 500 µg of DNA, 0.2 µl of 10 U/ml phi29 polymerase enzyme, 2.5 µl of 10X phi29 reaction buffer, 0.5 µl of 10 mg/ml bovine serum albumin (BSA) (New England Biolabs, England), 1 µl of 100 µM random primer with specific overhang (deRisiA), 1 µl of 100 µM dNTPs (Roche Applied Science, Switzerland) and 14.8 µl of nuclease free water. This reaction was performed at 30°C for 2 h and inactivated at 65°C for 10 min in PCR thermal cycler model 9800 Fast Thermal Cycler (Applied Biosystems, USA). The round B of amplification with labeling was performed in total 50 µl reaction including 10 µl of the product from round A, 1 µl of 5 U/µl *Taq* polymerase, 5 µl of 10X *Taq* reaction buffer (Roche Applied Science, Switzerland), 1 µl of 100 µM biotin-16-UTP (biotinylated primer-specific for overhang, deRisi B), 1 µl of 10 mM dNTPs. The condition for round B amplification was 55°C for 1 min, 72°C for 5 min and 95°C for 2 min; 35 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 1 min; and

72°C for 5 min in PCR thermal cycler. The products from the round B reaction were collected for hybridization.

Prior to hybridization, the 40 µl of labeling products were mixed with 60 µl of 3DNA buffer, pH 7.25 [250 mM of sodium phosphate, 4.5% sodium dodecyl sulfate, 1mM EDTA, 1X SSC buffer (1X SSC buffer consists of 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0)] and were denatured at 95 °C for 5 min before cooling down on ice for 2 min. The arrays were washed with 500 µl of sterile ultrapure water at 30°C, 550 rpm for 5 min and 500 µl of 3DNA buffer in the same condition using thermomixer (Eppendorf AG, Germany).

Custom-constructed array tubes model AMR+ve-3, which were kindly supported by Professor Dr Vincent Perreten, were used for detection of resistance genes in this study. The probe-denatured product was added in the array tube for hybridization at 55°C, 550 rpm for 3 h. After hybridization, the arrays were firstly washed with 500 µl of 2X SSC with 0.2% SDS at 25°C, 550 rpm for 5 min followed by 2X SSC and 0.2X SSC in the same condition, respectively. Then, 2% of milk powder in 100 µl of 6X SSPE buffer with 0.005% Triton X-100 (6X SSPE consists of 60 mM sodium phosphate, 1.08 M NaCl, 6 mM EDTA, pH 7.4) was added for blocking step at 30°C, 550 rpm for 10 min. For conjugation, 100 µl 6X SSPE containing 100 pg/µl of streptavidin-peroxidase polymer (Sigma Life Science, USA) was added, incubated at 30°C, 550 rpm for 20 min. The arrays were washed again with 500 µl 2X SSC solution with 0.01% Triton X-100, 2X SSC and 0.2X SSC in the condition as same as the first washing. Finally, 100 µl of tetramethylbenzidine peroxidase substrate was added to stain the hybridized probes, incubated at room temperature for 15 min. The image of arrays was documented by atr03 array tube reader (Alere Technologies GmBH, Germany) and was analyzed by Inoclust software (Alere Technologies GmBH, Germany). The spots presenting on arrays with signal $\geq$ 0.1 were interpreted as positive.

4.2.7 Detection of *lsa*(E)

The strains exhibiting resistance to pleuromutilin and streptogramin A were determined the presence of *lsa*(E) by PCR. Specific primers for detection were designed from the nucleotide sequence accession no. AF408195, shown in Table 4.7. Total 25 µL of PCR mixture consisted of 12.5 µl of 2X GoTaq Green master mix (Promega, USA), 0.5 µl of 10 µM of each primer, 1 µl of extracted DNA template and 10.5 µl of nuclease free water. The reaction was performed in condition as follows, an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, and a final extension at 72°C for 7 min. PCR products were inspected under UV illuminator after 1.0% agarose gel electrophoresis.

Table 4.7 Primers for *lsa*(E) detection

Primer names	Oligonucleotide sequences (5'→ 3')	Product size (bp)
lsaE-F	ACGGACGCGGTAAAACTACT	693
lsaE-R	TTGGCACGTTTCATCGCTTT	

4.3 Molecular typing

4.3.1 Multiplex PCR for SCC*mec* type I to V identification

The SCC*mec* type I to V was classified by approved multiplex PCR (Kondo et al., 2007). The scheme was incorporated with multiplex PCR panel 1 and 2 which were used for typing of *ccr* and *mec* complex, respectively. The sets of primers were illustrated in Table 4.8. For the panel 1, the total 50 µl of reaction mixture consisted of 25 µl of 2X GoTag Green master mix (Promega, USA), 1 µl of 10 µM of each primers, 3.4 µl of 25 µM of $MgCl_2$ (Promega, USA), 1 µl of extracted DNA template and 10.6 µl of nuclease free water, performing in condition as follows, an initial denaturation at 94°C for 2 min; 35

cycles of denaturation at 94°C for 2 min, annealing at 57°C for 1 min and extension at 72°C for 2 min; and a final extension at 72°C for 2 min. For the panel 2, the total 50 µl of reaction mixture consisted of 25 µl of 2X GoTag Green master mix, 1 µl of 10 µM of each primers, 2 µl of 25 µM of $MgCl_2$, 1 µl of extracted DNA template and 18 µl of nuclease free water, performing in condition as follows, an initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 2 min, annealing at 60°C for 1 min and extension at 72°C for 2 min; and a final extension at 72°C for 2 min. The PCR products were run in 1.0% agarose gel electrophoresis and were inspected under UV illuminator. The results from two reactions were interpreted together for identification of SCC*mec* type. The MRSP strains presenting SCC*mec* type III were included to specifically identify SCC*mec* type II-III. In this step, MRSA strains including NCTC 10442, N315, 8512082 and WIS, kindly provided by Professor Dr Keiichi Hiramatsu and Associate Professor Teruyo Ito, was used as positive controls for SCC*mec* type I, II, III and V, respectively. In addition, MRSA carrying SCC*mec* type IV, kindly provided by Assistant Professor Dr Chanwit Tribuddharat, was also used as a positive control.

Table 4.8 Primer sets of multiplex PCR for SCC*mec* type I to V typing (Kondo et al., 2007)

Primer for PCR	Oligonucleotide primer (5'→3')	Gene target	Gene detected by primer pair	Size of product (bp)
Multiplex PCR panel 1 for *ccr* complex identification with *mecA* confirmation				
mA1	TGCTATCCACCCTCAAACAGG	*mecA*	*mecA* (mA1-mA2)	286
mA2	AACGTTGTAACCACCCCAAGA	*mecA*		
α1	AACCTATATCATCAATCAGTACGT	*ccrA1*	*ccrA1-ccrB* (α1-βc)	695
α2	TAAAGGCATCAATGCACAAACACT	*ccrA2*	*ccrA2-ccrB* (α2-βc)	937
α3	AGCTCAAAAGCAAGCAATAGAAT	*ccrA3*	*ccrA3-ccrB* (α3-βc)	1,791
βc	ATTGCCTTGATAATAGCCITCT	*ccrB1,ccrB2 ,ccrB3*		
α4.1	GTATCAATGCACCAGAACTT	*ccrA4*	*ccrA3-ccrB* (α1-βc)	1,287
β4.2	TTGCGACTCTCTTGGCGTTT	*ccrB4*		
γR	CCTTTATAGACTGGATTATTCAAAATAT	*ccrC*	*ccrC* (γF-γR)	518
γF	CGTCTATTACAAGATGTTAAGGATAAT	*ccrC*		
Multiplex PCR panel 2 for *mecA* complex identification				
mI6	CATAACTTCCCATTCTGCAGATG	*mecI*	*mecA-mecI* (mA7-mI6)	1,963
IS7	ATGCTTAATGATAGCATCCGAATG	IS*1272*	*mecA*-IS*1272* upstream of *mecA* (mA7-IS7)	2,827
IS2 (iS-2)	TGAGGTTATTCAGATATTTCGATGT	IS*431*	*mecA*-IS*431* upstream of *mecA* (mA7-iS2(iS-20))	804
mA7	ATATACCAAACCCGACAACTACA	*mecA*		

4.3.2 SCC*mec* type II-III identification

To identify SCC*mec* type II-III, the specific primer set was used for amplification to detect the absence or presence of cadmium resistant operon (Descloux et al., 2008). The primers for SCC*mec* type II-III and interpretation were listed in Table 4.9. In total 25 µl of reaction, the mixture consisted of 12.5 µl of 2X GoTag Green master mix, 0.5 µl of 10 µM of each primers, 1 µl of extracted DNA template and 10 µl of nuclease free water, performing in condition as follows, an initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 2 min, annealing at 54°C for 1 min and extension at 72°C for 2 min and a final extension at 72°C for 2 min.

Table 4.9 Primers for identification of SCC*mec* type II-III (Descloux et al., 2008)

Primer name	Oligonucleotide primer (5'→3')	Size of product (bp)	Interpretation
sccmecIII-F4	AACAGCCATGACAAGCAC	831	absence of cadmium resistant operon (SCC*mec* type II or type II-III)
scc241-F6	AAGACTTAGCAGGAAAACGC	1,118	presence of cadmium resistant operon (SCC*mec* type III)
sccmecIII-R3	TAATGCCCATCATTTCAC		

4.3.3 Identification of non-typeable SCC*mec* in MRSP

The SCC*mec* of MRSP strains that could not be typed by the approved multiplex PCRs were included for novel ψSCC*mec*$_{57395}$ identification (Perreten et al., 2013). The entire ψSCC*mec* from *orfX* region to *attSCC* site was amplified by orfX-R3 and attSCC-R primers (Table 4.10) by using the Expand Long Template PCR System (Roche Applied

Science, Switzerland) according to manufacturer's recommendation in total 30 µl of reaction mixture. This reaction was performed in the specific condition including an initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 30 s and extension at 68°C for 13 min; and a final extension at 68°C for 13 min. To confirm by restriction analysis, the fragment from *orfX* to chromosomal region downstream to 3'-end of the cassette was amplified using the orfX-R3 and contig27-F3 primers (Table 4.10) in the condition as follows, an initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 30 s and extension at 68°C for 16 min; and a final extension at 68°C for 16 min. The PCR products were run in 0.8% agarose gel electrophoresis.

The fragment from *orfX* to *contig-27* was digested by *Bsu*36I restriction enzyme. The 5 µl of long-range PCR product was incubated with 5 U of *Bsu*36I restriction enzyme (New England Biolabs, England) in total 20 µl of reaction at 37°C for 2 h, and the enzyme was inactivated at 80°C for 20 min. MRSP 57395 carrying ψSCC*mec*$_{57395}$ was used as a positive control. The MRSP strains showing positive in both long-range PCRs and *Bsu*36I-digested pattern homology to prototype strain were ψSCC*mec*$_{57395}$-carrying MRSP.

Table 4.10 Primers for ψSCC*mec*$_{57395}$identification (Perreten et al., 2013)

Primer names	Oligonucleotide sequences (5'→ 3')	Product size (bp)
orfX-R3	AGATGAAAAAGCACCCGAAAC	
attSCC-R	ATATGCTTCTGCGTATCG	12,637
contig27-F3	CTTAAATGTCCAATATGTAAACACTC	21,677

4.3.4 Pulsed-fields gel electrophoresis (PFGE)

DNA fingerprints of bacteria were compared by macrorestriction analysis of bacterial chromosome by PFGE. The bacterial cells were grown on TSA with 5% sheep blood for 24 h and were suspended in 3 µl of cell suspension buffer (0.1 M Tris-HCl and 0.1 M EDTA, pH 8.0). The absorbance of bacterial suspension was measured using spectrophotometer at wavelength 610 nm in equivalent to 1.0-1.1. To prepare the cell-embedded agarose plugs, 200 µl of bacterial suspension ware transferred into 1.5 ml microcentrifuge tube and were centrifuged at 10,000xg for 4 min. Then, bacterial cell pellets were collected and were resuspended with 200 µl of TE buffer (0.1 M Tris-HCl and 0.01 M EDTA, pH 8.0). After incubation at 37°C for 15 min, 2 µl of 1 mg/ml lysostaphin solution (Sigma, USA) and 200 µl of 1.8% SeaKem Gold agarose gel (Lonza, USA) were immediately added, and all were mixed well before forming in plug molds (Biorad, USA). The cell-agarose plugs were incubated in 2.8 ml TE buffer with 0.2 µl of 10 mg/ml lyzozyme solution at 37°C for 2 h. Then, these were incubated in 3 ml of lysis buffer (6 mM Tris HCl, 1M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% Sodium deoxycholate and 0.5% Sodium laurylsarcosine) (Sigma, USA) with 15 µl of 20 mg/ml Proteinase K solution at 58°C for 4 h. After that, all plugs were washed in 20 ml of sterile ultrapure water at 58°C for 15 min, 2 times and 15 ml of TE buffer at 58°C for 15 min, 4 times. Before digestion with restriction enzyme, the plugs were cut at 2 mm in size by surgical blade. The chromosomal DNA of bacteria was digested by 30 U of *Sma*I restriction enzyme (New England Biolabs, England) in total volume 20 µl at 25°C for 5 h. For *Sma*I-indigestible strains, 40 U of *Cfr*9I restriction enzyme (Thermo Scientific, USA), the neoschizomer of *Sma*I, was used for chromosomal digestion at 37°C for 5 h. For electrophoresis, the plug slices were embedded in 1.0% PFGE grade agarose gel (Biorad, USA) and run in 0.5X TBE buffer in the specific condition (pulse time 5-40 s, voltage 6 V/cm, temperature 14°C, time 21 h) by using CHEF-DR III (Biorad, USA). Using

as a global standard marker, *Xba*I-digested chromosomal DNA of *Salmonella* Braenderup strain H9812 was used as a reference for pattern normalization.

The DNA in PFGE gels were stained with 300 ml of 1 µg/ml of ethidium bromide solution. The gel images were captured and were documented under UV illuminator by GelDoc 2000 (Biorad, USA). To analyze strain relatedness by pattern similarity, the dendrogram were constructed to group in each species and used enzyme by using UPGMA with optimization at 0.5% and position tolerance at 1.5% by InfoQuest version 4.5 software (Biorad, USA) (Black et al., 2009). The strains exhibiting $\geq$85% similarity were grouped into the same cluster.

4.3.5 Multilocus sequence typing (MLST) for MRSP

Two systems, including MLST-4 and MLST-7, were used for MLST analysis of MRSP. Firstly, 4 genes including *tuf*, *cpn60*, *pta*, and *agrD* were amplified and sequenced for MLST-4 analysis (Bannoehr et al., 2007). The specific primers and product sizes of each gene were shown in Table 4.11. In 50 µl of total volume, the reaction for each gene included 25 µl of 2X GoTaq Green master mix (Promega, USA), 1 µl of 10 µM of each primer, 1 µl of extracted DNA template and 22 µl of nuclease free water. The specific condition for *tuf*, *cpn60*, *pta* amplification was performed as follows, an initial denaturation at 95^{o}C for 2 min; 30 cycles of denaturation at 95 ^{o}C for 1 min, annealing at 55 ^{o}C for 1 min and extension at 72 ^{o}C for 1 min; and a final extension at 72^{o}C for 7 min. The amplification of *agrD* was uniquely performed in condition as follows, an initial denaturation at 94^{o}C for 2 min; 35 cycles of denaturation at 94 ^{o}C for 15 s, annealing at 45 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min; and a final extension at 72^{o}C for 7 minutes.

Table 4.11 Primers for MLST-4 of *S. pseudintermedius* (Bannoehr et al., 2007)

Primer name	Oligonucleotide sequence (5'→3')	Product size (bp)
tuf-F	CAATGCCACAAACTCG	500
tuf-R	GCT TCA GCGTAGTCTA	
cpn60-F	GCGACTGTACTTGCACAAGCA	550
cpn-60-R	AACTGCAACCGCTGTAAATG	
pta-F	GTGCGTATCGTATTACCAGAAGG	570
pta-R	GCAGAACCTTTTGTTGAGAAGC	
agrD-F	GGGGTATTATTACAATCATTC	300
agrD-R	CTGATGCGAAAATAAAGGATTG	

Seven housekeeping genes including *ack*, *cpn60*, *fdh*, *purA*, *pta*, *sar* and *tuf* were used for MLST-7 analysis (Solyman et al., 2013). The *ack*, *fdh*, *purA* and *sar* were additionally amplified in the same condition as *tuf*, *cpn60* and *pta* by specific primers listed in Table 4.12. All genes were sequenced by BigDye Terminator (Qiagen, USA). Nucleotide sequences were submitted to www.pubmlst.org via Professor Dr Vincent Perreten for allelic number and ST assignations.

Table 4.12 Primers for MLST-7 of *S. pseudintermedius* (Solyman et al., 2013)

Primer name	Oligonucleotide sequence (5'→3')	Product size (bp)
ack-F	CACCACTTCACAACCCAGCAAACT	680
ack-R	AACCTTCTAATACACGCGCACGCA	
fdh-F	TGCGATAACAGGATGTGCTT	408
fdh-R	CTTCTCATGATTCACCGGC	
purA-F	GATTACTTCCAAGGTATGTTT	490
purA-R	TCGATAGAGTTAATAGATAAGTC	
sar-F	GGATTTAGTCCAGTTCAAAATTT	521
sar-R	GAACCATTCGCCCCATGAA	

4.3.6 MLST for MRSA

For MRSA, MLST was conducted according to the previous study (Enright et al., 2000). Approved seven housekeeping genes were amplified by PCR with specific primers listed in Table 4.13. The reaction was performed in a specific condition including an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 10 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The amplification of each gene was performed in 50 µl total volume which consisted of 25 µl of 2X GoTaq Green master mix (Promega, USA), 1 µl of 10 µM of each primer, 1 µl of extracted DNA template and 22 µl of nuclease free water. The amplified products at 450-550 bp in range were run in 1.5% agarose gel electrophoresis and were inspected under UV illuminator.

Table 4.13 Primers for MLST of *S. aureus* (Enright et al., 2000)

Primer names	Oligonucleotide sequences (5'→3')
arcC-Up	TTGATTCACCAGCGCGTATTGTC
arcC-Dn	AGGTATCTGCTTCAATCAGCG
aroE-Up	ATCGGAAATCCTATTTCACATTC
aroE-Dn	GGTGTTGTATTAATAACGATATC
glpF-Up	CTAGGAACTGCAATCTTAATCC
glpF-Dn	TGGTAAAATCGCATGTCCAATTC
gmk-Up	ATCGTTTTATCGGGACCATC
gmk-Dn	TCATTAACTACAACGTAATCGTA
pta-Up	GTTAAAATCGTATTACCTGAAGG
pta-Dn	GACCCTTTTGTTGAAAAGCTTAA
tpi-Up	TCGTTCATTCTGAACGTCGTGAA
tpi-Dn	TTTGCACCTTCTAACAATTGTAC
yqiL-Up	CAGCATACAGGACACCTATTGGC
yqiL-Dn	CGTTGAGGAATCGATACTGGAAC

DNA products were purified by Nucleospin® DNA purification kit (Macherey-Nagel, Germany) for nucleotide sequencing that was conducted by BigDye Terminator (Qiagen, USA). Nucleotide sequences were compared with online database at www.mlst.net for allelic number and ST assignations.

CHAPTER V

RESULTS

5.1 Development of biochemical scheme for canine CoPS identification

5.1.1 Verification of CoPS standard strains from animal origins by gene sequencing

By using *sodA* and *cpn60* sequences, the verified standard strains showed >98% nucleotide identity by comparison with type strain of each species. The *sodA* sequence of two *S. aureus* strains from canine origin showed 100% homology to that of *S. aureus* ATCC 12600^{T}. Four *S. pseudintermedius* strains (3 from dogs and 1 from human) had 100% and 98% homology of *sodA* and *cpn60*, respectively, by comparison with those of *S. pseudintermedius* LMG 22219^{T}. For *S. intermedius* from pigeon, this showed 100% nucleotide identity of both genes compared with that of *S. intermedius* ATCC 29663^{T}. The three strains of *S. delphini* from pigeons shared 98-100% similarity of *sodA* and 99% similarity of *cpn60* with those of *S. delphini* ATCC 49171^{T}. All *S. schleiferi* subsp. *coagulans* showed 99% homology of *sodA* to that of *S. schleiferi* subsp. *coagulans* ATCC 49545^{T}. The percentage of nucleotide identity of each strains and GenBank accession numbers are listed in Table 5.1. The phylogenetic trees illustrating relationships among species and strains are shown in Figure 5.3.

Table 5.1 Sequenced genes, origins and GenBank accession no. and percentages of nucleotide identity compared with type strains of coagulase-positive staphylococci isolated from dog, pigeon and human, used as standard controls.

Species	Strains	Animal origins	Gene	GenBank Accession No.	%nucleotide identity with type strain*
Staphylococcus aureus	CVMC 0509	Dog	*sodA*	GU930723	100%
	CVMC 0609	Dog	*sodA*	GU930724	100%
S. pseudintermedius	CVMC 0108	Dog	*sodA*	GU930731	100%
			cpn60	GU930715	98%
	CVMC 0608	Dog	*sodA*	GU930732	100%
			cpn60	GU930716	98%
	CVMC 0708	Dog	*sodA*	GU930733	100%
			cpn60	GU930717	98%
	CVMH 0808	Human	*cpn60*	GU930718	98%
S. schleiferi subsp. *coagulans*	CVMC 0208	Dog	*sodA*	GU930725	99%
	CVMC 0308	Dog	*sodA*	GU930726	99%
	CVMC 0408	Dog	*sodA*	GU930727	99%
	CVMC 0508	Dog	*sodA*	GU930728	99%
	CVMP 0609	Pigeon	*sodA*	GU930729	99%
	CVMP 0709	Pigeon	*sodA*	GU930730	99%
S. intermedius	CVMP 0309	Pigeon	*sodA*	GU930734	100%
		Pigeon	*cpn60*	GU930719	100%

Species	Strains	Animal origins	Gene	GenBank Accession No.	%nucleotide identity with type strain*
S. delphini	CVMP 0109	Pigeon	*sodA*	GU930735	100%
		Pigeon	*cpn60*	GU930720	98%
	CVMP 0209	Pigeon	*sodA*	GU930736	99%
		Pigeon	*cpn60*	GU930721	99%
	CVMP 0409	Pigeon	*cpn60*	GU930722	98%

*type strains, *S. aureus* ATCC 12600^T; *S. pseudintermedius* LMG22219^T; *S. schleiferi* subsp. *coagulans* ATCC 49545^T; *S. intermedius* ATCC 29663^T; *S. delphini* ATCC 49171^T.

5.1.2 Canine CoPS isolates for determination of biochemical properties

Totally isolated from 66 dogs, 337 CoPS isolates (164 from the nasal mucosa of 61 dogs, 124 from perineal area of 47 dogs and 49 from the groin of 15 dogs) were included for determination of biochemical properties. After incubation at 37 °C for 24 h, all CoPS colonies exhibited colony morphology of staphylococci and various hemolytic patterns. The 300 isolates showed double hemolytic zones comprising an outer zone of α-hemolysis and an inner zone of β-hemolysis. The other 37 isolates only showed α-hemolysis. Within 48 h, all showed strong β-hemolysis with variations of diameter sizes without the double zone. All selected CoPS were gram-positive cocci, positive for catalase production and glucose fermentation but were negative in the motility test and the oxidase production. Additionally, all CoPS isolates were positive in the tube coagulase test.

5.1.3 Genotypic species identification by multiplex PCR

Using the DNA extraction kit, five isolates demonstrated a specific band at 359 bp for *S. aureus*, 69 isolates presented a specific band at 529 bp for *S. schleiferi* subsp. *coagulans* and the other 263 was identified as *S. pseudintermedius* on the basis of exhibiting a band at 926 bp of *nuc* gene (Figure 5.1). Using the boiling method, the results of all *S. aureus* and the 50 isolates of *S. schleiferi* subsp. *coagulans* were consistent with those obtained with the extraction kit, but the other 283 isolates did not demonstrate only the expected bands and showed non-specific bands. Ambiguous results also were carried out using the species-specific single PCRs. The numbers of positive results are shown in Table 5.2. On the other hand, the control strains of *S. aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans* and *S. delphini* but not *S. pseudintermedius* showed correct DNA bands using extracted DNA by both techniques. Negative results were obtained with *S. epidermidis* and the blank control.

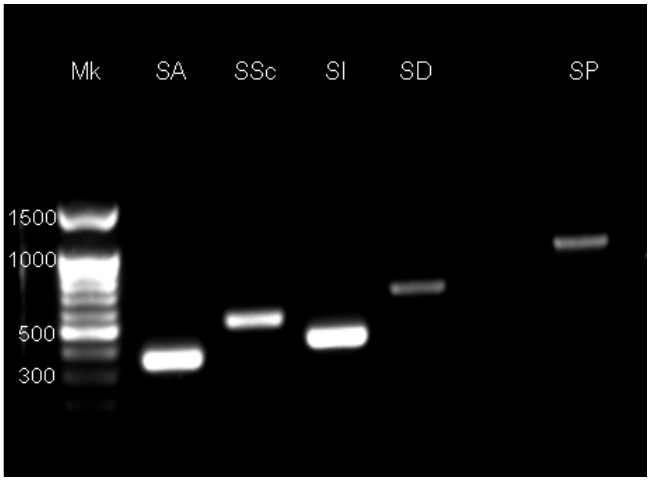


Figure 5.1 Product sizes of *nuc* fragment amplified by multiplex PCR for CoPS identification (Mk, 100 bp marker; SA, *S. aureus* CVMC 0609 (359 bp); *S. schleiferi* subsp. *coagulans* CVMC0208 (526 bp); *S. intermedius* CVMP 0309 (430 bp); *S. delphini* CVMP 0109 (661 bp); and *S. pseudintermedius* CVMC0108 (926 bp)

5.1.4 Biochemical properties

The percentages of positive result of the biochemical tests for the 337 canine CoPS are shown in Table 5.2. Variable results obtained for biochemical properties using in-house methods and commercial kits from previous reports are also shown in the Table 5.2. The 74 canine CoPS were positive in acetoin production, whereas the *S. intermedius* group (SIG) including *S. pseudintermedius*, *S. delphini* and *S. intermedius* were negative. All the canine CoPS were positive in the galactose assimilation test. The 263 *S. pseudintermedius* and five *S. aureus* isolates were positive in the maltose, trehalose and lactose assimilation tests. The positives were only detected using purple broth, but all showed negative results by using the agar base for assimilation of all carbohydrates. Moreover, the reactions in broth base showed strong positive reactions within 48 h of incubation. All *S. pseudintermedius* were negative in mannitol fermentation, but 11.6% of *S. schleiferi* subsp. *coagulans* showed positive in this test. The proposed scheme of biochemical set for canine CoPS identification is demonstrated in Table 5.3.

Table 5.2 Numbers of positive isolate tested by biochemical tests and multiplex PCR for CoPS identification, and comparison with the results of previous studies

				No. of positive isolates (%)		
	S. aureus ATCC 25923	*S. intermedius* (n = 1)	*S. delphini* (n = 2)	*S. pseudintermedius* (n=263)	*S. schleiferi* subsp. *coagulans* (n=69)	*S. aureus* (n=5)
PCR (DNA extracted by boiling method)						
M-PCR	+	+	+	0[a]	50 (72.5)[b]	5 (100)
Single PCR	+	+	+	263 (100)	69 (100)	5 (100)
M-PCR (DNA extracted by Nucleospin® tissue for nucleic acid and protein purification)	+	+	+	263 (100)	69 (100)	5 (100)

					No. of positive isolates (%)		
		S. aureus ATCC 25923	*S. intermedius* (n = 1)	*S. delphini* (n = 2)	*S. pseudintermedius* (n=263)	*S. schleiferi* subsp. *coagulans* (n=69)	*S. aureus* (n=5)
Primary test							
	Gram-positive staining	+	+	+	263 (100)	69 (100)	5 (100)
	Motility	-	-	-	0	0	0
	Oxidation-fermentation (F)	+	+	+	263 (100)	69 (100)	5 (100)
	Catalase	+	+	+	263 (100)	69 (100)	5 (100)
	Oxidase	-	-	-	0	0	0
Secondary test							
	Coagulase	+	+	+	263 (100)	69 (100)	5 (100)
	Acetoin production	+	-	-	0	69 (100)	5 (100)
	Maltose Broth	+	+	+	263 (100)	0	5 (100)
	Agar	-	-	-	0	0	0
	Galactose Broth	+	-	+	263 (100)	69 (100)	5 (100)
	Agar	-	-	-	0	0	0
	Trehalose Broth	+	+	+	263 (100)	0	5 (100)
	Agar	-	-	-	0	0	0
	Lactose Broth	+	+	+	263 (100)	0	5 (100)
	Agar	-	-	-	0	0	0
	Mannitol fermentation test	+	+	-	0	8 (11.6)	4 (80.0)

Identification kits**	*S. aureus* ATCC 12598	No. of positive isolates (%)			
Total		(n=12)	(n=17)	(n=83)	(n=16)
Glucose	+	12 (100)	17 (100)	83 (100)	16 (100)
Fructose	+	12 (100)	17 (100)	83 (100)	16 (100)
Mannose	+	12 (100)	17 (100)	83 (100)	16 (100)
Maltose	+	11 (91.7)	16 (94.1)	75 (90.4)	0
Lactose	+	7 (75.0)	16 (94.1)	83 (100)	0
Trehalose	+	12 (100)	16 (94.1)	83 (100)	0
Mannitol	+	12 (100)	12 (70.6)	38 (45.8)	0
Raffinose	+	0	0	0	0
Alkaline phosphatase	+	12 (100)	17 (100)	82 (98.8)	16 (100)
Acetoin	+	0	0	8 (9.6)	16 (100)
Nitrate reduction	+	12 (100)	17 (100)	83 (100)	16 (100)
Urease	+	12 (100)	15 (88.2)	83 (100)	16 (100)
Galactose	+	0[c]	16 (94.1)[c]	76 (91.6)[c]	0
Mannitol fermentation***	ND	0	0	0	ND

[a] negative, presence of at least three multiple bands of approximately 900, 700 and 550 bp

[b] negative; presence of non specific band of approximately 350 bp

[c] galactose assimilation tested by microbroth

*control strains in this study

**results from previous studies: *S. aureus* and *S. schleiferi* subsp. *coagulans* (Zdovc et al., 2004), *S. pseudintermedius*, *S. delphini* and *S. intermedius* (Sasaki et al., 2007)

*** by conventional technique

ND, not determined

Table 5.3 Proposal for biochemical patterns to be used for routine canine CoPS identification

Species	Primary test				Secondary test									
									Carbohydrate assimilation test in broth					
	Gram-positive cocci staining	Motility	Oxidation-fermentation	Catalase	Oxidase	Coagulase	Acetoin prodction	maltose	galactose	trhalose	lactose	Mannitol fermentation
S. aureus	+	-	F	+	-	+	+	+	+	+	+	+*
S. pseudintermedius	+	-	F	+	-	+	-	+	+	+	+	-
S. schleiferi subsp. *coagulans*	+	-	F	+	-	+	+	-	+	-	-	+**

*over 80% positive

**over 85% negative

5.1.5 Protein pattern analysis

Each CoPS including *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans*, *S. aureus*, *S. intermedius* and *S. delphini* had a unique whole cell protein profile (Figure 5.2). The molecular weights and similarities of protein bands are shown in Table 5.4. By cluster analysis from protein pattern, the CoPS species were categorized in different clades that were consistent to those of phylogenetic tree based on *sodA* and *cpn60* gene analysis (Figure 5.3). The three canine CoPS were clearly differentiated from *S. intermedius* and *S. delphini*, and prominent interesting bands ranging from 32 to 62 kDa had high discrimination for species specific (Figure 5.2). Overall, marked intra-species

variations of protein bands were not observed. This phenotypic characteristic for the 337 canine CoPS was strongly relevant to the results obtained from biochemical tests and multiplex PCR.

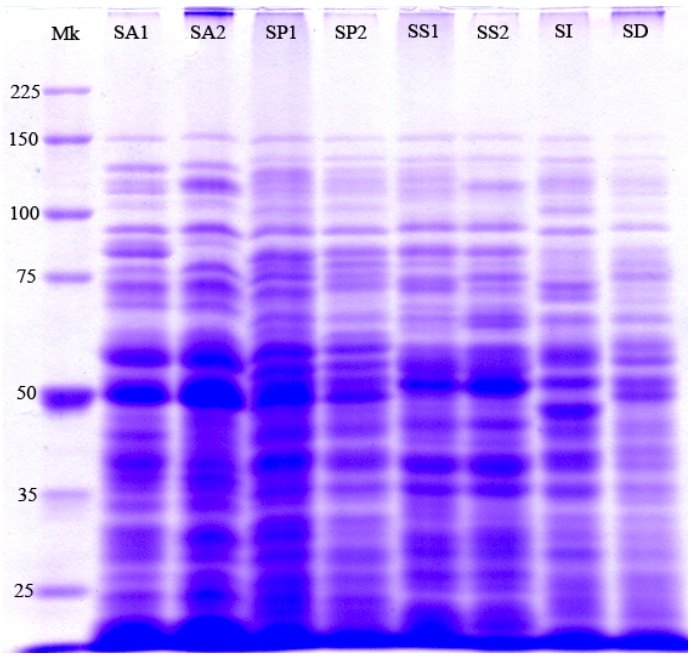
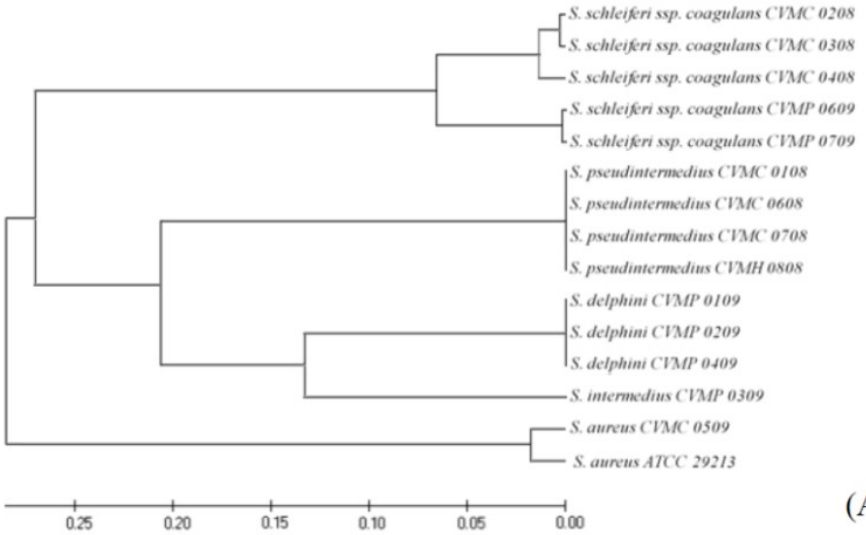


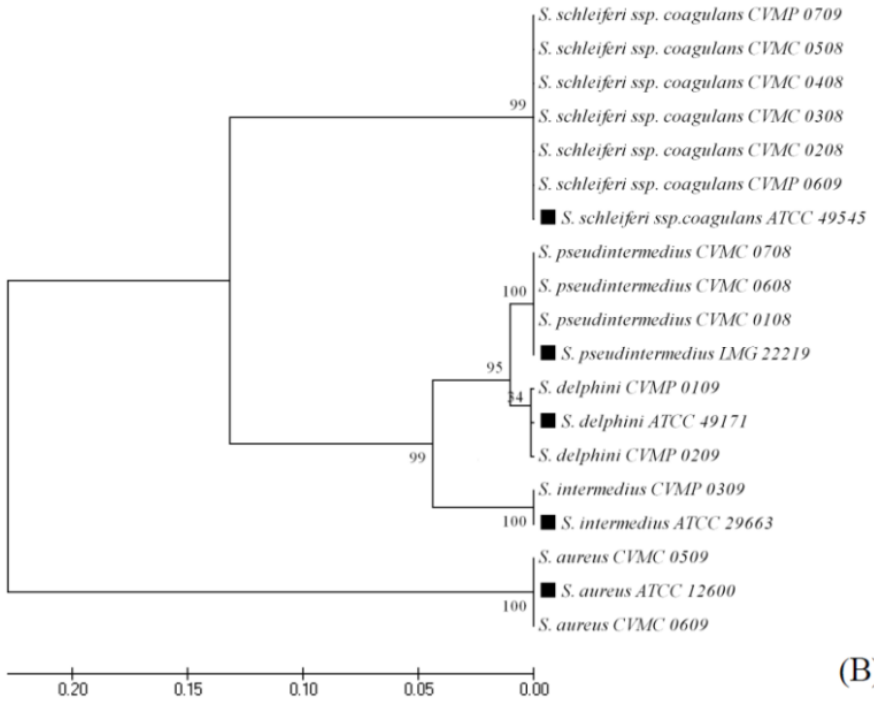
Figure 5.2 The comparison of protein patterns among canine CoPS on dog skin by SDS-PAGE. Mk indicates molecular weight makers (kDa). Lanes: (SA1) *S. aureus* ATCC 29213; (SA2) *S. aureus* CVMC 0609; (SP1) *S. pseudintermedius* CVMC 0108; (SP2) *S. pseudintermedius* CVMC 0708; (SS1) *S. schleiferi* subsp. *coagulans* CVMC 0208; (SS2) *S. schleiferi* subsp. *coagulans* CVMC 0408; (SI) *S. intermedius* CVMP 0309; and (SD) *S. delphini* CVMP 0109.

Table 5.4 Molecular weight of protein bands (kDa) of CoPS illustrated in Figure 5.2.

Lanes / Band no.	SA1	SA2	SP1	SP2	SS1	SS2	SI	SD
1	149.1	149.1	149.1	149.1	149.1	149.1	149.1	149.1
2	128.9	128.9	135.0	135.0	135.0	135.0	135.0	135.0
3	117.4	117.4	124.7	124.7	126.4	115.8	126.4	126.4
4	110.4	110.4	118.2	118.2	119.0	92.4	119.0	119.8
5	104.7	104.7	111.9	111.9	111.2	84.1	113.5	113.5
6	92.4	92.4	100.0	100.0	92.4	80.0	102.0	102.0
7	87.5	87.5	91.9	91.9	84.1	75.0	92.4	93.9
8	83.2	77.5	83.2	83.2	80.0	72.3	84.1	84.1
9	77.5	72.8	78.7	78.7	74.2	68.3	79.6	79.6
10	72.8	68.5	74.7	74.7	72.3	66.2	75.0	75.0
11	68.5	62.2	72.0	72.0	68.3	64.3	72.0	72.0
12	62.2	57.9	66.4	66.4	65.9	59.1	70.8	70.8
13	57.9	50.0	62.2	62.2	64.3	57.4	65.6	65.6
14	50.0	45.9	59.1	59.1	59.1	55.7	57.6	59.6
15	45.9	43.3	55.7	55.7	57.4	52.1	52.8	56.9
16	43.3	39.6	52.8	52.8	55.7	47.4	47.4	52.8
17	39.6	37.2	50.0	50.0	52.1	45.1	44.2	50.0
18	37.2	35.5	47.4	47.4	47.4	41.9	43.9	45.1
19	35.5	33.7	43.9	43.9	45.1	38.7	39.2	43.7
20	33.7	30.7	39.0	39.0	41.9	35.5	35.5	40.4
21	30.7	29.6	35.5	35.5	38.7	33.4	32.2	39.0
22	29.6	28.5	34.1	34.1	35.5	32.1	30.3	35.5
23	28.5	26.4	32.2	32.2	33.4	30.1	28.5	34.1
24	26.4	24.6	30.7	30.7	32.1	28.5	26.4	32.2
25	24.6	23.6	28.5	28.5	30.1	26.4	24.4	30.3
26	23.6		26.4	26.4	28.5	25.6	23.3	28.5
27			24.4	24.4	26.4	23.6		26.4
28			23.3	23.3	25.6	22.7		24.4
29					23.55			23.31
30					22.71			


S. schleiferi ssp. coagulans CVMC 0208
S. schleiferi ssp. coagulans CVMC 0308
S. schleiferi ssp. coagulans CVMC 0408
S. schleiferi ssp. coagulans CVMP 0609
S. schleiferi ssp. coagulans CVMP 0709
S. pseudintermedius CVMC 0108
S. pseudintermedius CVMC 0608
S. pseudintermedius CVMC 0708
S. pseudintermedius CVMH 0808
S. delphini CVMP 0109
S. delphini CVMP 0209
S. delphini CVMP 0409
S. intermedius CVMP 0309
S. aureus CVMC 0509
S. aureus ATCC 29213
0.25
0.20
0.15
0.10
0.05
0.00

(A)


S. schleiferi ssp. coagulans CVMP 0709
S. schleiferi ssp. coagulans CVMC 0508
S. schleiferi ssp. coagulans CVMC 0408
99
S. schleiferi ssp. coagulans CVMC 0308
S. schleiferi ssp. coagulans CVMC 0208
S. schleiferi ssp. coagulans CVMP 0609
S. schleiferi ssp.coagulans ATCC 49545
S. pseudintermedius CVMC 0708
100
S. pseudintermedius CVMC 0608
S. pseudintermedius CVMC 0108
S. pseudintermedius LMG 22219
95
S. delphini CVMP 0109
84
S. delphini ATCC 49171
S. delphini CVMP 0209
99
S. intermedius CVMP 0309
100
S. intermedius ATCC 29663
S. aureus CVMC 0509
S. aureus ATCC 12600
100
S. aureus CVMC 0609
0.20
0.15
0.10
0.05
0.00

(B)

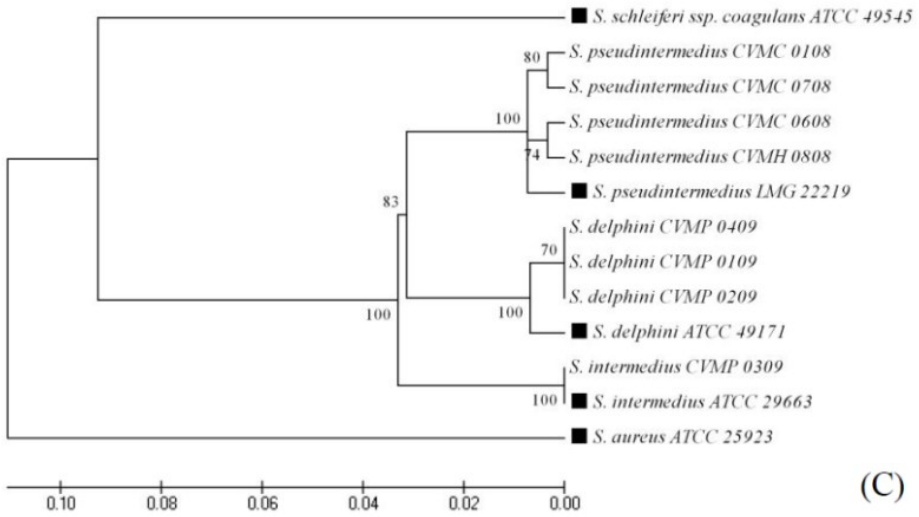


Figure 5.3 The cluster analysis of CoPS (A) Dendrogram based on protein pattern analysis (B) Phylogenetic tree based on *sodA* sequence analysis (C) Phylogenetic tree based on *cpn*60 sequence analysis. (■, type strains from GenBank database)

5.2 Prevalence of CoPS in dogs and dog-associated people

5.2.1 Descriptive characteristics of populations

Totally, 100 owned-dogs were included in dog population, and the characteristics of dogs were illustrated in Table 5.5. The control group of human population included 100 Thai people without dog and cat in their households and without contact with dog and cat during 3-6 months. The ages of this group were 22 to 25 years old in range. For dog's owners, 100 dog's owners were included, and the pair sampling was performed in the 58 owners from 50 households together with their dogs. Additionally, 200 Thai small animal practitioners were included in group of veterinarian. The characteristics of dog's owners and veterinarians were shown in Table 5.6 and 5.7, respectively.

Table 5.5 Descriptive characteristics of dogs

	No. (%)	
Characteristics of dogs (n=100)	Positive	No data
Sex		14 (14.0)
Female	42 (42.0)	
Male	44 (44.0)	
Age		15 (15.0)
<3 months	2 (2.0)	
3-12 months	6 (6.0)	
1-5 years	41 (41.0)	
6-10 years	30 (30.0)	
>10 years	6 (6.0)	
Breed		11 (11.0)
Basset Hound	1 (1.0)	
Beagle	1 (1.0)	
Boxer	1 (1.0)	
Chihuahua	2 (2.0)	
Dalmatian	2 (2.0)	
English Cocker Spaniel	10 (10.0)	
French Bulldog	3 (3.0)	
Golden Retriever	7 (7.0)	
Jack Russell	1 (1.0)	
Miniature Pincher	4 (4.0)	
Mongrel	22 (22.0)	
Pomeranian	5 (5.0)	
Poodle	11 (11.0)	
Pug	2 (2.0)	
Shih Tzu	9 (9.0)	
Siberian Husky	2 (2.0)	
Saint Bernard	1 (1.0)	
Thai Bangkaew	3 (3.0)	
West Highland White Terrier	2 (2.0)	

Characteristics of dogs (n=100)	No. (%)	
	Positive	No data
No antimicrobial exposure	39 (39.0)	0
Last antimicobial exposure within 6 months	42 (42.0)	0
Amoxycillin-clavulanic acid	3 (3.0)	
Clindamycin	1 (1.0)	
Doxycycline	1 (1.0)	
First generation cephalosprins	39 (39.0)	
Fluoroquinolones (enrofloxacin and marbofloxacin)	9 (9.0)	
Last antimicrobial exposure during 6-12 months	19 (19.0)	0
Amoxycillin-clavulanic acid	5 (5.0)	
Clindamycin	5 (5.0)	
Doxycycline	4 (4.0)	
First generation cephalosporins	12 (12.0)	
Fluoroquinolones (enrofloxacin, marbofloxacin and norfloxacin)	7 (7.0)	
Sulfamethoxazole	2 (2.0)	

Table 5.6 Descriptive characteristics of dog's owners

	No. (%)	
Characteristics of dog's owners (n=100)	Positive	No data
Sex		0
Female	63 (63.0)	
Male	37 (37.0)	
Age (year)		0
≤25	49 (49.0)	
26-30	12 (12.0)	
31-35	8 (8.0)	
36-40	5 (5.0)	
41-45	6 (6.0)	
46-50	2 (2.0)	
≥51	18 (18.0)	
Sharing area in household with dog		3 (3.0)
Allow dog living outside the house only	26 (26.0)	
Allow dog living in house	44 (44.0)	
Allow dog to rest in bedroom	27 (27.0)	
Pattern of contact		3 (3.0)
No usual contact	4 (4.0)	
Touching	23 (23.0)	
Allow licking hand	38 (38.0)	
Allow licking face and/or kissing	32 (32.0)	

Table 5.7 Descriptive characteristics of veterinarians

Characteristics of veterinarians (n=200)	No. (%)	
	Positive	No data
Sex		0
Female	115 (57.5)	
male	85 (42.5)	
Age (year)		0
≤25	35 (17.5)	
26-30	92 (46.0)	
31-35	30 (15.0)	
36-40	26 (13.0)	
41-45	7 (3.5)	
46-50	4 (2.0)	
≥51	6 (3.0)	
Job		0
Full time	156 (78.0)	
Part time	44 (22.0)	
Dog case(s)/day		0
≤1	9 (4.5)	
2-5	16 (8.0)	
6-10	76 (38.0)	
≥10	99 (49.5)	
Hygiene		0
Mask	18 (9.0)	
Gloves	32 (16.0)	
Hand washing	182 (91.0)	

5.2.2 Prevalence canine CoPS colonization and their distribution on dog skin

All dogs were colonized with CoPS at least one site of sampling. CoPS could be isolated from 100% of dog at nasal mucosa and groin. The *S. pseudintermedius* was the most prevalence in dog that could be obtained from at least one site of sampling. *S. schleiferi* subsp. *coagulans* was moderately isolated in prevalence at 28%. On the other hand, *S. aureus* could be recovered from only nasal mucosa of dogs at 4%. Regarding methicillin resistance, dogs were colonized with MRSP, MRSSc and MRSA in prevalence at 45%, 17% and 1%, respectively. Also, MRSP and MRSSc could be isolated from all sites of collection, but MRSA was only isolated from nasal mucosa. The prevalence and numbers of dog with CoPS colonization are shown in Table 5.8.

5.2.3 Prevalence of CoPS colonization in humans

CoPS could be isolated from 25%, 37% and 25% of small animal veterinarian, dog's owner and people without dog and cat association, respectively. Only veterinarians and dog's owners were colonized with *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans*, but these two species could not be isolated from control people. Concerning methicillin-resistant trait, MRCoPS could be isolated from veterinarians and dog's owners but not from control people also. Therefore, only methicillin-susceptible *S. aureus* was obtained from control people. The prevalences and numbers of people and dog with CoPS colonization categorized into species are shown in Table 5.9.

Table 5.8 Prevalence of CoPS colonization in dogs and distribution on different anatomical sites

Species*	Dog, no. (%)			
	All sites	Nasal mucosa	Perineum	Groin
Total	100	100	90	16
CoPS	100 (100)	86 (86.0)	78 (86.7)	16 (100)
Single MSCoPS	52 (52.0)	47 (47.0)	40 (44.4)	4 (25.0)
Single MRCoPS	29 (29.0)	32 (32.0)	30 (33.3)	9 (56.3)
Mixed MRCoPS and MSCoPS	19 (19.0)	7 (7.0)	8 (8.9)	3 (18.75)
S. pseudintermedius	100(100)	83 (83.0)	73 (81.1)	16 (100)
Single MSSP	55 (55.0)	47 (47.0)	37 (41.1)	4 (25.0)
Single MRSP	29 (29.0)	32 (32.0)	29 (32.2)	9 (56.3)
Mixed MRSP and MSSP	16 (16.0)	4 (4.0)	7 (7.8)	3 (18.8)
S. schleiferi subsp. *coagulans*	29 (29.0)	18 (18.0)	18 (20.0)	6 (37.5)
Single MSSSc	12 (12.0)	6 (6.0)	6 (6.7)	2 (12.5)
Single MRSSc	17 (17.0)	12 (12.0)	12 (13.3)	4 (25.0)
Mixed MRSSc and MSSSc	0	0	0	0
S. aureus	4 (4.0)	4 (4.0)	0	0
Single MSSA	3 (3.0)	3 (3.0)	0	0
Single MRSA	1 (1.0)	1 (1.0)	0	0
Mixed MRSA and MSSA	0	0	0	0

*CoPS, coagulase-positive staphylococci; MSCoPS, methicillin-susceptible coagulase-positive staphylococci; MRCoPS, methicillin-resistant coagulase-positive staphylococci, MRSP, methicillin-resistant *S. pseudintermedius*; MSSP, methicillin-susceptible *S. pseudintermedius*, MRSSc, methicillin-resistant *S. schleiferi* subsp. *coagulans*; MSSSc, methicillin-susceptible *S. schleiferi* subsp. *coagulans*; MRSA, methicillin-resistant *S. aureus*; and MSSA, methicillin-susceptible *S. aureus*

Table 5.9 Prevalence and species distribution of CoPS categorized into species and methicillin-resistant traits in Thai dogs and dog-associated people

	Dog, no. (%)	Human, no. (%)		
		Control people	Dog-associated people	
			Veterinarian	Dog's owner
Total	100	100	200	100
S. pseudintermedius	100 (100)	0	21(10.50)	13 (13.00)
Single MSSP	55 (55.00)	0	5 (2.50)	10 (10.00)
Single MRSP	29 (29.00)	0	16 (8.00)	2 (2.00)
Mixed MR and MSSP	16 (16.00)	0	0	1 (1.00)
S. schleiferi subsp. *coagulans*	29 (29.00)	0	8 (4.00)	4 (4.00)
Single MSSSc	12 (12.00)	0	2% (4)	2 (2.00)
Single MRSSc	17 (17.00)	0	2% (4)	2 (2.00)
Mixed MR and MSSP	0	0	0%	0
S. aureus	4 (4.00)	25 (25.00)	24 (12.00)	20 (20.00)
Single MSSA	3 (3.00)	25 (25.00)	21 (10.50)	20 (20.00)
Single MRSA	1 (1.00)	0	3 (1.50)	0
Mixed MR and MSSA	0	0	0	0

*CoPS, coagulase-positive staphylococci; MSCoPS, methicillin-susceptible coagulase-positive staphylococci; MRCoPS, methicillin-resistant coagulase-positive staphylococci, MRSP, methicillin-resistant *S. pseudintermedius*; MSSP, methicillin-susceptible *S. pseudintermedius*, MRSSc, methicillin-resistant *S. schleiferi* subsp. *coagulans*; MSSSc, methicillin-susceptible *S. schleiferi* subsp. *coagulans*; MRSA, methicillin-resistant *S. aureus*; and MSSA, methicillin-susceptible *S. aureus*

5.3 Antimicrobial resistance of MRCoPS isolated from dogs and dog-associated people

5.3.1 Antimicrobial resistance of MRCoPS by MIC determination

Totally, the 113 MRCoPS [83 MRSP (64 from dogs, 16 from veterinarians and 3 from dog's owners), 26 MRSSc (20 from dogs, 4 from veterinarians and 2 from dog's owners) and 4 MRSA (1 from a dog and 3 from veterinarians) were included for characterization of antimicrobial resistance and genetic features.

The 82 [(98.8%); 63 from dogs, 16 from veterinarians and 3 from owners] of 83 MRSP exhibited resistance to oxacillin and penicillin. For aminoglycoside resistance, 81 MRSP [(97.6%); 62 from dogs, 16 from veterinarians and 3 from owners] resisted to gentamicin and kanamycin, and 80 MRSP [(96.4%); 61 from dogs, 16 from veterinarians and 3 from owners] also resisted to streptomycin. Tetracycline and sulfamethoxazole resistance was detected in 82 MRSP [(98.8%); 63 from dogs, 16 from veterinarians and 3 from owners]. The 80 MRSP [(96.4%); 61 from dogs, 16 from veterinarians and 3 from owners] exhibited erythromycin and clindamycin resistance, and 10 isolates of these [(12.0%); 8 from dogs and 2 from veterinarians] expressed inducible clindamycin resistance. Resistance to ciprofloxacin, chloramphenicol and trimethoprim was detected in 69 isolates [(83.1%); 50 from dogs, 16 from veterinarians and 3 from owners], 46 isolates [(55.4%); 39 from dogs and 7 from veterinarians] and 39 isolates [(47%); 25 from dogs, 11 from veterinarians and 3 from owners], respectively. Only 3 canine MRSP isolates were mupirocin resistance. No MRSP exhibited resistance to vancomycin, linezolid, rifampicin, fusidic acid, tiamulin and quinupristin/dalfopristin. The MIC values and numbers of resistant isolates of MRSP are summarized in Table 5.10.

For MRSSc, oxacillin and penicillin resistance was detected in 24 isolates [(92.3%), 20 from dogs, 3 from veterinarians and 1 from an owner] and 13 isolates [(50%); 9 from dogs, 2 from veterinarian and 2 from owners], respectively. The 20 MRSSc [(76.9%); 15 from dogs, 3 from veterinarians and 2 from owners] exhibited

tetracycline resistance. Kanamycin, streptomycin, erythromycin and clindamycin resistance was detected in 10 MRSSc [(38.5%); 7 from dogs, 1 from a veterinarian and 2 from owners]. Gentamicin and sulfamethoxazole resistance was detected in 8 isolates [(30.8%); 7 from dogs, 1 from an owner]. Mupirocin and ciprofloxacin resistance was detected in 6 isolates [(23.1%); 5 from dogs and 1 from an owner] and 1 canine isolates (3.8%), respectively. No MRSSc showed resistance to chloramphenicol, trimethoprim, vancomycin, linezolid, rifampicin, fusidic acid, quinupristin/dalfopristin and tiamulin. The MIC values and numbers of resistant isolates of MRSSc are summarized in Table 5.11.

All MRSA (100%) exhibited resistance to oxacillin, penicillin, tetracycline, gentamicin, kanamycin, streptomycin, clindamycin, trimethoprim and quinupristin/dalfopristin. Chloramphenicol was detected in 2 isolates [(50%); 1 from a dog and 1 from a veterinarian], and mupirocin resistance was detected in 1 isolates (25%) from a dog. All MRSA were susceptible to erythromycin, vancomycin, rifampicin, linezolid and fusidic acid. Interestingly, MRSA showed macrolide-susceptible and lincosamide-resistant (M^S/L^R) phenotype. All isolates presented high level of MIC value of tiamulin (>4 mg/l) and viginiamycin M1 (512 mg/l). The MIC values of MRSA are shown in Table 5.12.

5.3.2 Presence of genes mediating resistance to various antimicrobial classes

Using microarray, *mecA* and *blaZ* were detected in all MRSP (100%). Mediating tetracycline resistance, *tet*(M), *tet*(K) and *tet*(L) were detected in 81 isolates [(97.6%); 62 from dogs, 16 from veterinarians and 3 from owners], 7 isolates [(7.2%), 6 from dogs and 1 from a veterinarian] and 1 isolate from a dog (1.2%), respectively. For aminoglycoside resistance, bifunctional *aac(6')-Ie-aph(2')-Ia* was detected in 81 MRSP expressing gentamicin and kanamycin resistance, and *aph(3')-III* and *ant(6')-Ia* were detected in 80 MRSP expressing kanamycin and streptomycin resistance. Regarding

erythromycin and lincosamide resistance, *erm*(B) was carried by these 80 MRSP (80.6%), and *erm*(C) was detected in only 1 isolate from a dog (1.2%). Additionally, cat_{pC221} was detected in 46 MRSP [(55.4%); 39 from dogs and 7 from dogs] with chloramphenicol resistance, and *dfrG* was detected 39 MRSP expressing trimethoprim resistance. Only 3 isolates from dogs expressing mupirocin resistance were identified as *mupR*-positive MRSP. The numbers of MRSP and the resistance genes and phenotypes are summarized in Table 5.16.

For MRSSc, *mecA* was detected in 26 isolates (100%), and *blaZ* was detected in only 12 isolates with penicillin resistance. The *aph(3')-III*, *ant(6')-Ia* and *erm*(B) were detected in 10 MRSSc (38.5%) presenting resistance to kanamycin, streptomycin, erythromycin and clindamycin that 1 canine isolate (3.9%) also carried *erm*(C). The *aac(6')-Ie-aph(2')-Ia* was identified in 8 isolates (30.8%) with gentamicin resistance. Of 20 tetracycline-resistant MRSSc, *tet*(K) was detected in 12 isolates [(46.2%); 10 isolates from dogs and 2 isolates from veterinarians), and *tet*(O) was detected in 10 isolates [(38.5%); 7 from dogs, 1 from a veterinarian and 2 from owners). Of these, only 2 canine isolates were carried both *tet*(K) and *tet*(O). The *mupR* was detected in 8 isolates (30.8%). The numbers of MRSSc isolates with the resistance genes and the resistant phenotypes are summarized in Table 5.17.

The *mecA* and *blaZ* were detected in all MRSA (100%). As regards to aminoglycoside resistance, bifunctional *aac(6')-Ie-aph(2')-Ia* and *ant(6')-Ia* were detected in all MRSA as same as MRSP and MRSSc. Additionally, *ant(4')-Ia* was identified in all MRSA, but *aph(3')-III* could not be detected in MRSA. Both *tet*(M) and *tet*(L) were carried in all isolates. Mediating resistance to lincosamide, *lnu*(B) were detected at all. As for phenicol resistance, *fexA* was detected in 2 MRSA exhibiting chloramphenicol resistance. The *mupR* was also detected in 1 canine isolate. By specific PCR, all MRSA (100%) carried *lsa*(E) mediating resistance to streptogramin A

and pleuromutilin. The numbers of MRSA with the resistance genes and the resistant phenotypes were summarized in Table 5.18. The positive resistance genes are demonstrated in Figure 5.4.

Table 5.10 MIC of 19 antimicrobial drugs of MRSP isolates with the presence of resistance genes

	Resistance Breakpoint (mg/l)*	Range tested (mg/l)	MIC (mg/l)															Number of resistant isolates (%)	Resistance-mediating gene(s)	Numbers of isolates with the presence of resistance genes
			≤0.015	0.03	0.06	0.12	0.25	0.5	1	≥2	≥4	≥8	≥16	≥32	≥64	≥128	≥256	≥512			
Oxacillin with 2% NaCl	>0.25	0.25-8					1	11	13	1		57							82 (98.8%)	*mecA*	83
Penicillin	>0.25	0.012-2				1				82									82 (98.8%)	*blaZ*	83
Ciprofloxacin	>1	0.25-8					2	10	2	1		68							69 (83.1%)	ND	ND
Tetracycline	>2	0.5-16						1					82						82 (98.8%)	*tet*(M); *tet*(K); *tet*(L)	81; 7; 1
Gentamicin	>1	1-16							2	3	8	9	61						81 (97.6%)	*aac(6')-Ie-aph(2')-Ia*	81
Kanamycin	>16	4-64									2				81				81 (97.6%)	*aac(6')-Ie- aph(2')-Ia*; *aph(3')-III*	81; 80
Streptomycin	>16	4-32									3			80					80 (96.4%)	*ant(6')-Ia*	80
Erythromycin	>2	0.25-8					3					80							80 (96.4%)	*erm*(B); *erm*(C)	80; 1
Clindamycin	>0.5	0.12-4				10	2	3			70								80* (96.4%)	*erm*(B); *erm*(C)	80; 1
Chloramphenicol	>8	4-64									14	23			46				46 (55.4%)	cat_{pC221}	46
Sulfamethoxazole	>256	64-512															1	82	82 (98.8%)	ND	ND
Trimethoprim	>4	2-32								30	14			39					39 (47.0%)	*dfrG*	39
Mupirocin	>256	0.5-2 and 256						79		1							3		3 (3.6%)	*mupR*	3
Vancomycin	>2	1-16							83										0	0	0
Rifampicin	>0.5	0.015-0.5	83																0	0	0
Linezolid	>4	1-8							83										0	0	0
Fusidic acid	>1	0.5-4						83											0	0	0
Quinupristin/dalfopristin	>2	0.5-4						83											0	0	0
Tiamulin	NA	0.5-4						83											0	0	0

*Breakpoints were recommended by EUCAST version 2.0. The breakpoints kanamycin, streptomycin and sulfamethoxazole were recommended by French Society for Microbiology in 2010.

Highlight, level of concentration more than clinical breakpoints referring resistance

NA, not available

ND, not determined

*10 isolates presenting inducible clindamycin resistance by D test

Table 5.11 The MIC of 19 antimicrobial drugs for MRSSc with the presence of resistance genes

	Resistance Breakpoint (mg/l)*	Range tested (mg/l)	MIC (mg/l)															Number of resistant isolates (%)	Resistance-mediating genes()	Numbers of isolates with the presence of resistance genes
			≤0.015	0.03	0.06	0.12	0.25	0.5	1	≥2	≥4	≥8	≥16	≥32	≥64	≥128	≥256	≥512			
Oxacillin with 2% NaCl	>0.25	0.25-8					2	14	10										24 (92.3%)	*mecA*	26
Penicillin	>0.125	0.0125-2				13	1	12											13 (50.0%)	*blaZ*	12
Ciprofloxacin	>1	0.25-8					9	6	10	1									1 (3.8%)	ND	ND
Tetracycline	>2	0.5-16						6			1		19						20 (76.9%)	*tet*(K); *tet*(O)	12; 10
Gentamicin	>1	1-16							18		1	1	6						8 (30.8%)	*aac(6')-Ie-aph(2')-Ia*	8
Kanamycin	>16	4-64									16				10				10 (38.5%)	*aac(6')-I-aph(2')-Ia*; *aph(3')-III*	8; 10
Streptomycin	>16	4-32									16			10					10 (38.5%)	*ant(6')-Ia*	10
Erythromycin	>2	0.25-8					16					10							10 (38.5%)	*erm*(B); *erm*(C)	10; 1
Clindamycin	>0.5	0.12-4				16					10								10 (38.5%)	*erm*(B); *erm*(C)	10; 1
Sulfamethoxazole	>256	64-512													1	9	8	8	8 (30.8%)	ND	ND
Mupirocin	>256	0.5-2 and 256						18		2							6		6 (23.1%)	*mupR*	8
Chloramphenicol	>8	4-128									26								0	0	0
Trimethoprim	>4	2-32								21	5								0	0	0
Vancomycin	>2	1-16							12	14									0	0	0
Rifampicin	>0.5	0.015-0.5	26																0	0	0
Linezolid	>4	1-8							26										0	0	0
Fusidic acid	>1	0.5-4						26											0	0	0
Quinupristin/dalfopristin	>2	0.5-4						26											0	0	0
Tiamulin	NA	0.5-4						26											0	0	0

*Breakpoints were recommended by EUCAST version 2.0. The breakpoints of kanamycin, streptomycin and sulfamethoxazole were recommended by French Society for Microbiology in 2010.

Highlight, level of concentration more than clinical breakpoints referring resistance

Table 5.12 The MIC of 19 antimicrobial drugs of MRSA isolates with the presence of resistance genes

	Resistance Breakpoint (mg/l)*	Range tested (mg/l)	MIC (mg/l)															Number of resistant isolates (%)	Resistance-mediating gene(s)	Numbers of isolates with the presence of resistance genes
			≤0.015	0.03	0.06	0.12	0.25	0.5	1	≥2	≥4	≥8	≥16	≥32	≥64	≥128	≥256	≥512			
Oxacillin with 2% NaCl	>0.25	0.25-8										4							4 (100%)	*mecA*	4
Penicillin	>0.25	0.012-2								4									4 (100%)	*blaZ*	4
Ciprofloxacin	>1	0.25-8										4							4 (100%)	ND	ND
Tetracycline	>2	0.5-16											4						4 (100%)	*tet*(M); *tet*(L)	4; 4
Gentamicin	>1	1-16									1	2	1						4 (100%)	*aac(6')-Ie-aph(2')-Ia*	4
Kanamycin	>16	4-64												2	2				4 (100%)	*aac(6')-Ie- aph(2')-Ia*; *ant(4')-Ia*	4; 4
Streptomycin	>16	4-32												4					4 (100%)	*ant(6')-Ia*	4
Erythromycin	>2	0.25-8					2	2											0	0	0
Clindamycin	>0.5	0.12-4									4								4 (100%)	*lnu*(B)	4
Chloramphenicol	>8	4-64										2			2				2 (50.0%)	*fexA*	2
Sulfamethoxazole	>256	64-512													3	1			0	0	ND
Trimethoprim	>4	2-32												4					4 (100%)	*dfrA*	4
Mupirocin	>256	0.5-2 and 256						3									1		1 (25.0%)	*mupR*	1
Vancomycin	>2	1-16							4										0	0	0
Rifampicin	>0.5	0.015-0.5	4																0	0	0
Lizenolid	>4	1-8							2	2									0	0	0
Fusidic acid	>1	0.5-4						4											0	0	0
Quinupristin/dalfopristin	>2	0.5-4									4								1 (25.0%)	*lsa*(E)	4
Tiamulin	NA	0.5-4									4								NA	*lsa*(E)	4
Virginiamycin M1	NA	0.12-512															4		NA	*lsa*(E)	4

*Breakpoints were recommended by EUCAST version 2.0. The breakpoints of kanamycin, streptomycin and sulfamethoxazole were recommended by French Society for Microbiology in 2010.

Highlight, level of concentration more than clinical breakpoints referring resistance

NA, not available

ND, not determined

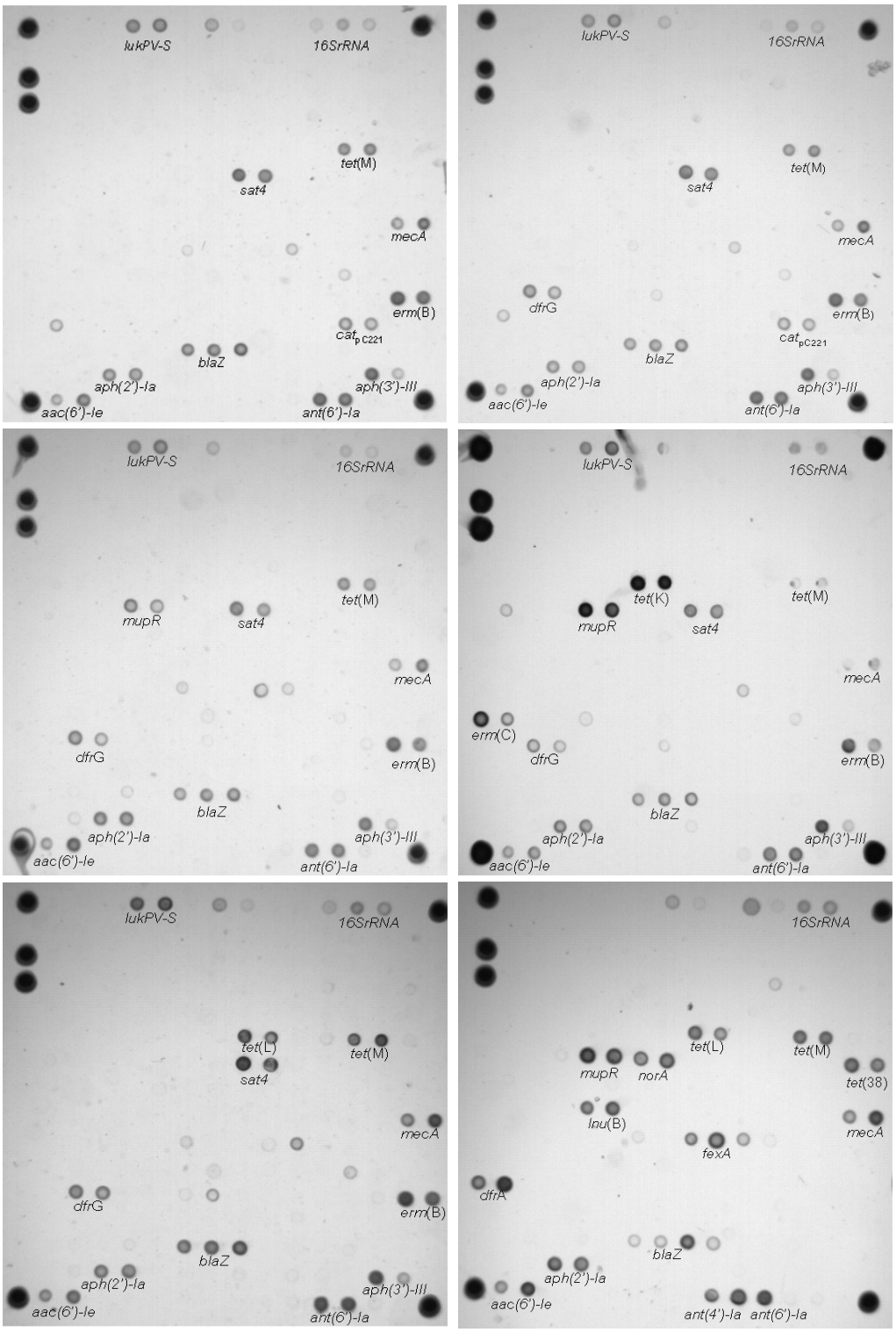


Figure 5.4 Example of arrays with positive resistance genes found in MRCoPS

5.4 Molecular characteristics of MRCoPS isolated from dogs and dog-associated people

5.4.1 SCC*mec* types in MRCoPS

5.4.1.1 Various SCC*mec* types carried by MRSP

Of 83 MRSP, SCC*mec* of 35 isolates [(42.2%); 23 from dogs, 9 from veterinarians and 3 from owners] could be classified by multiplex PCR that showed positive the amplified fragments of *ccr* complex and *mec* complex in both panels (Figure 5.5 and 5.6). One canine MRSP was positive only for class A *mec* complex amplification, and other 47 MRSP [(56.7%); 40 from dogs and 7 from veterinarians] were negative for PCR amplification of both panels. As an internal control, all MRSP presented band of *mecA* at 286 bp in the panel 1.

Classified by multiplex PCR, the 22 MRSP [(26.5%); 13 from dogs, 6 from veterinarians and 3 from owners] carried SCC*mec* type V which presented fragments of *ccrC* (518 bp) and class C *mec* complex (804 bp). The 11 MRSP [(13.3%); 9 from dogs and 2 from veterinarians) carried non-classified SCC*mec* which was positive for *ccrA1B1* (698 bp) and class A *mec* complex (1,963 bp), named SCC*mec* A1. The SCC*mec* of two isolates [(2.4%); 1 from a dog and 1 from a veterinarian] was identified as SCC*mec* type III showing 2 bands of *ccrA3B3* (1,791 bp) and *ccrC* (518 bp) in panel 1 and a band of class A *mec* complex (1,963 bp) in panel 2. These two isolates were included for specific identification of SCC*mec* type II-III. As a result, these showed positive for SCC*mec* type II-III because of the absence of cadmium resistance operon in this cassette presenting PCR product at 831 bp.

The 47 MRSP resulting in negative for SCC*mec* typing by multiplex PCR were included for ΨSCC*mec*$_{57395}$ identification by long-range PCR. The 46 MRSP [(55.4%); 40 from dogs and 6 from veterinarians] gave a positive band of the fragment from *orfX* to *attSCC* of ΨSCC*mec*$_{57395}$ at 12,677 bp. Furthermore, these 46 isolates were also positive for amplification of *orfX* to 3' chromosomal region downstream to *attSCC* resulting in

21,677 bp. This amplicon was digested by *Bsu*36I for restriction analysis resulting in 5 fragments at 627 bp, 1,866 bp, 3,207 bp, 5,406 bp and 10,571 bp in size (Figure 5.7). The restriction pattern of all isolates was identical to MRSP 57395 (Figure 5.8).

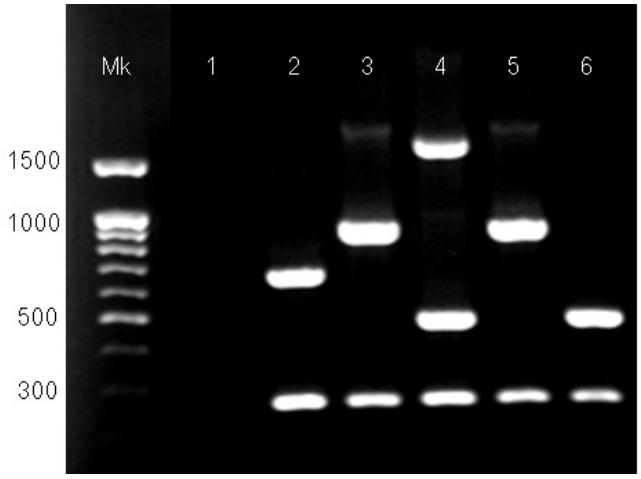


Figure 5.5 Amplified products of type 1 to 5 *ccr* complex of SCC*mec* type I to V and internal control of *mecA* (286 bp) by multiplex PCR panel 1 for SCC*mec* typing [Mk, 100 bp ladder marker; lane 1, *S. aureus* ATCC 25923 as a negative control; lane 2, MRSA NCTC 10442 (type 1 *ccr* complex, 695 bp); lane 3 MRSA N315 (type 2 *ccr* complex, 937 bp); lane 4 MRSA 8512082 (type 3 *ccr* complex, 1793 bp and type 5 *ccr* complex, 518 bp); lane 5 MRSA SCC*mec* type IV (type 4 *ccr* complex, 1, 287 bp); and lane 6 MRSA WIS (type 5 *ccr* complex, 518 bp)]

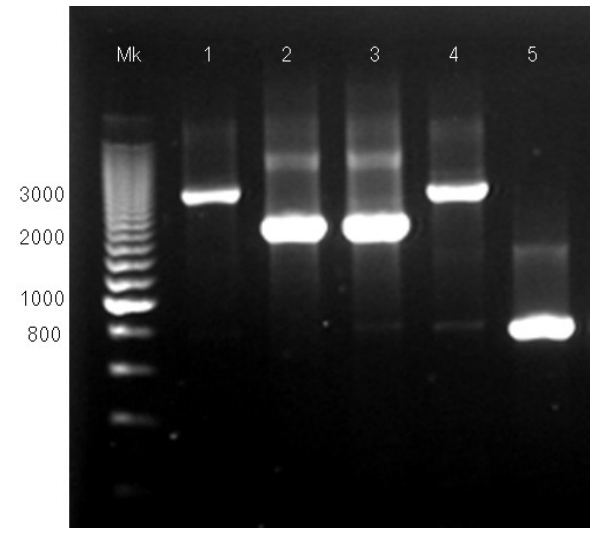


Figure 5.6 Amplified products of class A, B and C of *mec* complex of SCC*mec* type I to V and by multiplex PCR panel 2 for SCC*mec* typing [Mk, 200 bp ladder marker; lane 1, MRSA NCTC 10442 (class B *mec* complex, 2,827 bp); lane 2 MRSA N315 (class A *mec* complex, 1,963 bp); lane 3 MRSA 8512082 (class A *mec* complex, 1,963 bp); lane 4 MRSA SCC*mec* type IV (class B *mec* complex, 2,827 bp); and lane 5 MRSA WIS (class C *mec* complex, 804 bp)]

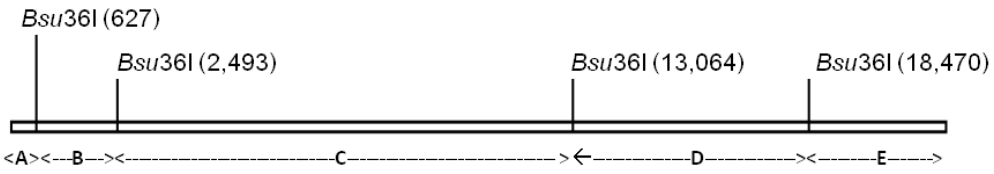


Figure 5.7 Schematic diagram of restriction sites for *Bsu*36I of fragment from *orfX* to chromosomal region downstream to ψSCC*mec*$_{57395}$ of MRSP 57395 (A, 627 bp; B, 1,866 bp; C, 10,571 bp; D, 5,406 bp and E, 3,207 bp)

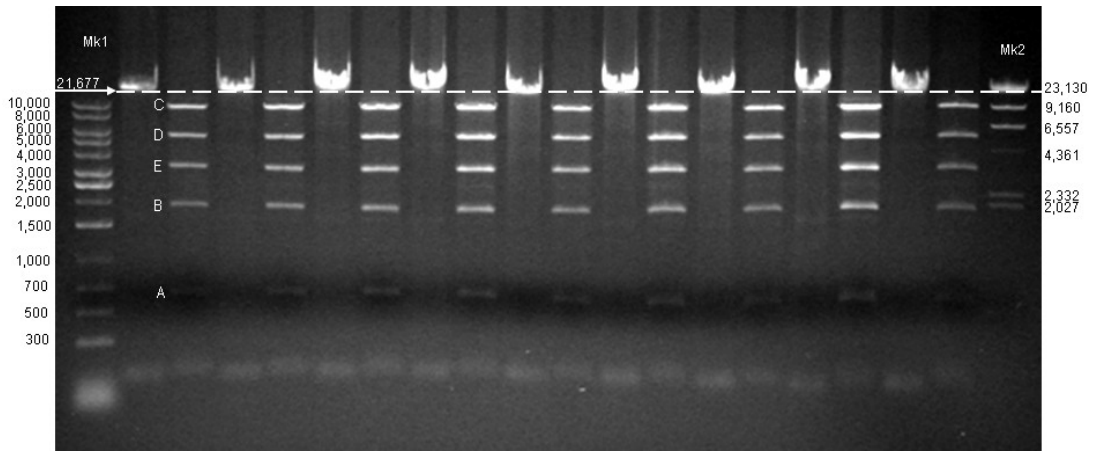


Figure 5.8 Amplified fragment from *orfX* to *contig27* and digested product by *Bsu*36I [Mk1, 1 kb ladder marker; Mk2, *Hind*III-digested λ marker; lane 1, *orfX* to *contig27* amplicon (21,677 bp) of MRSP 57395; lane 2 digested products of MRSP 57395; lane 3-18 *orfX* to *contig27* amplicon (21,677 bp) of MRSP samples with their digested products (A, 627 bp; B, 1,866 bp; C, 10,571 bp; D, 5,406 bp and E, 3,207 bp)]

5.4.1.2 SCC*mec* type V carried by MRSA and MRSSc

For MRSSc and MRSA, all isolates were positive for multiplex PCR for SCC*mec* typing. These carried SCC*mec* type V showing fragment of *ccrC* at 518 bp in panel 1 and fragment of class C *mec* complex at 804 bp in panel 2. As an internal control, all isolates resulted in positive for *mecA* amplification also.

5.4.2 MLST analysis of MRSP and MRSA

5.4.2.1 Sequence types of MRSP by MLST-4

The 83 MRSP were clonally analyzed by MLST-4 resulting in 13 sequence types (ST_4). The 6 STs of MRSP, including $ST_4$5, 26, 29, 71, 131, 133 and 191, could be isolated from both dogs and dog-associated people. Interestingly, members of MRSP $ST_4$133 and 191 could be isolated from dogs and owners in the same households. The

numbers of MRSP in each ST_4 and sources are shown in Table 5.13, and allelic profiles of each ST by MLST-4 are illustrated in Table 5.15.

Table 5.13 Sequence types of MRSP from different sources by MLST-4

Sequence type by MLST-4 (ST_4)	No., isolate			No., isolate (%)
	Dog	veterinarian	Dog's owner	
Total	64	16	3	83
2	1	0	0	1 (1.2)
4	0	1	0	1 (1.2)
5	9	2	0	11 (13.3)
6	3	0	0	3 (3.6)
26	1	2	0	3 (3.6)
29	3	2	0	5 (6.0)
71	1	1	0	2 (2.4)
100	2	0	0	2 (2.4)
103	2	0	0	2 (2.4)
131	34	4	0	38 (45.8)
133	5	4	1	10 (12.1)
163	1	0	0	1 (1.2)
191	2	2	0	4 (4.8)

5.4.2.2 Sequence types of MRSP by MLST-7

By comparison with MLST-4, MLST-7 can provide higher discriminatory power resulting in 17 sequence types (ST_7). The 6 STs of MRSP including $ST_7$45, 112, 169, 178, 181 and 183 could be isolated from both dogs and dog-associated people. Also, members of MRSP $ST_7$181 and 183 could be isolated from dogs and owners in the same households. The numbers of MRSP in each ST_7 and sources are shown in Table 5.14. Allelic profiles of each ST using MLST-7 are illustrated in Table 5.15.

Table 5.14 Sequence types of MRSP from different sources by MLST-7

Sequence type by MLST-7 (ST_7)	No., isolate			No., isolate (%)
	Dog	veterinarian	Dog's owner	
Total	64	16	3	83
45	36	6	0	42 (50.6)
55	1	0	0	1 (1.2)
110	0	1	0	1(1.2)
111	1	0	0	1(1.2)
112	8	2	0	10 ()
113	0	1	0	1 (1.2)
114	1	0	0	1 (1.2)
115	0	1	0	1 (1.2)
116	1	0	1	1 (1.2)
121	1	0	1	1 (1.2)
125	1	0	1	1 (1.2)
169	1	1	0	2 (2.4)
178	1	1	0	2 (2.4)
181	5	3	1	9 (1.1)
182	3	0	0	3 (3.6)
183	2	0	2	4 (4.8)
185	2	0	0	2 (2.4)

Table 5.15 Sequence types and allelic profiles of MRSP by MLST-4 and MLST-7 techniques

MLST-7	*ack*	*cpn60*	*fdh*	*pta*	*purA*	*sar*	*tuf*	*agrD**	MLST-4
45	3	2	2	1	2	1	2	2	131
45	3	2	2	1	2	1	2	1	29
55	16	2	2	1	13	1	2	1	29
110	3	9	2	1	2	1	2	1	6
111	2	2	1	1	1	2	1	2	5
112	2	2	2	1	1	2	1	2	5
113	2	11	3	1	11	2	2	3	4
114	2	7	2	1	2	5	2	3	100
115	3	11	2	24	11	1	1	3	133
116	3	7	2	1	2	2	2	3	100
121	2	9	3	1	1	2	1	1	2
125	2	10	2	1	1	1	1	4	163
169	2	9	1	2	1	1	1	1	71
178	3	9	4	1	1	1	2	1	6
181	3	11	2	24	20	1	1	3	133
182	7	10	1	1	11	1	2	4	26
183	1	45	2	1	22	1	1	4	191
185	1	9	2	1	2	1	2	3	103

Highlight, genes used for MLST-4 analysis

**agrD* used for only MLST-4 analysis

5.4.2.3 Sequence types of MRSA

Using MLST scheme for *S. aureus*, only one ST was identified in MRSA isolated from a dog and 3 veterinarians. All MRSA were the same ST398, which has allelic profile as 3-35-19-2-20-26-39.

5.4.3 PFGE analysis of MRCoPS

5.4.3.1 *Sma*I-PFGE analysis of MRSP

All 83 MRSP were firstly included for *Sma*I-PFGE analysis. Interestingly, chromosomal DNA of 39 of 83 isolates [(47.0%); 27 from dogs, 9 from veterinarians and 3 from owners] could be digested by *Sma*I and were analyzed resulting in 16 clusters (*Sma*I A to P) (Figure 5.9). The *Sma*I-A cluster was the major group consisting of 14 isolates [(16.9%); 10 from dogs, 2 from veterinarians and 2 from owners]. Secondly, the *Sma*I-N cluster consisted of 6 isolates [(7.2%); 3 from dogs and 3 from veterinarians]. The *Sma*I-E cluster had 3 isolates (3.6%) from dogs. Each *Sma*I-L and O cluster consisted of 2 isolates (2.4%) from dogs. Notably, *Sma*I-P and O cluster also consisted of 2 isolates (2.4%) obtained from a dog and an owner in the same household. The other 10 clusters, including *Sma*I-B, C, D, F, G, H, I, J, K and M, had only 1 canine isolate in each cluster.

5.4.3.2 *Cfr*9I-PFGE analysis of MRSP

The other 44 MRSP [(53.0%); 37 from dogs and 7 from veterinarians] needed *Cfr*9I for PFGE analysis resulting in 3 clusters (*Cfr*9I A, B and C) (Figure 5.10). The predominance was *Cfr*9I-A cluster including 34 isolates [(41.0%); 31 from dogs and 3 from veterinarians]. The *Cfr*9I-B cluster included 7 isolates [(8.4%); 4 from dogs and 3 from veterinarians] and *Cfr*9I-C cluster included 3 isolates [(3.6%); 2 from dogs and 1 from a veterinarian].

5.4.2.3 *Sma*I-PFGE analysis of MRSSc

All 26 MRSSc could be analyzed by *Sma*I-PFGE resulting in 4 clusters ($SmaI_{SSc}$-A to D) (Figure 5.11). The 13 MRSSc [(50%); 8 from dogs, 3 from veterinarians and 2 from owners] were grouped in the major $SmaI_{SSc}$-A cluster. The $SmaI_{SSc}$-A cluster included 7

isolates [(26.9%); 6 from dogs and 1 from a veterinarian]. The *Sma*I$_{SSc}$-B and C clusters included 5 and 1 isolates from dogs, respectively.

5.4.2.1 *Cfr*9I-PFGE analysis of MRSA

All MRSA (100%) could not be typed by *Sma*I-PFGE, so these needed *Cfr*9I for DNA fingerprint analysis. As a result, all MRSA exhibited very similar pattern between canine and human isolates which had only 3 distinct bands, but all human isolates were identical by naked eye visualization (Figure 5.12).

5.4.4 Compatibility of genetic and antimicrobial resistance characteristics

The genetic characteristics including MLST-7, PFGE clusters and SCC*mec* types with the resistance profiles of MRSP are shown in Table 5.16. The 42 MRSP ST$_7$45 (ST$_4$29 and 131) shared the same characteristics including *Sma*I indigestible characteristics and ψSCC*mec*$_{57395}$ carriage except only 1 MRSP with non-typeable SCC*mec* from a veterinarian (Figure 5.9). All isolates in ST$_7$45 were consistently resistant to oxacillin, penicillin, tetracycline, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, chloramphenicol and sulfamethoxazole. The 4 MRSP ST$_7$45 which were ST$_4$29 exhibited additional resistance to trimethoprim. Also, the *Sma*I-indigestible characteristics and ψSCC*mec*$_{57395}$ could be found in ST$_7$113 (ST$_4$6) and 116 (ST$_4$100). Additionally, MRSP ST$_7$114 (ST$_4$100) and ST$_7$185 (ST$_4$103) also carried ψSCC*mec*$_{57395}$, but these could be typed by *Sma*I-PFGE.

Regarding the MRSP with *Sma*I-digestible characteristic, SCC*mec* type V was identified in isolates belonged to 8 STs by MLST-7 including ST$_7$55, 110, 115, 121, 178, 181, 182 and 183. The SCC*mec* A1 was specifically associated only in MRSP ST$_7$111 and 112 (ST$_4$5) and inducible clindamycin resistance could be specifically detected in these STs. Also, the SCC*mec* type II-III was also specifically associated in MRSP ST$_7$169

($ST_4$71). One MRSP $ST_7$125 ($ST_4$163) carried non-typeable SCC*mec* which presented only class A *mec* complex and negative *ccr* by multiplex PCR.

All MRSSc carried SCC*mec* type V, and *Sma*I-PFGE could be used for DNA fingerprint analysis for this species. The isolates which belonged to $SmaI_{SSc}$-D exhibited multidrug resistance (more than 3 groups), while the isolates in cluster *Sma*I-A, B and C were resistant to only 1 to 3 antimicrobial classes. The genetic characteristics and resistance profiles of MRSSc are shown in Table 5.17 and Figure 5.11.

All MRSA ST398 carried SCC*mec* type V and needed *Cfr*9I for PFGE analysis. These were commonly resistant to oxacillin, penicillin, tetracycline ciprofloxacin, gentamycin, kanamycin, streptomycin, clindamycin, trimethoprim and quinupristin/dalfopristin. The genetic characteristics and resistance profiles of MRSA are shown in Table 5.18 and Figure 5.12.

5.4.5 Common and various clones MRCoPS distributing in dogs and dog-associated people.

The predominant clone distributing in Thailand was MRSP $ST_7$45(MLST)-*Cfr*9I-A(PFGE)-ψSCCmec_{57395}(SCC*mec*) colonizing in 27 dogs (27%) and 3 veterinarians (1.5%). MRSP $ST_7$112-*Sma*I-A-A1 also colonized in 8 dogs (8%) and 2 veterinarians (1%). MRSP $ST_7$181, which had 3 related PFGE clusters (*Sma*I-N, O and P) and carried SCC*mec* type V, could be isolated from 5 dogs (5%), 3 veterinarians (1.5%) and 1 owner (1%). Also, MRSP $ST_7$183-*Sma*I-A-V was isolated from 2 dogs and 2 owners. Remarkably, each of MRSP $ST_7$181 and 183 was isolated from 2 pairs of dog and owner in the same households that demonstrated the very similar to identical PFGE patterns (Table 5.16). The MRSP $ST_7$169 and 178 carried SCC*mec* type II-III and V, respectively, colonizing in 1 dog and 1 veterinarian of each ST. Other various STs of MRSP including $ST_7$55, 111, 114, 116, 121, 125, 182 and 185 could be sporadically found in Thai dogs.

Otherwise, MRSP $ST_7$110, 113 and 115 could be isolated only from veterinarians. The numbers of dogs and dog-associated people colonized with various characteristics of MRSP are shown in Table 5.16.

The 10 multidrug-resistant MRSSc in *Sma*I_{SSc}-D (PFGE) cluster could be isolated from 7 dogs, 2 owners and a veterinarian. Regarding co-colonization, the concurrent MRSP and MRSSc or difference of MRSP STs colonization in the same dog were found, shown in Table 5.19. The number of dogs and dog-associated people colonized with various characteristics of MRSSc are shown in Table 5.17. The resistance characteristics and gene carriage of MRSA ST398-*Cfr*9I_{SA}-V isolated from a dog and 3 veterinarians are summarized in Table 5.18.

Table 5.16 Numbers of isolate, molecular characteristics and antimicrobial resistance profiles of MRSP from Thai dogs, dog's owners and veterinarians

No of isolates	Positive no. (%)			ST_7	ST_4	PFGE type	SCC*mec* type	Antimicrobial resistance phenotypes (breakpoints) (mg/l) and resistance genes												
	Dog	Veterinarian	Owner					OXA (>0.25) *mecA*	PEN (>0.125) *blaZ*	TET (>2) *tet*	GEN/KAN (>1/>16) *aac(6')-Ie-aph(2')-Ia*	KAN (>16) *aph(3')-III*	STR (>16) *ant(6')-Ia*	STH* *sat4*	ERY/CLI (>2/>0.5) *erm*	TMP (>4) *dfrG*	CHL (>8) cat_{pC221}	MUP (>256) *mupR*	CIP (>1) ND	SMX (>256) ND
Total, N=83	N=100	N=200	N=100																	
34	31 (31.00)	3 (1.50)	0	45	131	*Cfr*9I-A	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	-	+	-	+	+
1	1 (1.00)	0	0	45	131	*Cfr*9I-B	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	-	+	-	+	+
1	1 (1.00)	0	0	45	131	*Cfr*9I-B	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	-	+	-	-	+
1	0	1 (0.50)	0	45	131	*Cfr*9I-C	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	-	+	-	+	+
1	1 (1.00)	0	0	45	131	*Cfr*9I-C	ΨSCC*mec*$_{57395}$	+	+	-	+	+	+	+	+(B)	-	+	-	+	+
3	2 (2.00)	1 (0.50)	0	45	29	*Cfr*9I-C	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	+	+	-	+	+
1	0	1 (0.50)	0	45	29	*Cfr*9I-C	NT	+	+	+(M)	+	+	+	+	+(B)	+	+	-	+	+
1	1 (1.00)	0	0	55	29	*Sma*I-M	V	+†	+†	+(M)	+	+	+	+	+(B)	-	+	-	+	+
1	0	1 (0.50)	0	110	4	*Sma*I-G	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
1	1 (1.00)	0	0	111	5	*Sma*I-C	A1	+	+	+(K)	+	+	+	+	+(B)§	+	-	-	-	+
4	4 (4.00)	0	0	112	5	*Sma*I-A	A1	+	+	+(M),(K)	+	+	+	+	+(B)§	+	-	-	-	+
3	3 (3.00)	0	0	112	5	*Sma*I-A	A1	+	+	+(M)	+	+	+	+	+(B)§	+	-	-	-	+
1	0	1 (0.50)	0	112	5	*Sma*I-A	A1	+	+	+(M)	+	+	+	+	+(B)§	+	-	-	+	+
1	1 (1.00)	0	0	112	5	*Sma*I-A	A1	+	+	+(M),(K)	+	+	+	+	+(B),(C)	+	-	+	-	+
1	0	1 (0.50)	0	112	5	*Sma*I-A	A1	+	+	+(M)	+	+	+	+	+(B)§	+	-	+	+	+
1	0	1 (0.50)	0	113	6	*Cfr*9I-B	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	-	+	-	+	+
1	1 (1.00)	0	0	114	100	*Sma*I-P	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
1	0	1 (0.50)	0	115	133	*Sma*I-H	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
1	1 (1.00)	0	0	116	100	*Cfr*9I-E	ΨSCC*mec*$_{57395}$	+	+	+(M)	+	+	+	+	+(B)	-	+	-	-	+
1	1 (1.00)	0	0	121	2	*Sma*I-B	V	+	+	+(M)	+	+	+	+	+(B)	+	+	-	+	-
1	1 (1.00)	0	0	125	163	*Sma*I-D	A	+	+	+(M),(L)	+	+	+	+	+(B)	+	-	-	+	+
1	0	1 (0.50)	0	169	71	*Sma*I-I	II-III	+	+	+(M),(K)	+	+	+	+	+(B)	+	-	-	+	+
1	1 (1.00)	0	0	169	71	*Sma*I-F	II-III	+	+	+(M)	+	-	-	-	-	-	-	-	-	-
1	1 (1.00)	0	0	178	6	*Sma*I-J	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
1	0	1 (0.50)	0	178	6	*Sma*I-K	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
6	3 (3.00)	3 (1.50)	0	181	133	*Sma*I-N	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
2	2 (2.00)	0	0	181	133	*Sma*I-O	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
1	0	0	1 (1.00)	181	133	*Sma*I-P	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
2	2 (2.00)	0	0	182	26	*Sma*I-E	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
1	1 (1.00)	0	0	182	26	*Sma*I-E	V	+	+	+(M)	+	+	+	+	+(B)	+	-	+	+	+
4	2 (2.00)	0	2 (2.00)	183	191	*Sma*I-A	V	+	+	+(M)	+	+	+	+	+(B)	+	-	-	+	+
2	2 (2.00)	0	0	185	103	*Sma*I-N	ΨSCC*mec*$_{57395}$	+	+	+(M)	-	-	-	-	-	-	-	-	-	+
Positive isolate no. (%)								83 (100)	83 (100)	81 (97.59)	81 (97.59)	80 (96.38)	80 (96.38)	80 (96.38)	80 (96.38)	39 (46.98)	46 (55.42)	3 (3.61)	69 (83.13)	81 (97.59)

All MRSP isolates were susceptible to vancomycin, rifampicin, linezolid, fusidic acid, quinupristin/dalfopristin and tiamulin.

+, positive; -, negative; ND, the resistance mechanisms were not determined; NT, non-typeable; A1, present class A *mec* complex and type 1 *ccr*; A, present only class A *mec* complex

Highlight, MRSP $ST_7$181 ($ST_4$133) was isolated from dog and their owners in the same households as well as MRSP $ST_7$183 ($ST_4$191).

*only the presence of *sat4* by microarray

†presence of the resistance gene without expression the resistance phenotype

§presence of inducible-clindamycin resistance

Table 5.17 Numbers of isolate, molecular characteristics and antimicrobial resistance profiles of MRSSc from Thai dogs, dog's owners and veterinarians

No of isolates	Positive no. (%)			PFGE type	SCC*mec* type	Antimicrobial resistance phenotypes (breakpoints (mg/l) and resistance genes										
	Dog	Veterinarian	Owner			OXA (>0.25)	PEN (>0.125)	TET (>2)	GEN/KAN (>1/>16)	KAN (>16)	STR (>16)	STH *	ERY/CLI (>2/>0.5)	MUP (>256)	CIP (>1)	SMX (>256)
Total, N=83	N=100	N=200	N=100			*mecA*	*blaZ*	*tet*	*aac(6')-Ie-aph(2')-Ia*	*aph(3')-III*	*ant(6')-Ia*	*sat4*	*erm*	*mupR*	ND	ND
5	5 (5.00)	0	0	A	V	+		+(K)								
2	1 (1.00)	1 (0.50)	0	A	V	+†		+(K)								+
1	1 (1.00)	0	0	B	V	+	+									
4	4 (4.00)	0	0	C	V	+										+
1	1 (1.00)	0	0	C	V	+		+(K)								+
2	0	1 (0.50)	1 (1.00)	D	V	+	+	+(O)		+	+	+	+(B)			
1	0	0	1 (1.00)	D	V	+†	+	+(O)	+	+	+	+	+(B),(C)	+		
1	1 (1.00)	0	0	D	V	+	+	+(O)	+	+	+	+	+(B)	+§	+	+
1	0	1 (0.50)	0	D	V	+		+(K)								
1	0	1 (0.50)	0	D	V	+	+									
4	4 (4.00)	0	0	D	V	+	+	+(O)	+	+	+	+	+(B)	+§		
2	2 (2.00)	0	0	D	V	+	+	+(K),(O)	+	+	+	+	+(B)	+		
1	1 (1.00)	0	0	D	V	+	+¶	+(K)								
Positive isolate no. (%)						26 (100)	13 (50.00)	20 (76.92)	8 (30.76)	10 (38.46)	10 (38.36)	10 (38.46)	10 (38.46)	8 (30.76)	1 (3.84)	8 (30.76)

All MRSSc isolates were susceptibile to chloramphenicol, trimethoprim, vancomycin, rifampicin, linezolid, fusidic acid, quinupristin/dalfopristin and tiamulin.

+, positive; -, negative; ND, the resistance mechanisms were not determined

*only the presence of *sat4* by microarray

† human isolates did not express resistance to oxacillin

§showed *mupR*-positive but mupirocin MIC = 2 mg/L

¶showed penicillin resistance but *blaZ*-negative

Table 5.18 Numbers of isolate, molecular characteristics and antimicrobial resistance profiles of MRSA from Thai dogs and veterinarians

No of isolates	Prevalence (No.)			MLST	PFGE type	SCC*mec* type	Antimicrobial resistance phenotypes (breakpoints (mg/L) and resistance genes												
	Dog	Veterinarian	Owner				OXA (>0.25)	PEN (>0.125)	TET (>2)	GEN/KAN (>1/>16)	KAN (>16)	STR (>16)	CLI (>0.5)	TIA (NA)	TMP (>4)	CHL (>8)	MUP (>256)	CIP (>1)	SYN (>2)
Total, N=4	N=100	N=200	N=100				*mecA*	*blaZ*	*tet*	*aac(6')-Ie-aph(2')-Ia*	*ant(4')-Ia*	*ant(6')-Ia*	*lnu*(B)	*lsa*(E)	*dfrA*	*fexA*	*mupR*	ND	ND
2	0	2 (1.00)	0	398	*Cfr*9I	V	+	+	+(L),(M)	+	+	+	+	+	+	-	-	+	+
1	1 (1.00)	0	0	398	*Cfr*9I	V	+	+	+(L),(M)	+	+	+	+	+	+	+	+	+	+
1	0	1 (0.50)	0	398	*Cfr*9I	V	+	+	+(L),(M)	+	+	+	+	+	+	+	-	+	+
Positive isolate no. (%)							4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	2 (50.00)	1 (25.00)	4 (100)	4 (100)

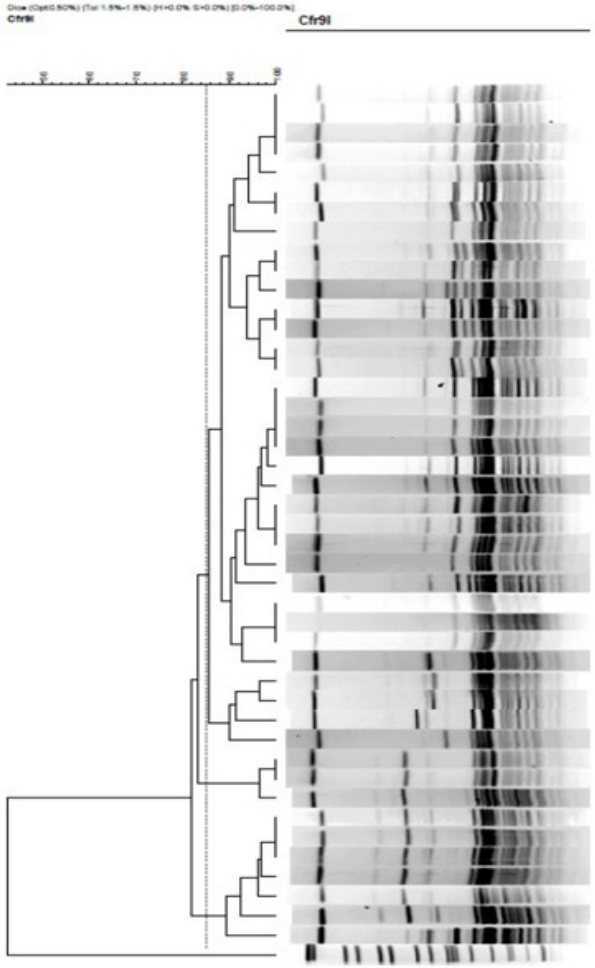
All MRSA were susceptible to erythromycin, vancomycin, rifampicin, linezolid and fusidic acid.

+, positive; -, negative; ND, the resistance mechanisms were not determined.

NA, the breakpoint is not available.

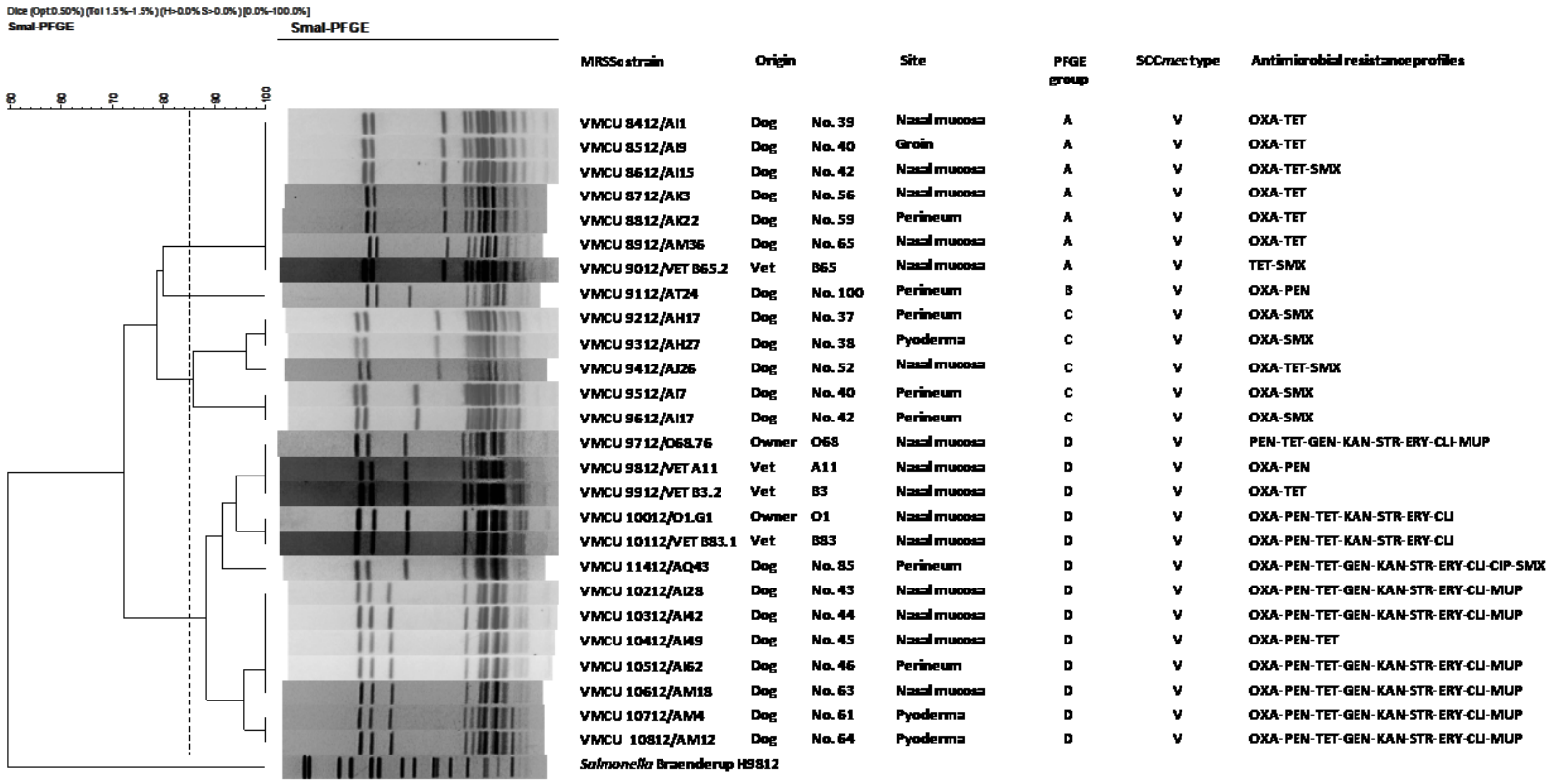
MRSP strain	Origin		Site	MLST-4	MLST-7	PFGE group	SCC*mec* type	Antimicrobial resistance profiles
VMCU 6312/AI3	Dog	No. 39	Nasal mucosa	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 5812/AI6	Dog	No. 40	Nasal mucosa	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 5912/AI16	Dog	No. 41	Groin	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 6012/AI46	Dog	No. 45	Perineum	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 6112/AK9	Dog	No. 56	Groin	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 6212/AK23	Dog	No. 59	Perineum	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 5712/AK5	Dog	No. 56	Nasal mucosa	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 5312/VET A26	Vet	A26	Nasal mucosa	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-CIP-SMX
VMCU 5512/AJ1	Dog	No. 47	Perineum	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-MUP-SXT
VMCU 5412/VET B16.4	Vet	B16	Nasal mucosa	5	112	A	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-MUP-TMP-CIP-SMX
VMCU 6712/AQ17	Dog	No. 82	Nasal mucosa	191	183	A	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SXT
VMCU 6512/AQ25	Owner	O100	Nasal mucosa	191	183	A	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SXT
VMCU 6612/AQ30	Owner	O101	Nasal mucosa	191	183	A	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SXT
VMCU 6812/AH22	Dog	No. 38	Perineum	191	183	A	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SXT
VMCU 6412/AH1	Dog	No. 35	Nasal mucosa	2	121	B	V	OXA-PEN-TET-GEN-KAN-STR-TMP-CHL-CIP
VMCU 5612/AM33	Dog	No. 64	Perineum	5	111	C	A1	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI(i)-TMP-SMX
VMCU 4812/AP54	Dog	No. 77	Nasal mucosa	163	125	D	A	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7012/AM6	Dog	No. 61	Perineum	26	182	E	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7112/AM35	Dog	No. 65	Nasal mucosa	26	182	E	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7212/AM21	Dog	No. 63	Nasal mucosa	26	182	E	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-MUP-CIP-SMX
VMCU 8312/AM25	Dog	No. 63	Perineum	71	169	F	II-III	OXA-PEN-TET-GEN-KAN
VMCU 6912/VET A36	Vet	A36	Nasal mucosa	4	110	G	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 8212/VET B62.1	Vet	B62	Nasal mucosa	133	115	H	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 11312/VET B66.1	Vet	B66	Nasal mucosa	71	169	I	II-III	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 5012/VET A77	Vet	A77	Nasal mucosa	6	178	J	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 4912/AE1	Dog	No. 30	Nasal mucosa	6	178	K	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 4512/A2	Dog	No. 1	Perineum	103	185	L	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-SMX
VMCU 4612/B5	Dog	No. 2	Nasal mucosa	103	185	L	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-SMX
VMCU 5112/AP47	Dog	No. 76	Perineum	29	55	M	V	TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 7612/AQ45	Dog	No. 85	Perineum	133	181	N	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7412/VET B45.1	Vet	B45	Nasal mucosa	133	181	N	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7512/VET B98.1	Vet	B98	Nasal mucosa	133	181	N	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7712/VET A82	Vet	A82	Nasal mucosa	133	181	N	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 8112/AI14	Dog	No. 40	Groin	133	181	N	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7312/AT21	Dog	No. 100	Perineum	133	181	N	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7912/AP64	Dog	No. 70	Perineum	133	181	O	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 8012/AP84	Dog	No. 77	Perineum	133	181	O	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 7812/AP20	Owner	O91	Nasal mucosa	133	181	P	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
VMCU 4412/AP11	Dog	No. 69	Nasal mucosa	100	114	P	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CIP-SMX
Salmonella Braenderup H9812								

Figure 5.9 DNA fingerprint patterns by *Sma*I-PFGE with molecular characteristics and antimicrobial resistance profiles of MRSP



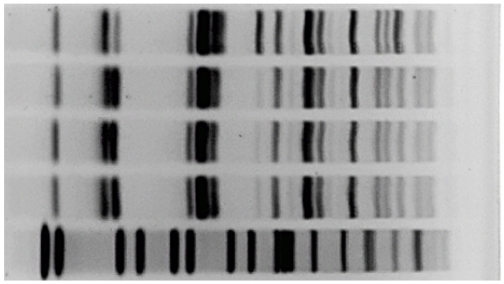
MRSP strain	Origin		Site	MLST-4	MLST-7	PFGE group	SCC*mec* type	Antimicrobial resistance profiles
VMCU 3212 /AQ16	Dog	No. 82	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 0712/AI39	Dog	No. 44	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2912/AQ8	Dog	No. 79	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 3012/AQ12	Dog	No. 80	Perineum	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 3512/AK2	Dog	No. 55	Perineum	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 0312/U4	Dog	No. 18	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 0812/AC1	Dog	No. 25	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2212/AD1	Dog	No. 28	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2512/AI55	Dog	No. 46	Groin	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1512/AH10	Dog	No. 36	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 3812/AM43	Dog	No. 65	Groin	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1612/AH23	Dog	No. 38	Groin	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2812/AK39	Dog	No. 60	Perineum	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 3112/AJ18	Dog	No. 51	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 5212/AH16	Dog	No. 37	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 0512/AI13	Dog	No. 40	Groin	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1012/AK12	Dog	No. 57	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1712/AK16	Dog	No. 58	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1112/AK30	Dog	No. 60	Groin	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 0612/AI25	Dog	No. 42	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 0212/VET B71.1	Vet	B71	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1312/AJ14	Dog	No. 50	Perineum	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2612/AJ25	Dog	No. 52	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 3412/AP6	Dog	No. 68	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 3312/AM38	Dog	No. 65	Perineum	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2012/VET B4.1	Vet	B4	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2712/AI47	Dog	No. 45	Groin	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1212/AJ7	Dog	No. 49	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 0112/AJ28	Dog	No. 53	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2112/VET B88.1	Vet	B88	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1812/AJ6	Dog	No. 48	Perineum	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2312/AH12	Dog	No. 36	Perineum	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 2412/AH19	Dog	No. 37	Nasal mucosa	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1912/AM11	Dog	No. 61	Groin	131	45	A	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 1212/AP62	Dog	No. 69	Perineum	131	45	B	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-SMX
VMCU 0412/AP65	Dog	No. 70	Perineum	131	45	B	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 4712/VET A23	Vet	A23	Nasal mucosa	6	113	B	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 4112/VET A8	Vet	A8	Nasal mucosa	29	45	C	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CHL-CIP-SMX
VMCU 3712/VET B31.1	Vet	B31	Nasal mucosa	131	45	C	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
VMCU 3912/AH18	Dog	No. 37	Perineum	29	45	C	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CHL-CIP-SMX
VMCU 4012/S4	Dog	No. 15	Nasal mucosa	29	45	C	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CHL-CIP-SMX
VMCU 4312/AM27	Dog	No. 64	Nasal mucosa	100	116	C	ΨSCC*mec*$_{57395}$	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CHL-SMX
VMCU 4212/VET B58.1	Vet	B58	Nasal mucosa	29	45	C	Non typeable	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-TMP-CHL-CIP-SMX
VMCU 36/12AK20	Dog	No. 59	Nasal mucosa	131	45	C	ΨSCC*mec*$_{57395}$	OXA-PEN-GEN-KAN-STR-ERY-CLI-CHL-CIP-SMX
Salmonella Braenderup H9812								

Figure 5.10 DNA fingerprint patterns by *Cfr*9I-PFGE with molecular characteristics and antimicrobial resistance profiles of MRSP



MRSSc strain	Origin		Site	PFGE group	SCC*mec* type	Antimicrobial resistance profiles
VMCU 8412/AI1	Dog	No. 39	Nasal mucosa	A	V	OXA-TET
VMCU 8512/AI9	Dog	No. 40	Groin	A	V	OXA-TET
VMCU 8612/AI15	Dog	No. 42	Nasal mucosa	A	V	OXA-TET-SMX
VMCU 8712/AK3	Dog	No. 56	Nasal mucosa	A	V	OXA-TET
VMCU 8812/AK22	Dog	No. 59	Perineum	A	V	OXA-TET
VMCU 8912/AM36	Dog	No. 65	Nasal mucosa	A	V	OXA-TET
VMCU 9012/VET B65.2	Vet	B65	Nasal mucosa	A	V	TET-SMX
VMCU 9112/AT24	Dog	No. 100	Perineum	B	V	OXA-PEN
VMCU 9212/AH17	Dog	No. 37	Perineum	C	V	OXA-SMX
VMCU 9312/AHZ7	Dog	No. 38	Pyoderma	C	V	OXA-SMX
VMCU 9412/AJ26	Dog	No. 52	Nasal mucosa	C	V	OXA-TET-SMX
VMCU 9512/AI7	Dog	No. 40	Perineum	C	V	OXA-SMX
VMCU 9612/AI17	Dog	No. 42	Perineum	C	V	OXA-SMX
VMCU 9712/O68.76	Owner	O68	Nasal mucosa	D	V	PEN-TET-GEN-KAN-STR-ERY-CLI-MUP
VMCU 9812/VET A11	Vet	A11	Nasal mucosa	D	V	OXA-PEN
VMCU 9912/VET B3.2	Vet	B3	Nasal mucosa	D	V	OXA-TET
VMCU 10012/O1.G1	Owner	O1	Nasal mucosa	D	V	OXA-PEN-TET-KAN-STR-ERY-CLI
VMCU 10112/VET B83.1	Vet	B83	Nasal mucosa	D	V	OXA-PEN-TET-KAN-STR-ERY-CLI
VMCU 11412/AQ43	Dog	No. 85	Perineum	D	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-CIP-SMX
VMCU 10212/AI28	Dog	No. 43	Nasal mucosa	D	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-MUP
VMCU 10312/AI42	Dog	No. 44	Nasal mucosa	D	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-MUP
VMCU 10412/AI49	Dog	No. 45	Nasal mucosa	D	V	OXA-PEN-TET
VMCU 10512/AI62	Dog	No. 46	Perineum	D	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-MUP
VMCU 10612/AM18	Dog	No. 63	Nasal mucosa	D	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-MUP
VMCU 10712/AM4	Dog	No. 61	Pyoderma	D	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-MUP
VMCU 10812/AM12	Dog	No. 64	Pyoderma	D	V	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-MUP
Salmonella Braenderup H9812						

Figure 5.11 DNA fingerprint patterns by *Sma*I-PFGE with SCC*mec* types and antimicrobial resistance profiles of MRSSc



Cfr9I-PFGE	MRSA strain	Origin		Site	MLST	SCC*mec* type	Antimicrobial resistance profile
	VMCU 10912/AF1	Dog	31	Nasal mucosa	398	V	OXA-PEN-TET-GEN-KAN-STR-CLI-CHL-TMP-CIP-SYN-TIA-MUP
	VMCU 11012/VET B50.1	Vet	B50	Nasal mucosa	398	V	OXA-PEN-TET-GEN-KAN-STR-CLI-TMP-CIP-SYN-TIA-MUP
	VMCU 11112/ VET B51.1	Vet	B51	Nasal mucosa	398	V	OXA-PEN-TET-GEN-KAN-STR-CLI--TMP-CIP-SYN-TIA
	VMCU 11212 VET B77.3	Vet	B77	Nasal mucosa	398	V	OXA-PEN-TET-GEN-KAN-STR-CLI-CHL-TMP-CIP-SYN-TIA-SMX
	Salmonella Braenderup H9812						

Figure 5.12 DNA fingerprint patterns by *Cfr*9I-PFGE with molecular characteristics and antimicrobial resistance profiles of MRSA

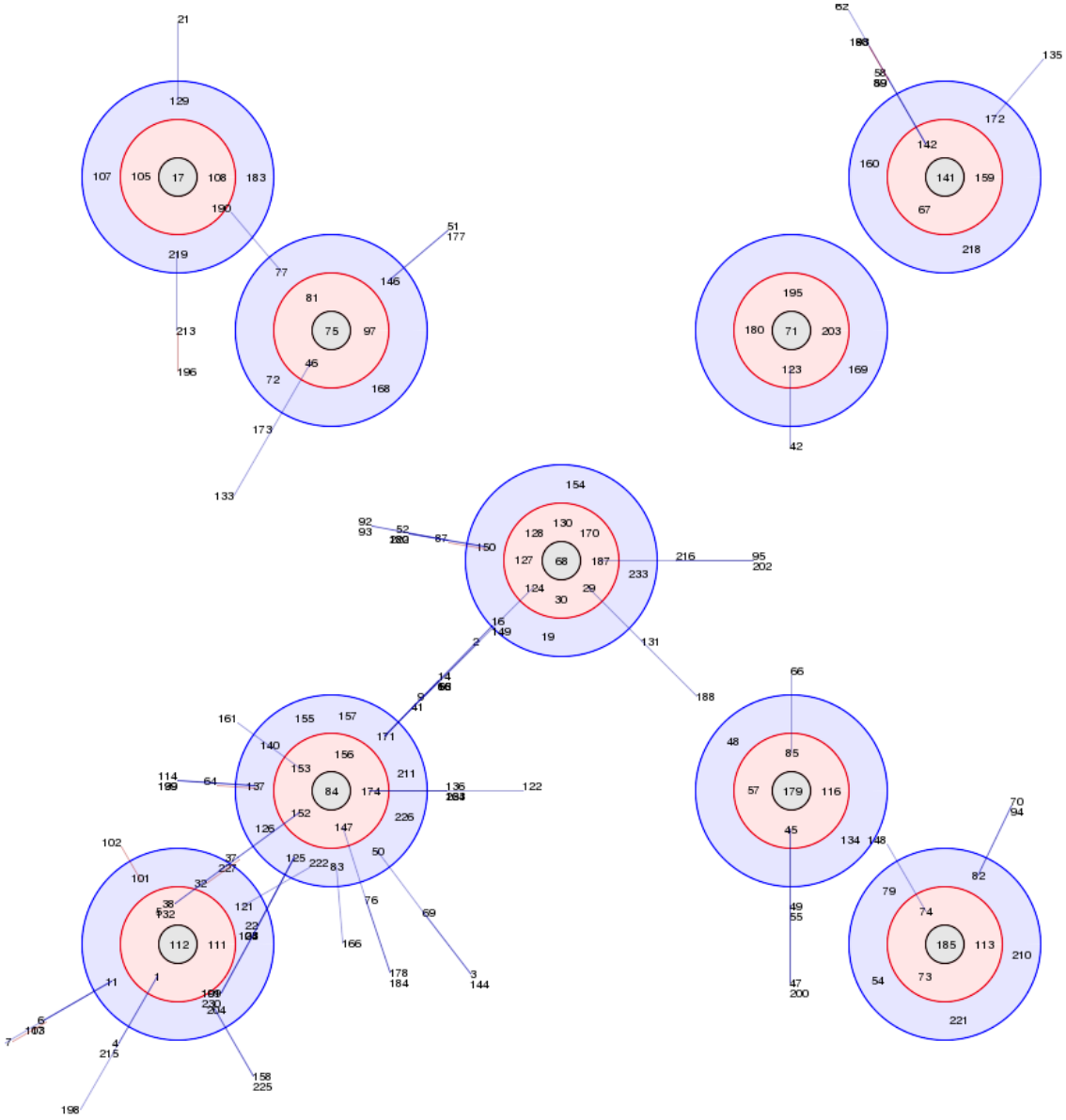


Figure 5.13 Evolutionary relationships and population snapshot of *S. pseudintermedius*. Cluster analysis of related STs (by MLST-7) was constructed based on all *S. pseudintermedius* database from www.pubmlst.org using BURST by setting group definition at 5 loci.

Table 5.19 Numbers of dogs colonized with multiple species and MRSP STs by MLST-7

Multiple species and STs of MRSP colonization	Positive no., dog (%)
Total	100
MRSP ST45 and MRSSc	5 (5%)
MRSP ST112 and MRSSc	3 (3%)
MRSP ST181 and MRSSc	1 (1%)
MRSP ST45, ST182 and MRSSc	2 (2%)
MRSP ST169, ST182 and MRSSc	1 (1%)
MRSP ST45, ST112/ST181 and MRSSc	1 (1%)
MRSP ST45 and ST112	1 (1%)
MRSP ST45 and ST114	1 (1%)
MRSP ST45 and ST181	1 (1%)
MRSP ST111 and ST116	1 (1%)
MRSP ST125 and ST181	1 (1%)
MRSP ST133 and ST181	1 (1%)

CHAPTER VI

DISCUSSION

6.1 Biochemical markers and protein pattern analysis for canine CoPS identification

The biochemical identification using commercial test kits cannot consistently differentiate the species of CoPS derived from animals, especially among SIG, *S. schleiferi* subsp. *coagulans* and *S. aureus* (Jousson et al., 2007; Sasaki et al., 2007; Zdovc et al., 2004). The multiplex PCR for identification was chosen for verifying species of CoPS in this study with validated positive control by gene sequencing. The *cpn60* and *sodA* sequences were approved for confirmation of species of staphylococci (Kwok and Chow, 2003; Poyart et al., 2001; Sasaki et al., 2007). The results from biochemical properties and protein pattern analysis were consensual for species identification and in accordance with genotypic identification. By biochemical markers, protein patterns and *nuc* amplification, all tested canine CoPS were ultimately identified into three species of *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus*. For routine diagnostic scheme, the biochemical tests and multiplex PCR are strongly recommended for identification of CoPS from canine origin. Additionally, quality of DNA template might affect the specificity of this multiplex PCR, because the presence of non-specific bands by using boiling method for DNA isolation.

Biochemical markers can be used for differentiation of CoPS from dogs. Secondary biochemical tests including acetoin production, assimilation of maltose, trehalose and lactose were consensual to distinguish *S. pseudintermedius*, *S. aureus* and *S. schleiferi* subsp. *coagulans*. Even though galactose assimilation could not differentiate among three canine CoPS species, this was able to differentiate *S. pseudintermedius* from *S. intermedius*. Regarding base media for carbohydrate

assimilation, broth medium gave more satisfactory results than agar for interpretation, because the positive reaction might be caused by fermentation in broth yielding acidity (Baird-Parker, 1963). The false negative results of carbohydrate utilization were presented by using agar medium according to the recommendation (Freney et al., 1999). Therefore, the broth medium was suggested for determination of carbohydrate assimilation tests. The negative mannitol fermentation can be used for a marker of *S. pseudintermedius* identification (Van Hoovels et al., 2006), but the positive of this was not the representative marker for *S. aureus* and *S. schleiferi* subsp. *coagulans*. In addition, the negative maltose, lactose and trehalose assimilations could be the markers of *S. schleiferi* subsp. *coagulans* identification. This scheme could not be indicated to differentiate among SIG. However, dogs are not the specific host for *S. intermedius* and *S. delphini* (Bannoehr et al., 2007). Indeed, the secondary biochemical markers consist of the acetoin production, the assimilations of maltose, galactose, trehalose and lactose in broth medium and mannitol fermentation are strongly proposed to be the reliable scheme for primary screening of CoPS from canine origins.

With a high agreement with biochemical properties and multiplex PCR, the protein patterns of *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus* using SDS-PAGE exhibited unique pattern within species and different patterns among three species. The protein pattern analysis is acceptable for identification of species of *S. aureus* and coagulase-negative staphylococci (Berber et al., 2003; Pierre et al., 1990), but this has not been proposed for *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* in previous studies. Each species of 337 CoPS exhibited distinct patterns corresponding to the standard controls. Although the minor variation of protein bands were found within species, more than 90% similarity of bands has been accepted to group the same species by quantitative analysis (Berber et al., 2003). Moreover, this tool could provide the information about different patterns of proteins of CoPS that could be

used for further study. Regarding advance proteomic technology, use of MALDI (matrix assisted laser desorption/ionization) TOF (time-of-flight) MS-MS (tandem mass spectrometry) has provided advanced schemes for identifying peptide sequence markers for bacterial speciation using coagulase-negative staphylococci model.

Indeed, this study proposed the consensual phenotypic differences of canine CoPS that originated from our in-house method including acetoin production, assimilation of maltose, galactose, trehalose and lactose in purple broth base and fermentation of mannitol. In addition, canine CoPS-specific protein patterns using SDS-PAGE were illustrated that can differentiate the species as a phenotypic confirmation.

6.2 Prevalence and distribution of CoPS and MRCoPS in dogs and dog-associated people

The CoPS isolated from dogs and humans in this study were classified into three species including *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus*. The results confirmed that *S. pseudintermedius* was the major species colonizing on dog skin at highest prevalence (Devriese et al., 2009). Also, *S. schleferi* subsp. *coagulans* could be moderately isolated but *S. aureus* was rare. Prevalence of *S. schleiferi* subsp. *coagulans* was agreed with the previous reports that were consistently lower than *S. pseudintermedius* (Griffeth et al., 2008; Hanselman et al., 2008; May et al., 2012; Morris et al., 2006). In contrast, *S. aureus* was found in very low prevalence that might be reversely transmitted from human (Manian, 2003; Simoons-Smit et al., 2000). The ability of *S. aureus* colonizing on canine skin is possibly anticipated by colonization of *S. pseudintermedius* because of the competitive situation by similar homologue adhesin (Geoghegan et al., 2009). Nasal mucosa, perineum and groin can be representative sites for sampling. These occlusive sites provided optimal conditions for bacterial growth such as humidity and nutritional supply (Mason et al., 1996). Especially

at nasal mucosa, the variety of CoPS could be obtained at this site compared other areas. However, the multiple sampling from all sites could enhance the sensitivity of sample collection of CoPS from canine skins.

Regarding methicillin resistance, high prevalence of dogs carrying MRSP in this study is close to the high prevalence in South China. The colonization of this has been concerned as PA-MRS that spreads in healthy dogs in community. Also, MRSSc can be recovered from healthy dogs and also associated with skin lesions (May et al., 2012). The coexistence of methicillin-resistant and methicillin-susceptible strains revealed the heterogeneous populations colonizing in dog skin (Fazakerley et al., 2010). On the other hand, MRSA was isolated from only one dog corresponding to an incidental occurrence. By antimicrobial susceptibility test, the criteria for identification between MRSP and MRSA were different (Bemis et al., 2009; Papich, 2010; Schissler et al., 2009). Therefore, the reliable species identification must be performed. However, the *mecA* detection should be conducted to accurately identify the MRS derived from animal sources (Leonard and Markey, 2008).

Concerning dog-associated people, three species of CoPS could be isolated from dog's owners and veterinarians. On the contrary, *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* could not be isolated from the control people. The results also confirmed that dog is the specific host for *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans*, and human is the specific host for *S. aureus*. Furthermore, no methicillin-resistant strain could be isolated from people without dog in their household. These could infer the risk of colonization of canine CoPS and MRCoPS in dog-associated people. Notably, carriage rate of MRSP in veterinarians was higher than that of dog's owners. Regarding an occupational risk for veterinary career, opportunities of exposure to dog patients and hospital environment might increase the occurrence of canine MRCoPS acquisition. It is speculated that the colonization of canine CoPS

species in human might need enough duration of exposure such as by sharing the same environment (Morris et al., 2010; Soedarmanto et al., 2011; Walther et al., 2012). Therefore, acquired colonization in risk groups should be aware in term of interspecies transmission, especially methicillin-resistant strains. The prevalence of MRSSc in dog-associated people was lower than MRSP that were correlated with the prevalence in dog. However, the persistent colonization of canine CoPS in humans has not been clearly revealed and needs to be clarified by longitudinal study. To decrease the risk of colonization in human, the hygienic management and decontamination should be further evaluated and performed.

6.3 Antimicrobial resistance and molecular characteristics of MRCoPS in dogs and dog-associated people

Molecular characteristics revealed the diversity of population genetics of MRCoPS distributing in Thai dogs and dog-associated people. By MLST, various clones of MRSP were identified with novel STs. By comparison with MLST-4, MLST-7 can provide higher discriminatory power by additional four housekeeping genes (Solyman et al., 2013). Moreover, the sequence types analyzed by MLST-7 resulted in more consistent correlation and validity with PFGE analysis and SCC*mec* typing. Various SCC*mec* types were detected in Thai MRSP, but the majorities were ψSCCmec_{57395} and SCC*mec* type V. With the highest prevalence, the novel ψSCCmec_{57395} was prominently harbored by MRSP $ST_7$45, while SCC*mec* type V was widely distributed in many STs. The specific association of ψSCCmec_{57395} with MRSP $ST_7$45 might be caused by the lack of *ccr* in this genetic element (Perreten et al., 2013). Furthermore, MRSP $ST_7$45 carrying ψSCCmec_{57395} specifically presented *Sma*I-indigestible characteristic because of a presence of restriction-modification systems (Perreten et al., 2013). Thus, *Cfr*9I-PFGE was capable to demonstrate the strain identity and variations within this clone and

showed the relationships among human and canine isolates. The MRSP $ST_7$45-*Cfr*9I-A-ψSCC*mec*$_{57395}$ was proposed as the predominant clone of MRSP distributing in Thai dogs and dog-associated people that was mostly isolated from dogs and veterinarians but not from dog's owner. This clone possibly related to the isolates in the Netherlands that was previously designated as MRSP $ST_4$29 and $ST_4$131 (Laarhoven et al., 2011). Other ST of MRSP contained ψSCC*mec*$_{57395}$ might be originated from common ancestor, and this cassette could be found in clonal complex 179 (Perreten et al., 2013). The SCC*mec* A1 should be proposed as a new recombination that has never been classified in MRSA. The MRSP $ST_7$111 and 112 ($ST_4$5) in Thailand specifically contained SCC*mec* A1 but MRSP $ST_4$5 isolated in China harbor other SCC*mec* (Feng et al., 2012; Wang et al., 2012). The suspicion of variation in the MRSP $ST_4$5 might be elucidated by MLST-7 analysis. In addition, SCC*mec* type II-III was also specifically incorporated in MRSP $ST_7$169 ($ST_4$71). The MRSP $ST_7$71 ($ST_4$71) carrying SCC*mec* type II-III is recognized as a major European clone. (Descloux et al., 2008; Perreten et al., 2010). Similar to this study, this clone was isolated from dogs in China and Hong Kong referring intercontinental spread MRSP clone (Boost et al., 2009; Wang et al., 2012). In contrast to other types, SCC*mec* type V was widely associated in various STs of MRSP and also found in all MRSSc and MRSA. These inferred the extent dissemination of this cassette in staphylococci. The SCC*mec* type V was also harbored by MRSP ST68, the predominant clone in North America (Black et al., 2009; Perreten et al., 2010). In *S. aureus*, SCC*mec* type V frequently associates in community-acquired MRSA and also associates in MRSA derived from animals (Argudin et al., 2009; Ito et al., 2004). All MRSA isolated from a dog and dog-associated people showed the characteristics as same as those of livestock-associated MRSA (LA-MRSA) by ST398, a *Sma*I-indigestible feature and SCC*mec* type V carriage (Argudín et al., 2010). This could be an evidence of distribution of LA-MRSA ST398 in companion animal and veterinarians in Thailand.

Nevertheless, no HA-MRSA and CA-MRSA was detected in populations in this study. Additionally, the human and canine MRSSc isolates consistently carried SCC*mec* type V that were different from MRSSc carrying SCC*mec* type IV in a previous report (Roberts et al., 2005). Because MLST scheme of *S. schleiferi* subsp. *coagulans* has not been established, only PFGE and SCC*mec* typing were conducted for typing of MRSSc. The MRSSc in *Sma*I$_{SSc}$-D group carrying SCC*mec* type V was a major cluster that might be clonally related. Indeed, the various molecular characteristics could demonstrate the clone of MRSA ST 398, diversity of MRSP clones and possible-related MRSSc clones distributing in Thai dogs and dog-associated people.

Regarding antimicrobial resistance, over 80% of MRSP isolates could mediate multidrug resistance (more than 3 classes). The identified genes using microarray can describe the mechanisms by the presence of resistance genes. These showed the accumulation of genes encoding the resistance in Thai MRSP clones that were mostly contained on mobile genetic elements (Kadlec and Schwarz, 2012). Thai MRSP and MRSSc also carried Tn*5405*-like element by the concurrent detection of *aph(3')-III*, *ant(6')-Ia*, *sat4* and *erm*(B). This element contained a genetic linkage of aminoglycoside and macrolide resistance that was commonly found in *S. pseudintermedius* (Boerlin et al., 2001; Perreten et al., 2010). Many genes encoding the resistance were widely distributed in many clones of MRSP that might be a factor rendering clonal selection. The plasmid-borne resistance genes such as *tet*(K), *cat*$_{pC221}$ and *erm*(C) were rarely detected in MRSP that are possibly caused by the poor preference of acquired plasmid (Noble et al., 1996). By comparison of other STs, the presence of *cat*$_{pC221}$ and the lack of *dfrG* could be a resistance characteristic of the predominant MRSP ST$_7$45. On the other hand, the *dfr*G can be found in many clones of MRSP (Feng et al., 2012; Perreten et al., 2010). The multidrug-resistant MRSSc also exhibited resistance patterns close to MRSP and might contain common mobile genetic element. On the contrary, the genes

mediating resistance in MRSA were different from MRSP and MRSSc. The *tet*(L), *fex*A, *lsa*(E) and *lnu*(B), frequently found in LA-MRSA, were recognized as uncommon genes (Kadlec et al., 2012). The resistance to quinupristin/dalfopristin was concerned as an emergence. As a human-specific species, LA-MRSA can cause infection in farm workers and outbreak in human hospital (Kock et al., 2009; Lozano et al., 2011). These animal-derived MRCoPS are concerned in term of zoonosis and can rapidly develop resistance to last-resort drugs after antimicrobial exposure by gene acquisition and mutation (Kadlec et al., 2011; Schwendener and Perreten, 2011). Therefore, the investigation and monitoring should be continued to obtain the current information on the emergence of antimicrobial-resistant bacteria.

Common genetic and antimicrobial resistance features of MRCoPS isolated from Thai dogs and dog-associated people could support the interspecies transmission of the antimicrobial-resistant bacteria between dog and human. Many clones of MRSP were shared among dogs, veterinarians and dog's owners. This could be also confirmed by the similarity of DNA fingerprint pattern, especially isolates from dogs and dog's owner in the two same households. Also, the multiple clonal expansions of MRCoPS were revealed by isolation of different STs and species in same dogs. In summary, the common and various clones of MRSP sharing in Thai dogs and dog-associated people were illustrated with the proposed of predominant MRSP $ST_4$45 and various novel STs by MLST-7. The evidence of LA-MRSA colonizing in Thai dog and veterinarian was demonstrated and this study also showed the clonal relation of MRSSc between Thai dog and dog-associated people.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

This study found three CoPS species colonizing in dogs and dog-associated people, including *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans* and *S. aureus*. With the concordance phenotypes and genotypes, the species of these could be identified by using the proposed biochemical scheme and protein profile analysis. According to no database of animal-derived staphylococci, the conventional biochemical tests consisting of primary test, coagulase test, acetoin production, assimilation of maltose, galactose, trehalose and lactose in broth medium and mannitol fermentation can be used as a phenotypic screening in routine diagnosis of canine CoPS. The reliable result of species identification is necessary for phenotypic detection between MRSA and MRSP because of the different criteria. Regarding zoonosis, the species identification is primarily needed for clarification of interspecies transmission also. *S. pseudintermedius* is a major species-specific CoPS exhibiting on dog skin and *S. aureus* is a human-specific species. *S. schleiferi* subsp. *coagulans* is a minor CoPS population on canine skin. The colonization of *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans* in human is concerned as unusual occurrences that could be supported by no prevalence of canine species in control people. Focused on methicillin resistance, dog-associated people were a risk group that prone to acquire MRSP and MRSSc by cross-species transmission, especially veterinarian. The molecular features revealed the various clones of MRCoPS colonizing in Thai dogs and dog-associated people. These also confirmed the transmission between dogs and humans by common genetic characteristics. The MRSP $ST_7$45 carrying ΨSCC*mec*$_{57395}$ was a predominant clone and other various clones were also shared among Thai dogs, veterinarians and

dog's owners. Many novel STs of MRSP with unclassified SCC*mec* were identified in this study. Also, the evidence of LA-MRSA colonization in Thai dog and veterinarians was illustrated. With multidrug-resistant characteristics, MRCoPS exhibited most of approved basic drug for veterinary and human medicine and the mechanisms of resistance were identified by the detected genes. Concerning drug for MRSA decolonization and treatment, mupirocin resistance could be detected in all species. Additionally, all MRSA could mediate quinupristin/dalfopristin resistance.

As an emerging problem, continuing investigation is needed to conduct for monitoring the spread and development of these antimicrobial-resistant bacteria. The linkage of transmission of LA-MRSA among human, dog and other animals should be clarified in large cohort. Also, the longitudinal study and fitness cost for persistent colonization of MRCoPS should be carried out, especially in case of MRSP colonization in human. By MLST-7, the MRSP in previous studies should be re-identified for global standardization. With the increased significance, the development of MLST scheme can properly describe the clonal relation of MRSSc. To date, the recommended protocol for MRCoPS eradication in animals have not been evaluated and established in veterinary practice. The proper hygiene and decontamination should be performed to decrease the zoonotic transmission. Therefore, prudent use of antimicrobial, intensive hygienic management and monitoring must be promoted as primary strategies to cope with the challenge problem.

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APPENDIX

PREPARATION OF MEDIA, SOLUTIONS AND BUFFERS

1. Media for biochemical tests

1.1 Semisolid medium for oxidation-fermentation test

Tryptone 1%	10.0 g
Yeast extract	1.0 g
Agar	2.0 g
Bromcresol purple	0.01 g
Distilled water	1000.0 ml

1.2 Voges-Prausker test medium for acetoin production

Peptone	7.0 g
Glucose	5.0 g
NaCl	5.0 g
Distilled water	1000.0 ml

1.3 Purple agar medium for carbohydrate assimilation test

Proteose peptone No.3	10.0 g
Beef extract	1.0 g
NaCl	5.0 g
Agar	15.0 g
Bromcresol purple	0.02 g
Distilled water	1000.0 ml

1.4 Purple broth medium for carbohydrate assimilation test

Proteose peptone No.3	10.0 g
Beef extract	1.0 g
NaCl	5.0 g
Bromcresol purple	0.02 g

Distilled water	1000.0 ml

2. Buffer and solution for SDS-PAGE

2.1 Tris-HCl 1.125 M, pH 8.8

Tris-base	136.2357 g
Add distilled water	700.0 ml

Adjust pH by 5 N HCl to pH=8.8 and add distilled water to be 1000ml

2.2 Tris-HCl 0.625 M, pH 6.8

Tris-base	75.69 g
Add distilled water	700.0 ml

Adjust pH by 5 N HCl to pH=6.8 and add distill water to be 1000ml

2.3 Acrylamide:Bis-acrylamide 30:0.8

Acrylamide	30.0 g
Bis-acrylamide	0.8 g

Add distilled water to be 100 ml

2.4 10% SDS

Sodium dodecyl sulfate	10.0 g
Distilled water	100.0 ml

2.5 10% APS

Ammonium persulfate	1.0 g
Distilled water	10.0 ml

2.6 Saturated butanol

Butyl alcohol:distilled water	1:1

2.7 Stacking gel

Tris-HCl 0.625 M, pH 6.8	1.25 ml
Distilled water	3.0 ml
Acrylamide:bis acrylamide 30:0.8	0.75 ml

10% APS	22.5 µl
TEMED	12.5 µl

2.8 Separating gel

Tris-HCl 1.125 M, pH 8.8	5.0 ml
Distilled water	5.0 ml
Acrylamide:bis acrylamide 30:0.8	5.0 ml
10% SDS	150.0 µl
10% APS	30.0 µl
TEMED	15.0 µl
Saturated butanol	70.0 µl

2.9 Denaturing buffer

Tris-base	0.15 g
SDS	0.4 g
2-mercaptoethanol	1.0 g
Glycerol	2.0 ml
Distilled water	7.0 ml
Bromphenol blue	0.02 g

2.10 1X running buffer

Glycine	13.95 g
Tris-base	3.0 g
SDS	1.0 g
Add distilled water to be	1000.0 ml

2.11 Coomassie brilliant blue staining

Methanol	900.0 ml
Acetic acid	180.0 ml
Distilled water	900.0 ml

Mixed by magnetic stirror for 24 h and filtrated by filter paper no.2

2.12 Destaining buffer

Methanol	160.0 ml
Acetic acid	240.0 ml
Distilled water	875.0 ml

3. Buffer for PFGE

3.1 1 M Tris-HCl, pH 8.0

Tris base	121.0 g
Ultrapure water	700.0 ml

Adjust pH to 8.0 by 6 N HCl

Add ultrapure water to be 1000 ml and sterilize by autoclaving

3.2 10 N NaoH

NaOH	400.0 g
Add sterile ultrapure water	800.0 ml

Cool solution to room temperature

Add sterile ultrapure water to be 1000 ml

3.3 0.5 M EDTA, pH 8.0

$Na_2EDTA.2H_2O$	186.1 g
Ultrapure water	800.0 ml

Adjust pH to 8.0 by 10 N NaoH

Add ultrapure water to be 1000 ml and sterilize by autoclaving

3.4 10 mg/ml lysozyme stock solution

Lysozyme	100.0 mg
Sterile ultrapure water	10.0 ml

Mix and dispense in 500 µl in microcentrifuge tubesand store at -20^{o}C

3.5 20% SDS

SDS	20.0 g
Sterile ultrapure water	80.0 ml

3.6 20 mg/ml Proteinase K stock solution

Proteinase K	100.0 mg
Sterile ultrapure water	5.0 ml

Mix and dispense in 500 µl in microcentrifuge tubesand store at -20°C

3.7 10% N-lauryl sarcosine, Sodium salt (Sarcosyl)

Sodium lauryl sarcosine	10.0 g
Sterile ultrapure water	90.0 ml

3.8 10X Tris-Borate EDTA (TBE) buffer, pH 8.3

Tris base	108.0 g
Boric acid	55.0 g
0.5 M EDTA, pH 8.0	40.0 ml

Dilute to 1000 ml with sterile ultrapure water and sterilize by autoclaving

3.9 Ethidium Bromide stock solution

Ethidium bromide	10.0 mg
Sterile ultrapure water	1.0 ml

For staining, dilute 10 µl in 100 ml distilled water

3.10 Tris-EDTA (TE) buffer, pH 8.0

1M Tris-HCl, pH 8.0	10.0 ml
0.5 M EDTA, pH 8.0	2.0 ml

Dilute to 1000 ml with sterile ultrapure water

3.11 Cell suspension buffer, pH 8.0

1M Tris-HCl, pH 8.0	10.0 ml
0.5 M EDTA, pH 8.0	20.0 ml

Dilute to 100 ml with sterile ultrapure water

3.12 Cell lysis buffer for *Salmonella* Braenderup strain H 9812

1M Tris-HCl, pH 8.0	25.0 ml
0.5 M EDTA. pH 8.0	50.0 ml
10% Sodium lauryl sarcosine	50.0 ml

Dilute to 500 ml with sterile ultrapure water

3.13 EC lysis buffer for CoPS

1M Tris-HCl, pH 8.0	10.8 ml
5 M NaCl	360.0 ml
0.5 M EDTA, pH 8.0	360.0 ml
Brij-58 (Polyoxyethylene 20 Cetyl Ether)	9.0 ml
Sodium deoxycholate	3.6 g
Sodium lauryl sarcosine	9.0 g
Ultrapure water	700.0 ml

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SCHOLARSHIPS

Chulalongkorn University Graduate Scholarship to (2010-2012)

Commemorate the 72nd Anniversary of His Majesty King

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The 90th Anniversary of Chulalongkorn University Fund (2011)

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