สปีชี่ส์และการตอบสนองต่อยาต้านจุลชีพของเชื้อ Methicillin - resistant coagulase-positive *Staphylococcus spp.* (MRCPS) ที่แยกได้จากสุนัขในประเทศไทย

นางสาว พรรณพิชญา ฟุ้งวิทยา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต
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SPECIES AND ANTIMICROBIAL SUSCEPTIBILITY OF METHICILLIN-RESISTANT COAGULASE-POSITIVE *STAPHYLOCOCCUS SPP.* (MRCPS) ISOLATED FROM DOGS IN THAILAND

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN VETERINARY PHARMACOLOGY

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Thesis Title	SPECIES AND ANTIMICROBIAL SUSCEPTIBILITY OF METHICILLIN-RESISTANT COAGULASE-POSITIVE *STAPHYLOCOCCUS SPP.* (MRCPS) ISOLATED FROM DOGS IN THAILAND
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พรรณพิชญา ฟุ้งวิทยา : สปีชี่ส์และการตอบสนองต่อยาต้านจุลชีพของเชื้อ Methicillin - resistant coagulase-positive *Staphylococcus spp.* (MRCPS) ที่แยกได้จากสุนัขในประเทศไทย อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ภญ. สุพัตรา ศรีไชยรัตน์. อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.ภญ. พิณทิพย์ พงษ์เพ็ชร, 115 หน้า.

วัตถุประสงค์ของการวิจัยครั้งนี้เพื่อศึกษาอุบัติการของเชื้อกลุ่ม Coagulase positive *Staphylococcus spp.* (CPS) ที่แยกจากสุนัขที่เข้ามารับการรักษาที่โรงพยาบาลสัตว์เล็ก คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และทดสอบความไวรับของเชื้อกลุ่มนี้ที่มีต่อยาต้านจุลชีพ แยกชนิดของเชื้อดังกล่าวด้วยวิธีตรวจสอบพันธุกรรมและตรวจสอบพันธุกรรมที่เกี่ยวข้องกับกลไกการดื้อต่อยาเมธิซิลลินของเชื้อดังกล่าว จากการเพาะแยกเชื้อจากช่องจมูก แผลผิวหนัง และช่องหูอักเสบจำนวน 200 ตัวอย่างที่แยกได้จากสุนัข 100 ตัว พบว่ามีเชื้อ *Staphylococcus spp.* จำนวน 72 ตัวอย่าง (36%) ในจำนวนนี้พบว่าเป็นเชื้อ CPS 49 ตัวอย่าง (68.05%) โดยพบมากที่จมูก, แผล และ ช่องหูอักเสบตามลำดับและ *S. pseudintermedius* เป็นเชื้อที่พบมากที่สุด (85.71% ของ CPS)

ผลการทดสอบความไวรับต่อยาต้านจุลชีพ พบว่าเชื้อในกลุ่ม CPS มีความไวรับต่อ mupirocin มากที่สุดถึง 93.88% และ cefalexin เป็นยาเชื้อมีความไวรับต่อยา (2.04%) นอกจากนี้ยังพบว่าเชื้อส่วนใหญ่ (88%)มีการดื้อยาร่วมกันมากกว่า 3 กลุ่ม (multidrug resistant) และเชื้อที่ดื้อยาส่วนใหญ่ยังดื้อต่อยามากกว่า 4 ชนิดขึ้นไป ค่า MIC_{50} และ MIC_{90} ของยา Oxacillin ที่ต่อเชื้อกลุ่ม CPS ที่แยกได้มีค่าเท่ากันที่ 256 µg/ml เชื้อ *S. pseudintermedius* ที่แยกได้ส่วนใหญ่มีค่า MIC สูงกว่า 4 µg/ml ส่วน *S. aureus* และ CPS ตัวอื่นๆ ยังคงไวรับต่อ oxacillin โดยมีค่า MIC ของ oxacillin ในระดับที่ต่ำกว่า 2 µg/ml

ไม่พบว่ามีความสัมพันธ์กันจากการทดสอบคัดกรองหาการสร้างเอนไซม์เบต้าแลคแทมเมสกับการปรากฏของสารพันธุกรรมดื้อยา เชื้อที่ดื้อต่อ oxacillin ส่วนหนึ่ง (22จาก 49 เชื้อ) มีสารพันธุกรรมที่เกี่ยวข้องกับการดื้อยาทั้ง *blaZ* (สร้างเบต้าแลคแทมเมส) และ *mecA* (PBP2a) ซึ่งทำให้มีค่า MIC สูงกว่าเชื้อที่มีสารพันธุกรรมดื้อยา *blaZ* หรือ *mecA* เพียงอย่างเดียว เชื้อส่วนใหญ่ที่พบสารพันธุกรรมดื้อยา *blaZ* และ(หรือ) *mecA* เป็นเชื้อ *S. pseudintermedius* เชื้อ *S. aureus* เพียง 2 ในทั้งหมด 3 ไอโซเลตที่แยกได้มีพียงสารพันธุกรรมดื้อยา *blaZ* ในขณะที่ CPS อื่นๆไม่พบสารพันธุกรรมดื้อยาทั้งสองตัว จากผลการทดลองนี้แสดงให้เห็นว่าเชื้อในกลุ่ม CPS ที่พบในสุนัขมากที่สุดได้แก่ *S. pseudintermedius* ที่ดื้อต่อเมธิซิลลินและยังดื้อต่อยาหลายกลุ่ม โดยเฉพาะอย่างยิ่งดื้อต่อ cefalexin ซึ่งเป็นยาที่ใช้กันอย่างแพร่หลายในการรักษาทางสัตวแพทย์ การที่เชื้อที่มีสารพันธุกรรมดื้อยา *blaZ* และ *mecA* ทำให้อาจเกิดการถ่ายทอดสารพันธุกรรมดังกล่าวให้เชื้อในกลุ่ม *Staphylococcus spp.* อื่นซึ่งรวมถึง *S. aureus* อาจทำให้เกิดปัญหาแพร่กระจายของเชื้อ MRSA ในชุมชนได้

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PUNPICHAYAY FUNGWITHAYA : SPEICES AND ANTIMICROBIAL SUSCEPTIBILITY OF METHICILLIN-RESISTANT COAGULASE-POSITIVE *STAPHYLOCOCCUS SPP.* (MRCPS) ISOLATED FROM DOGS IN THAILAND. THESIS ADVISOR : ASSOC. PROF. SUPATRA SRICHIRAT, Ph.D. THESIS CO-ADVISOR : ASSOC. PROF. PINTIP PONGPECH, Ph.D., 115 pp.

The objective of this study was to screen prevalence and antimicrobial susceptibility of Coagulase positive *Staphylococcus spp.* (CPS) isolated from dogs visiting at the Small Animal Hospital of Chulalongkorn University. *Staphylococcus spp.* were identified by using of gene detection and PCR-REA technique. In addition, mechanism of methicillin resistance was determined by molecular detection of their resistance genes. 200 samples from both nostril, wound or otitis externa were obtained from 100 dogs. 72 isolates from 200 samples (36%) were found to be Staphyloccoci. Among ofthese , 49 isolates (68.05%) were identified as CPS. The highest prevalence occurrence of CPs found in this study was in nasal cavity, followed by wound and otitis externa, respectively. The PCR technique for molecular detection revealed that *S. pseudintermedius* was the most common CPS (85.71%) found in dogs.

The antimicrobial susceptibility test showed that most of CPS were susceptible to mupirocin (93.88%) whereas few of them were susceptible to cefalexin (2.04%). Moreover, 88% CPS isolates were multidrug resistant (more than 3 drugs) and most of the MDR-CPS resisted more than 4 antimicrobials. The MIC_{50} and MIC_{90} of oxacillin against MDR-CPS were 256 µg/ml. Most of *S. pseudintermedius* isolates resisted to oxacillin (MIC > 4 µg/ml) whereas no oxacillin resistance of *S. aureus* and other CPS were found (MIC < 2 µg/ml).

No correlation between beta-lactamase screening test and the presence of the resistant gene blaZ and mecA was found in this study. The molecular identification revealed that 22 out of 49 isolates carried both *blaZ* and *mecA* gene and their MIC of oxacillin were also higher than those against the isolates carried either *blaZ* or *mecA* genes. Most of *S. pseudintermedius* isolates carried both *blaZ* and *mecA* genes. Two out of three *S. aureus* isolates found in this study carried either *blaZ* while the other CPS did not carried *blaZ* or *mecA* genes. The results from this study showed that the most common of methicillin resistant CPS found in dogs was *S. pseudintermedius*. These isolates were highly resistant to many antimicrobials, including cefalexin which is commonly used in veterinary practice. Unfortunately, most of them were *blaZ* and *mecA* genes carrier. Eventhough, MRSP in people is only a minor concern, the methicillin resistant gene can transmit to the other *Staphylococcus spp.* including *S. aureus* that can be the cause of the MRSA spread in the community.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	Base pair
CA	Community-acquired methicillin-resistant *Staphylococcus aureus*
ºC	Degree Celcius
DNA	Deoxy ribonucleic acid
ET	Electrophoretic type
h	Hour
µg	Microgram (10^{-6} gram)
µl	Microlitre (10^{-6} litre)
M	Molar
MboI	*Moreaxella bovis* enzyme
mg	Milligram (10^{-3} gram)
min	Minute
ml	Millitre (10^{-3} litre)
mM	Millimolar (10^{-3} molar)
mm	Millimetre (10^{-3} metre)
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MR(P)IS	Methicillin-resistant *Staphylococcus (pseud)intermedius* (MR(P)IS)
MS(P)IS	Methicillin-susceptible *Staphylococcus (pseud)intermedius* (MS(P)IS)
MRSA	Methicillin-resistant *Staphylococcus aureus* (MRSA)
MSSA	Methicillin-susceptible *Staphylococcus aureus* (MSSA)
MRPS	Methicillin-resistant *Staphylococcus pseuintermedius* (MRPS)
MSPS	Methicillin-susceptible *Staphylococcus pseuintermedius* (MSPS)

PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PVL	Panton-Valentine Leukocidin
SA	*S. aureus*
SCC	Staphylococcal cassette chromosome
SIG	*Staphylococcus intermedius* group
SP	*S. pseudintermedius*
ST	Sequence type
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Staphylococcus spp. are Gram positive cocci. Two major groups of *Staphylococcus spp.* are coagulase-positive *Staphylococcus spp.* (CPS) and coagulase-negative *Staphylococcus spp.* (CNS) (Kloo and Bannerman, 1997; Songer and Post, 2004). CPS can be found in human and animals. They can spread by aerosol and contact with skin to skin and excretions (El-jakee et al., 2008). In dogs, *Staphylococcus aureus* (SA) and *Staphylococcus (pseud)intermedius* (S(P)I) are major coagulase-positive *Staphylococcus spp.* that cause infections. SA is normally found in human but in dogs is rare (Leonard and Markey, 2008). S(P)I is a member of the normal flora of the skin and mucosa and the major cause of skin infection including wound infection such as surgical wound infection, pyoderma and otitis externa in dogs (Hill et al., 2006).

In 1972, the first methicillin-resistant coagulase-positive *Staphylococcus spp.* from animals was reported (Devriese et al., 1972). After that both SA and S(P)I strains were reported with increasing frequency of methicillin-resistant *Staphylococcus aureus* (MRSA) (Pak et al., 1999; van Duijkeren et al., 2004; Bender et al., 2005; Anzai et al., 1996; Hartmann et al., 1997; Goni et al., 2004; Voss et al., 2005; Lee, 2003) and methicillin-resistant *Staphylococcus (pseud)intermedius* (MRS(P)I), (Gortel et al., 1999; Piriz et al., 1996; van Duijkeren et al., 2008; Vengust et al., 2006; Zubeir et al., 2007). MRSA is very dangerous for human more than MRS(P)I because MRSA can infect human and beacause of death in human who have a weaken immune system. *Staphylococcus spp.* have mechanism for antimicrobial resistance including production of Penicillinase enzyme and the PBP2a. The *blaZ* gene were encodes beta-lactamase enzyme. More than 90% of staphylococcal isolates now produce penicillinase, regardless of the clinical setting. The gene for beta-lactamase is part of a transposable element located on a large plasmid, often with additional antimicrobial resistance genes

(Lowy, 2003). *mecA* gene is a gene that expresses protein PBP2a (Mascarettii, 2003). Interestingly, *Staphylococcus spp.* can exchange *mecA* gene among different species. For this reason, MRS(P)I should be studied as same as the MRSA. MRS(P)I was found not only in animal or veterinary hospital but can be found in human who work in veterinary hospital such as veterinarians and nurses. In 2008, van Duijkeren et al. described that veterinary surgeon may play an important role for spreading MRS(P)I to surgery wound in animal.

In this study, CPS from dogs in a veterinary teaching clinic were screened and identified. The isolates were determined for antimicrobial susceptibility as well as studied for beta-lactam resistant mechanisms by screening for *blaZ* and *mecA* genes using by PCR method.

OBJECTIVES

- To study the prevalence of MRCPS in dogs
- To obtain antimicrobial susceptibility patterns of staphylococci isolates from dogs.
- To demonstrate presence of *blaZ* and *mecA* genes in staphylococcal isolates by PCR method

HYPOTHESIS

CPS can be isolated from dogs and are resistant bacteria which carry either *blaZ* or *mecA* genes or both.

EXPECTED BENEFITS

The prevalence of CPS and their antimicrobial susceptibility patterns can be used as the guildline in the treatment by veterinarian in Thailand. CPS carried either *blaZ* or *mecA* genes or both can be the source of such resistant genes that can be transferred to other *Staphylococcus spp.* including *Staphylococcus aureus* that in the major harmful bacteria in human. Veterinarians and owners who are colonized with these bacteria may be at risk for infection with methicillin-resistant *Staphylococcus aureus* (MRSA). Monitoring the status of bacteria by detecting resistant gene may be the good way to protect us from these harmful bacteria.

CHAPTER 2

LITERATURE REVIEWS

2.1 General characteristics of *Staphylococcus spp.*

Staphylococci are gram-positive spherical bacteria occurring in pairs, short chains, and clusters on shown in Figure 2-1. They are facultatively anaerobic, nonmobile, catalase positive, oxidase negative and fermentative. More than 47 species are in the genus *Staphylococcus.* Staphylococci occur frequently as commensals on the skin and mucous membranes of animals and human (Carter and Wise, 2004).

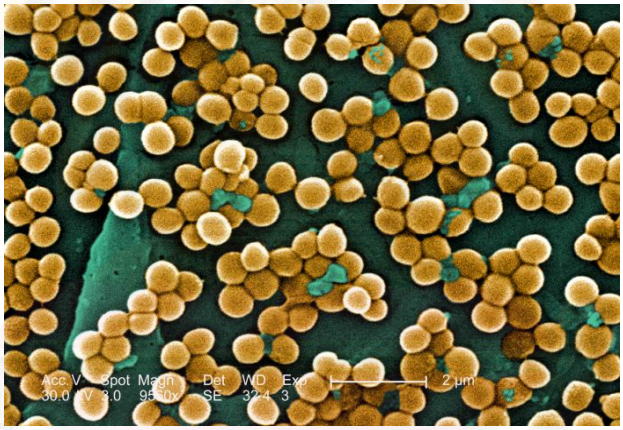


Figure 2-1 Colony of *Staphylococcus spp.*

Staphylococci are the most commonly isolated Micrococcaceae from veterinary clinical specimens. Member of the genus *Staphylococcus* can usually be differentiated from micrococci based on cell morphology and pigment production on solid media and they can be separated from each other by oxidase test. Micrococcus produces Cytochrome C Oxidase that oxidizes Kovac's oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride) and changes color of oxidase positive bacteria cells from clear to purple (Kloo and Bannerman, 1997; Songer and Post, 2004).

Staphylococcal group can produce catalase that converts hydrogen peroxide to oxygen and water. This enzyme can separate staphylococci from streptococci.

Coagulase, the enzyme that activates thrombin which subsequent conversion of fibrinogen to fibrin. Some staphylococci produce coagulase (*S. aureus*, *S. intermedius*, *S. pseudintermedius*, *S. delphini*, and *S. schleiferi* subsp. *coagulans*), while *S. hyicus* is variable in coagulase production. (Table 2-1).

Table 2-1 Differentiation among coagulase-positive staphylococci (Songer and Post, 2005)

organism	Tube coagulase	Voges-Proskauer test
S. aureus subsp. aureus	+	+
S. intermedius	+	-
S. hyicus	V	-
S. schleiferi subsp. *coagulans*	+	+

+, >90% of strains positive; >90% of strains negative; v, variable.

The coagulase-positive *Staphylococcus* (CPS) species are found frequently as etiologic agents of a variety of human and animal infections. *S. aureus* is the most commonly associated with human infections, while *S. (pseud)intermedius* is an important veterinary pathogens. Voges-Proskauer test (Acetoin Production) is useful for distinguishing *S. aureus* (positive) from another coagulase-positive species, *S. intermedius* and *S. hyicus* (negative) (Table 2-1).

2.2 The veterinary Coagulase Positive *Staphylococcus spp.*

2.2.1 *Staphylococcus aureus* (SA)

Staphylococcus aureus was discovered in 1880s. Since then, it has been shown to be a potential pathogenic Gram-positive bacterium, causing such infections as minor skin infections and post-operative wound infections. *S. aureus* can produce several toxins and enzymes mediating tissue invasion and survival at the infection site (Table 2-2). For example, leukocidin, hemolysins and other enzymes and toxins of *S. aureus*

damage blood cells, macrophages and epithelial and other cells. Serious infections produced by this strain including bacteremia, pneumonia, meningitis, mastitis, scalded skin syndrome and abcesses of the muscle (Murray et al., 2003).

Table 2-2 Virulence Factors of *Staphylococcus aureus* (Songer and Post, 2004)

Virulence factors	Effects
Capsule	Inhibits phagocytosis; promotes adherence
Peptidoglycan	Leukocyte chemoattractant; decomplementation
Teichoic acid	Fibronectin binding
Protein A	Immunoglobulin binding
Toxins (alpha, beta, other)	Antiphagocytic; cytotoxic
Exfoliative toxins	Serine proteases; split cellular bridges in stratum granulosum
Enterotoxin	Superantigens; nauseogenic, diarrheagenic
Toxic shock syndrome toxin	Superantigens; endothelial damage
Coagulase	Convert fibrinogen to fibrin
Hyaluronidase	Hydrolyzes hyaluronic acid in connective tissue
Lipase	Hydrolyzes lipids
Nuclease	Hydrolyzes deoxyribonucleic acid (DNA)

2.2.2 *Staphylococcus intermedius* group (SIG)

For many years we know *Staphylococcus spp*. have been the clinical problem in animals, especially *S. intermedius* which was previously identified to be a common cause of pyoderma and otitis, probably as a secondary any invader following skin injuries. A recent phylogenetic study has shown that *Staphylococcus pseudintermedius* is not S. intermedius is the common cause of canine pyoderma. (Hill et at., 2006). S(P)I is a common commensal of oral, nasal, and skin flora in healthy dogs, where it can also cause invasive disease (Hájek, 1976; Talan, 1989). In humans, it is recognized as an invasive zoonotic pathogen and has been isolated from 18% of canine infected wounds (Lee, 1994). In 2005, Devriese et al. found four staphylococcal isolates from clinical and

necropsy specimens from a cat, a dog, a horse and a parrot. 16S rRNA gene sequence analysis revealed that its closest phylogenetic relatives are *S. intermedius* and *S. delphini*. Growth characteristics, biochemical features and DNA–DNA hybridizations demonstrated that the strains differ from these and other known species and that they represent a single, novel *Staphylococcus* species for which the name *S. pseudintermedius* sp. nov. is proposed. However, using a multilocus sequencing approach, independent research groups have demonstrated that isolates phenotypically identified as *S. intermedius* consist of three distinct species, including *S. intermedius*, *S. pseudintermedius*, and *S. delphini*, which together represent the *S. intermedius* group (SIG). Nowaday, it was discovered that *S. pseudintermedius*, not *S. intermedius*, is the common canine pyoderma pathogen. However, *S. delphini* was isolated from a variety of different animals, may be more clinically important than was previously thought (Bannochr et al., 2009). *S. pseudintermedius* was confirmed by other techniques (van Hoovels et al., 2006) succeded in identifying a strain causing infection in a human as belonging to the new species by applying *tuf* gene sequencing. Moreover, a Japanese research group reported recently about methicillin-resistan *S. pseudintermedius* (MRSP) emergency in a veterinary teaching hospital (Sasaki et al., 2007a). A thorough investigation of 117 strains by the same group (Sasaki et al., 2007b) confirmed this observation by phylogenetic analysis of *sodA* and *hsp60* sequences. The new technique for identified *S. pseudinetermedius* were PCR-REA (Bannochr et al., 2009). *pta* gene of *Staphylococcus spp.* play an important role. The *pta* gene of *S. pseudintermedius* had a restriction of *MboI* enzyme (…GATC…) (Figure 2-2) but only one type of *S. aureus* (N315 and newman) has this restriction site.

GenBank: AM946758.1

Staphylococcus pseudintermedius partial *pta* gene for phosphate acetyltransferase, strain SD1071

Gtgcgtatcgtattaccagaaggagaagatgagcgtgtattaacagccgctgttgatttacaagcttctgactatgttgc
gccaatcgtattagggaatgttgacaaaattaaagcacttgctgcagaaaaatctttaaatattgaaggtttaaatatcatc
caacctgatacgagcgacctaaaagcaacactcgttgaacaatttgtagaacgtcgtaaagggaaagcgactgaa
gaacaagcacaatcgttattaaatgatgtgaactacttcggtacaatgcttgtttatgcaggtgtttagtgagcggtgcag
cccactcaacagcagacactgttcgtccagcgcttcaaattatcaaaacaaaaccaggtgtttctaagacatcaggtat
tttcttcatgattaaagaagatcaacaatttatttttcggtgactgtgcgattaaccctgaattagccgcatcagacttagctg
aaattgcggttgaaagcgcgaaaactgcacaaagcttcggcatggacccacgtgtagcgatgttaagcttctcaaca
aaaggttctgc

Figure 2-2 *Staphylococcus pseudintermedius* partial *pta* gene for phosphate acetyltransferase, strain SD1071 and restriction site of *Mbo*I (…GATC…)

Surprisingly, *S. intermedius* was only isolated from feral pigeons. The recent identified *S. pseudintermedius* is occasionally isolated from serious human infections. The emergence and spread of methicillin-resistant *S. pseudintermedius* strains are also major veterinary and public health issues (van Duijkeren et al., 2008).

2.2.3 Other coagulase positive *Staphylococcus spp.*

Staphylococci are mainly harmless commensals of the skin and mucous membranes of man and other mammals although species producing coagulase such as *S. aureus*, *S. hyicus*, *S. intermedius* and *S. schleiferi* subsp. *coagulans*, are potentially pathogenic for a wide variety of animals as well as humans (Werckenthin et al., 2001; Weese et al., 2006).

The occurrence of other coagulase-positive *Staphylococcus spp*. depend on species of animal. In dog, *S. schleiferi* subsp. *coagulans* can found in otitis externa

(Igimi, Takahashi, and Mitsuoka, 1990) whereas *S. hyicus* is the etiologic agent of exudative epidermitis in swine (Penny and Muirhead, 1981).

2.3 Identification and differentiation among CPS by PCR

All CPS can be identified on the basis of a variety of conventional physiological and biochemical characters (gram-positive cocci, catalase-positive, oxidase-negative, coagulase-positive). In addition, the species among CPS can be differentiated by PCR methods. In 2004, Baron et al. used *nuc* (encoding thermostable nuclease) gene to differentiate *S. aureus* and *S. intermedius*. *SAnuc* primer in this paper is very specific with *S. aureus* but *SInuc* primer matched with *S. intermedius* and *S. pseudintermedius* (Figure 2-3).

S. intermedius nuc **gene for thermonuclease (A)**

caatggagatggccctttaaaaagtcttcaacggatgtgagaggcgaatcatatctcgttaaacgcgtaat cgatgga gataccattattatcgataaagatgggcaagatgaacgtgtacgct

S. pseudintermedius nuc **gene for thermonuclease, complete cds (B)**

caatggagatggccctttaaaaagtcttcaacggatgtgagaggcgaatcatatctcgttaaacgcgtaat cgatggagataccattattatcgataaagatgggcaagatgaacgtgtacgct

Figure 2-3 Compared sites and base pairs that *SInuc* primer matched with *S. intermedius* (A) and *S. pseudintermedius* (B)

For identification of *S. intermedius* and *S. pseudintermedius*, *pta* gene which encodes the enzyme phosphoacetyl transferase, revealed the presence of an MboI restriction site in *S. pseudintermedius*, which was absent in all *S. intermedius* and *S. delphini* (Figure 2-4). In 2009, Bannoehr et al. described *pta* gene's primer that was suitable for used restriction enzyme *MboI*. A *pta* PCR product of 320 bp was successfully amplified from all SP and contained a single MboI site results in 2 restriction fragments of 213 bp and 107 bp, respectively.

acagccgctgttgatttacaagcttctgactatgttgcgccgatcgtattagggaatgttgacaaaattaaa
gcacttgctgcagaaaaatctttaaatattggaggtttaaatatcatccaacctgatacgagcgacctaaaagcaacac
tcgttgaacaatttgtagaacgtcgtaaagggaaagcgactgaagaacaagcacaatcgttattaaatgatgtgaact
acttcggtacaatgcttgtttatgc

Figure 2-4 *pta* gene of *S. pseudintermedius* and restriction site of *Mbo*I (5'…GATC…3')

2.4 Methicillin resistance in Coagulase Positive *Staphylococcus spp.*

Methicillin resistance strains of CPS, particularly *S. aureus*, *S. intermedius* group have increased the recovery rate in animals. Methicillin-resistant *S. aureus* (MRSA) is an important cause of human nosocomial infections worldwide. To date, there have been increasing reports of MRSA infections in dogs and cats both in veterinary hospitals and in the community (Baptiste et al., 2005; Loeffler et al., 2005; Morris et al., 2006; Tomlin et al., 1999; Weese et al., 2006). Besides common resistance to methicillin and beta-lactam in general, MRSA has also become resistant to multiple classes of antimicrobial agents including aminoglycosides, macrolide-lincosamides-streptogramins (MLS_B) and tetracyclines with many different resistance mechanisms (Table 2-3).

Table 2-3 Mechanisms of *S. aureus* resistance to antimicrobials (Lowy, 2003)

Antibiotic	Resistance gene	Gene product (s)	Mechanism(s) of resistance	Location(s)
Beta-lactams	1) *blaZ*	1) betalactamase	1) Enzymatic hydrolysis of β-lactam nucleus	1) Pl:Tn
	2) *mecA*	2) PBP2a	2) Reduced affinity for PBP	2) C:SCC*mec*
Glycopeptides	1) Unknown (VISA)	1) Altered peptidoglycan	1) Trapping of vancomycin in the cell wall	1) C
	2)	2) D-Ala-D-Lac	2) Synthesis of dipeptide with reduced affinity for vancomycin	2) Pl:Tn
Quinolones	1) *parC*	1) ParC (or GrlA) component of topoisomerase IV	1,2) Mutations in the QRDR region, reducing affinity of enzyme-DNA complex for quinolones	1) C
	2) *gyrA* or *gyrB*	2) GyrA or GyrB components of gyrase		2) C
Aminoglycosides (e.g., gentamicin)	Aminoglycoside-modifying enzymes (e.g., *aac*, *aph*)	Acetyltransferase, phosphotransferase	Acetylating and/or phosphorylating enzymes modify aminoglycosides	Pl, Pl:Tn
Trimethoprim-sulfamethoxazole (TMP-SMZ)	1) Sulfonamide: *sulA*	1) Dihydropteroate synthase	1) Overproduction of *p*-aminobenzoic acid by enzyme	1) C
	2) TMP: *dfrB*	2) Dihydrofolate reductase (DHFR)	2) Reduced affinity for DHFR	2) C
Oxazolidinones	*rrn*	23S rRNA	Mutations in domain V of 23S rRNA component of the 50S ribosome. Interferes with ribosomal binding	C
Quinupristin-dalfopristin (Q-D)	1) Q: *ermA*, *ermB*, *ermC*	1) Ribosomal methylases	1) Reduce binding to the 23S ribosomal Subunit	1) Pl, C
	2) D: *vat*, *vatB*	2) Acetyltransferases	2) Enzymatic modification of dalfopristin	2) Pl

[A]Examples of several of the *S. aureus* mechanisms of resistance to selected antibiotics (77, 95–97). Pl, plasmid; C, chromosome; Tn, transposon; QRDR, quinolone resistance–determining region.

Methicillin-resistant strain of *S. intermedius*, *Staphylococcus schleiferi* subsp. *coagulans* have also been identified in dogs, particularly in skin and soft tissue infection. (Jones et al., 2007; Morris et al., 2006)

2.4.1. Mechanism of penicillin resistant *Stapylococcus spp.*

Beta-lactamases are plasmid or chromosomally encoded bacterial enzymes that catalyze the hydrolysis of the beta-lactam C-N bond of beta-lactam antibiotics to give the corresponding beta-amino acid devoid of antibacterial activity. Beta-lactamases are produced in a constitutive or inducible manner and are secreted into the periplasmic space of gram-negative bacteria or into the outer medium of gram-positve bacteria. In 1940, Abraham and Chain first described a bacterial enzyme that destroyed penicillin; they termed it penicillinase. Various classification schemes have been proposed for beta-lactamase base on molecular weight or substrate specificities (Table 2-4). Pencillinase is classified as Amber class A or Bush, Jacoby and Medeiros Group 2a.

Table 2-4 beta-lactamase classification (Mascaretti, 2003)

Bush-Jacoby-Medeiros group	Molecular class	Preferred substrates	Inhibited by		Representative enzymes
			Clavulanic acid	EDTA	
1	C	Cephalosporins	-	-	AmpC beta-lactamases from gram-negative bacteria; MIR-1
2a	A	Penicillins	+	-	Penicillinases from gram-positive bacteria
2b	A	Penicillins, cephalosporins	+	-	TEM-1, TEM-2, SHV-1
2be	A	Penicillins, narrow-spectrum and extended-spectrum cephalosporins and monobactams	+	-	TEM-3 to TEM-26, SHV-2 to SHV-6, *K. oxytoca* K1
2br	A	Penicillins	±	-	TEM-30 to TEM-36, TCR-1
2c	A	Penicillins, carbenicillin	+	-	PSE-1, PSE-3, PSE-4
2d	D	Penicillins, cloxacillin	±	-	OXA-1 to OXA-11, PSE-2 (OXA-10)
2e	A	Cephalosporins	+	-	Inducible cephalosporinases from *Proteus vulgaris*
2f	A	Penicillins, cephalosporins, carbapenems	+	-	NMC-A from E. cloacae, Sme-1 from S. marcescens
3	B	Most beta-lactams including carbapenems	-	+	L1 from S. maltophilia, CcrA from *B. fragilis*
4	ND	Penicillins	-	?	Penicillinase from *P. cepacia*

ND = not determined

blaZ gene were encodes beta-lactamase enzyme. *blaZ* is under the control of two adjacent regulatory genes, the antirepressor *blaR1* and the repressor *blaI* (Kernodle, 2000). The signaling pathway responsible for beta-lactamase synthesis requires sequential cleavage of the regulatory proteins BlaR1 and BlaI. Following exposure to beta-lactams, BlaR1, a transmembrane sensor-transducer, cleaves itself (Gregory et al., 1997; Zhang et al., 2001). Zhang et al. (2001) hypothesize that the cleaved protein functions as a protease that cleaves the repressor BlaI, directly or indirectly (an additional protein, BlaR2, may be involved in this pathway) and allows *blaZ* to synthesize enzyme.

More than 90% of staphylococcal isolates now produce penicillinase, regardless of the clinical setting. The gene for beta-lactamase is part of a transposable element located on a large plasmid, often with additional antimicrobial resistance genes (e.g., gentamicin and erythromycin). Spread of penicillin resistance primarily occurs by spread of resistant strains (Lowy, 2003).

2.4.2. Mechanism of methicillin resistance

The most common mechanism of resistance to beta-lactams in Staphylococci is mediated by *blaZ* gene, which codes for staphylococcal beta-lactamase, primary penicillinase, the enzyme that degraded the drugs. Overexpression of beta-lactamase may also lead to low-level methicillin resistance.

Resistance to methicillin confer resistance to all penicillins and cephalosporins and requires the presence of the *mecA* gene, and encodes for the production of PBP2a. PBP2a is a transpeptidase that catalyzes the foundation of cross-bridges in bacterial cell wall peptidoglycan, and has a low affinity for all beta-lactam antibiotics. MRS may carry a gene or genes that regulates the production of both PBP2a and beta-lactamase.

Normally, *S. aureus* has five PBPs with molecular sizes of 87 (PBP1), 80 (PBP2), 75 (PBP3), 70 (PBP3'), and 41 (PBP4) kDa. PBP 1, 2 and 3 are essential and have high affinities for beta-lactam antibiotics. PBP1 may be the primary peptidoglycan transpeptidase, PBP2 (recently resolved into two components) is a transpeptidase functioning in non growing cells, PBP3 is a septation-associated transpeptidase, and PBP4 is a DD-carboxypeptidase and transpeptidase involved in secondary cross-linking of the peptidoglycan. MRSA have a special PBP that is PBP2a. PBP2a has a low affinity for beta-lactams and catalyze a penicillin-insensitive transpeptidation and transglycosylation. Then *S. aureus* that have PBP2a can produce their cell wall and survive (Mascarettii, 2003).

PBP2a from *S. aureus* contains an N-terminal transmembranous anchor, which can be removed without affecting the beta-lactam binding kinetics (Wu et al., 1992; Lu et al., 1999).

PBP2a were encoded by *mecA* gene on Staphylococcal chromosomal cassette (SCC*mec*). Most MRSA (>90%) harbor *mecA*. *mecA* gene were control by two regulator gene *mecI* and *mecR1* (Figure 2-5). Both genes are located immediately upstream of *mecA*, separated from *mecA* by promoter and operator, and are divergently transcribed. (Hiramastu, 1995) (Figure 2-5).

The *mecA* gene is carried on a mobile genetic element known as the Staphylococcal Cassette Chromosome (SCC) *mec*. (Figure 2-6) Until now, at least five different type of SCC*mec* have been described. Some SCC*mec* type also contain other genetic elements, such as Tn554 (which encodes resistance to macrolides, clindamycin and straptogramin B) and pT181 (which encodes resisitance to tetracyclines) (Ito et al., 2001). These SCC*mec* can spread horizontally across between different group of staphylococci, leading to potentially rapid resistance to multiple classes of antimicrobial agents (Epstein et al., 2009).

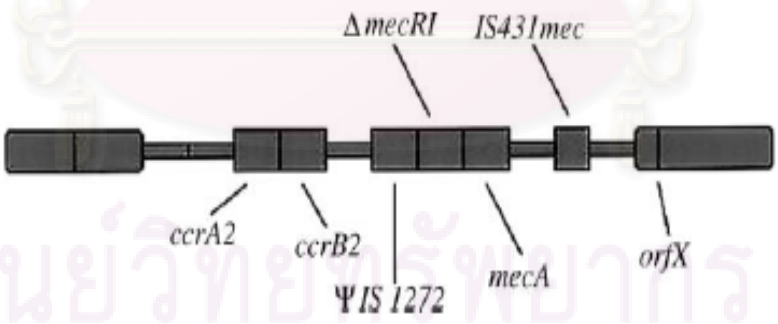


Figure 2-5 The staphylococcal cassette chromosome *mec* type IV (SCCmec type IV)

SCC*mec* type IV lacks antibiotic resistance elements directed at non-lactam antibiotics that are present in SCC*mec* types characteristic of hospital-acquired methicillin- resistant *Staphylococcus aureus*. *ccrA2* and *ccrB2* designate cassette chromosome recombinases. JIS 1272 designates IS431*mec* insertion sequences. *mecA* encodes PBP2a. *orfX* indicates an open reading frame. *mecR1* is a signal transducer gene whose activation by-lactam antibiotics inactivates the *mecI* repressor gene product, allowing expression of *mecA*.

mecA is highly conserved (>90% sequence identity) among staphylococcal species (Back, Berger-Bachi and Kayser, 1986; Ubukata et al., 1990). The origins of *mecA* are obscure. A *mecA* homolog with 88% amino acid similarity of MRSA has been found in *S. sciuri* (Wu et al., 1996). The acquisition of *mecA* by *S. aureus* is still mysterious, but transposition is a possible mechanism. The presence of one or more copies of the IS*431*element (also known as IS*257*) within SCC*mec* may play an important role in the integration of *mecA* gene and other antibiotic determinants (Skinner,1988; Stewart, 1994; Ito, 2004).

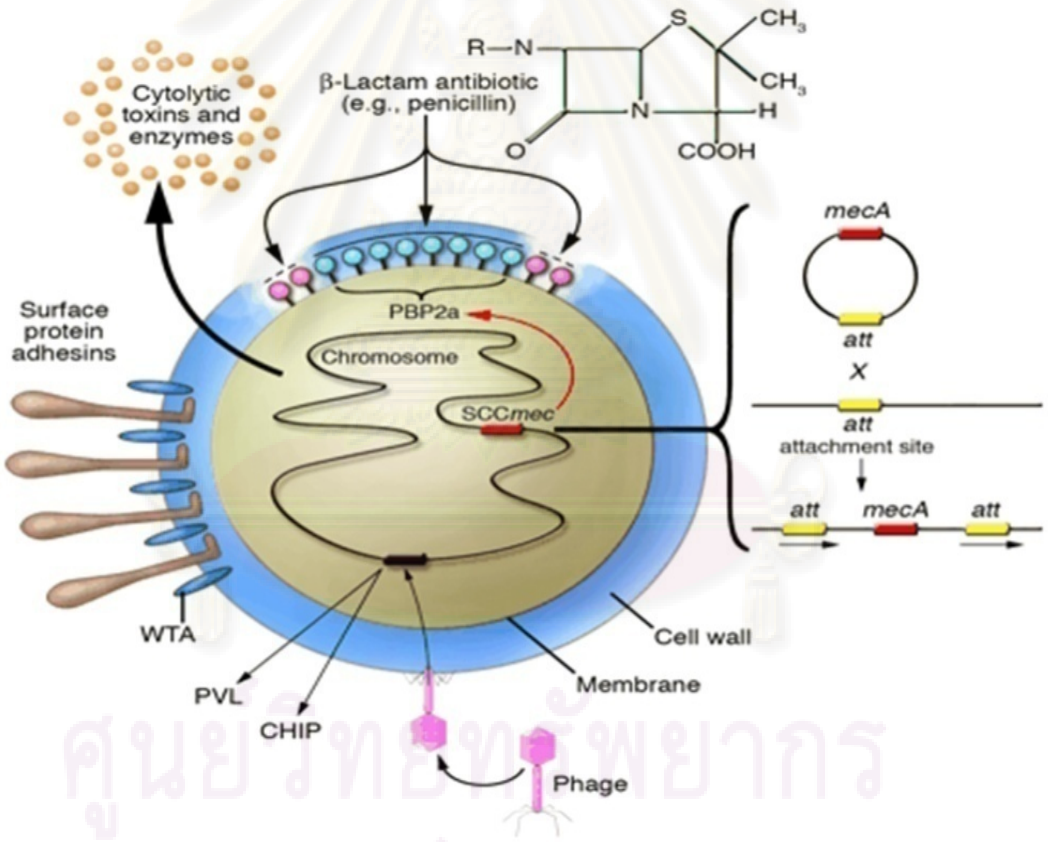


Figure 2-6 *mecA* positive *Staphylococcus spp.*

Regulation of *blaZ* and *mecA*

Unlike the *bla* regulatory gene system (*blaR1, blaI*) for beta-lactamase induction, the *mec* regulatory gene system (*mecR1, mecI*) does not respond well on many of the beta-lactam antibiotics (Figure 2-7), including methicillin.This has been proposed to be caused by the limited ligand-binding specificity of the penicillin-binding domain of MecR1 (Figure 2-7). In this regulatory condition, induced expression and production of PBP2a by beta-lactams such as ceftizoxime and cefoxitin, may not make the cells resistant to methicillin owing to the strong repressive function of *mecI* genes (Kuwahara-Arai et al., 1996). The complexity of *mecA* regulation by *mecI-mecR1* system leads to difference in phenotypic expression of MRSA strains. In some case, removal of MecI protein makes *S. aureus* cell express methicillin resistance (methicillin MIC >8 µg/ml or oxacillin >2 µg/ml) (Hiramatsu et al., 2001).

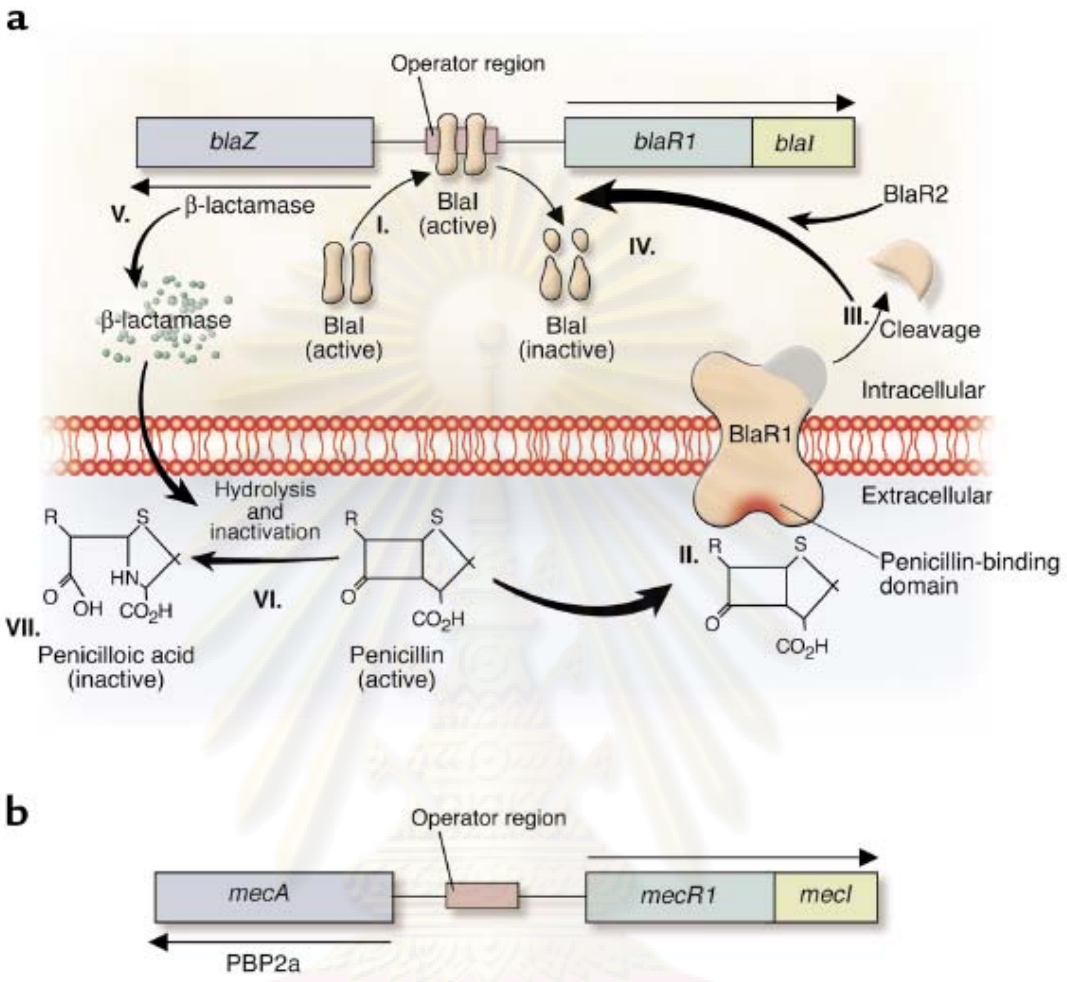


Figure 2-7 Mechanism of *blaZ* and *mecA* genes

(**a**) Induction of staphylococcal beta-lactamase synthesis in the presence of the beta-lactam antibiotic penicillin. I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR1-blaI*. In the absence of penicillin, beta-lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer *BlaR1* stimulates *BlaR1* autocatalytic activation. III–IV. Active *BlaR1* either directly or indirectly (via a second protein, *BlaR2*) cleaves *BlaI* into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI* to commence. V–VII. beta-lactamase, the extracellular enzyme encoded by *blaZ* (V), hydrolyzes the beta-lactam ring of penicillin (VI), thereby rendering it inactive (VII). (**b**) Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for beta-lactamase. Exposure of MecR1 to a beta-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. *MecI* and *BlaI* have coregulatory effects on the expression of PBP2a and beta-lactamase (Lowy, 2003).

Borderline methicillin resistant strain

Another type of methicillin resistance is exhibited in borderline or low level methicillin-resistant strain. Borderline strains may be classified into two categories on the basis of whether *mecA* is present. Borderline stains that contain *mecA* are tremendously heterogeneous methicillin-resistant strains that produce PBP2a (Gerberding, 1991). Borderline resistance in *mecA*-negative strains have hypothesis to result from modification of normal PBP genes or overproduction of staphylococcal beta-lactamase.

Alterations of penicillin binding by PBP1, 2 and 4 in beta-lactamase-negative, *mecA* negative borderline clinical isolates were documented by Tomasz et al. (1989). These binding alterations are the result of point mutations in the penicillin-binding domain (Hackbarth et al., 1995). Overproduction of PBPs also can produce low-level resistance (Henze and Berger-Bachi, 1996).

In the same manner, overproduction of beta-lactamase could result in borderline MICs and exhibit a lowering MICs into the susceptible rang upon addition of beta-lactamase inhibitors, such as clavulanate or sulbactam, or upon elimination of beta-lactamase plasmid (McDougal and Thornsberry, 1986).

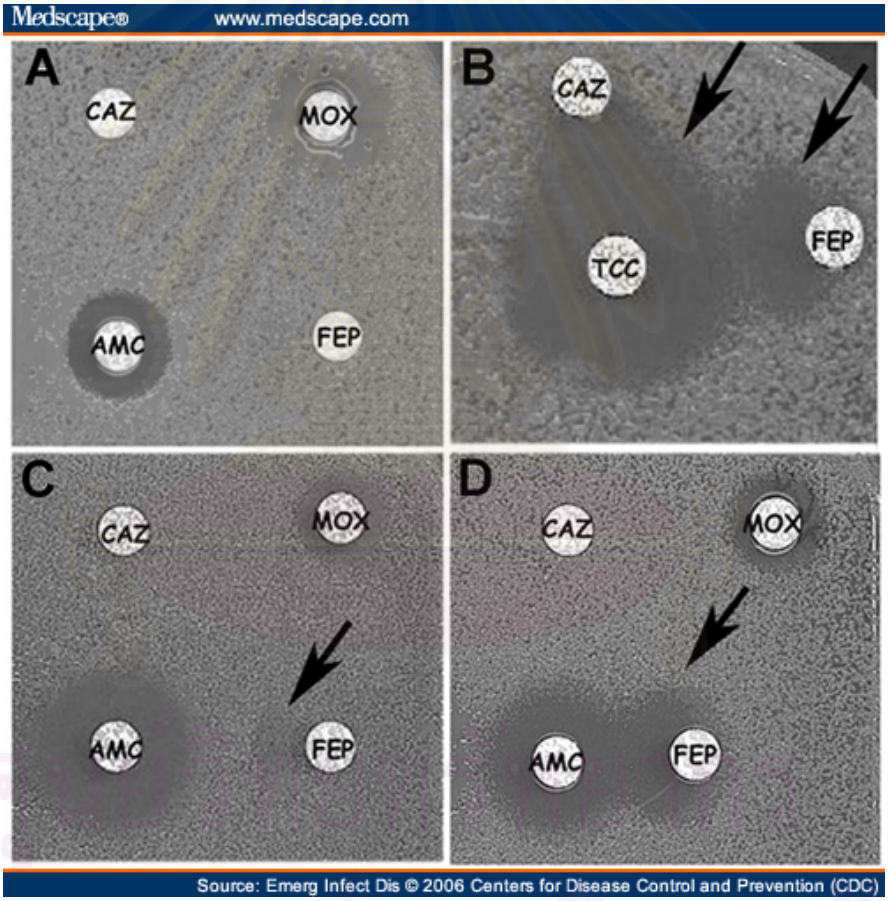
2.4.3 Detection of methicillin resistance in Staphylococci

1.) Minimum inhibition concentration (MIC) of *Staphylococcus spp.*

For the interpretation of MIC, *Staphylococcus spp.* is considered to be methicillin-resistant strain when MIC of methicillin and oxacillin is $\geq$ 8 mg/L and $\geq$ 4 mg/L, respectively (CLSI, 2007).

2.) Determination of beta-lactamase production in coagulase positive staphylococci (CPS) by Disk diffusion method

Determination of beta-lactamase production in coagulase positive staphylococci (CPS) was applied from double disk diffusion method for testing bacteria producing beta-lactamase as shown in Figure 2-8. Because of beta-lactamase production were inhibited by clavulanic acid placed near ampicillin, clear zone of ampicillin were synergy with amoxicillin/clavulanic acid (AMC) (Figure 2-8D).



AMC = amoxicillin/clavulanic acid, CAZ = ceftazidime, FEP = cefapime, MOX = moxalactam, TCC = ticacillin+ clavulanate

Figure 2-8 Synergy of clear zone of AMC and the other drug (Naas et al, 2006)

3.) PCR detection of drug resistant gene

The part of the DNA now most commonly used for taxonomic purposes for bacteria is the *16S rRNA* gene (Bottger, 1989; Garrity and Holt, 2001; Harmsen and Karch, 2004; Kolbert andPersing, 1999; Palys, Nakamura and Cohan, 1997; Tortoli, 2003). The *16S rRNA* gene is also designated *16S rDNA*, and the terms have been used interchangeably: current ASM policy is that "*16S rRNA* gene" be used. It was suggesed that there is little potential for finding novel genogroups within the well-studied major pathogenic species, since these taxonomic groups have been so well and accurately described that there is little confusion or variability in their *16S rRNA* gene sequence. Species with this genetically homogeneous characteristic that we have tested include *M. tuberculosis*, *H. ducreyi*, *H. influenzae* group b, *Streptococcus dysgalactiae*, *S. pneumoniae* (blood isolates), and *Staphylococcus aureus* (Clarridge, 2004).

PCR was gold standard for detection of resistance gene (i.e. *blaZ* and *mecA* gene) (Zhu et al., 2007). In 2004, Charpentier et al. designed *blaZ* primer to insert in vector. They found that the staphylococcal cadmium-inducible P_{cad}-*cadC* and constitutive P_{blaZ} promoters were designed and analyzed in transcriptional fusions to the staphylococcal beta-lactamase *blaZ*. In the same year, Strommenger et al. (2004) used *mecA* gene primer for detect antibiotic resistance gene in *S. aureus*.

2.5 Methicillin-resistant *Staphylococcus aureus* (MRSA) in animal

In 1972, MRSA were isolated from animals at first time (Devriese et al., 1972). Since then MRSA has been found in a variety of other domestic species including dogs (Pak et al., 1999; van Duijkeren et al., 2004), cats (Bender et al., 2005), horses (Anzai et al., 1996; Hartmann et al., 1997), sheep (Goni et al., 2004) pigs (Voss et al., 2005) and chickens (Lee, 2003) leading to an upsurge of reports and interest in MRSA colonization and infection in animals.

Epidemiological evidence, including phenotypic and molecular typing data, suggests that MRSA isolates from dogs and cats are indistinguishable from human

healthcare isolates, whereas strains of MRSA isolated from horses and associated personnel are different. There is evidence that transfer of MRSA strains can occur between animals and humans and vice versa (Leonard and Markey, 2008).

2.6 Methicillin-resistant *Staphylococcus (pseud)intermedius* (MRS(P)I)

As same as *S. aureus*, *S. (pseud)intermedius* isolates were reported to be susceptible to beta-lactam antibiotics. Recently, methicillin-resistant *S. (pseud)intermedius* (MRS(P)I) strains are being reported with increasing frequency (Gortel et al., 1999; Piriz et al., 1996; van Duijkeren et al., 2008; Vengust et al., 2006; Zubeir et al., 2007). The methicillin resistance of *S. pseudintermedius* is mediated by the *mecA* gene and found also in dogs, cats and humans (Hanselman et al., 2007; Sasaki et al., 2007a; Schwarz et al., 2008; Wettstein et al., 2008).

The prevalence of MRS(P)I colonization has been reported in various dog populations (Griffeth et al., 2008; Hanselman et al., 2007; Kania et al., 2004; Medleau et al., 1986; Sasaki et al., 2007a; Vengust et al., 2006) since Devriese et al. described *S. pseudointermedius* as a new species which was previously identified to be S. intermedius (Devriese et al., 2005). In 2007, Schwarz, Kadlec and Strommenger reported 2 strains of *S. pseudintermedius* isolated from canine carried the resistance gene *mecA*, *aacA/aphD* and *tet(K)*. In the same year, Sasaki et al. surveyed MRCPS strains from dogs and veterinary staff in a veterinary teaching hospital. The methicillin-resistant *S. pseudintermedius* (MRSP) found in this report showed high-level resistance to oxacillin (≥128 μg/ml, 15/18, 83.3%). In addition, 10 of 15 (66.7%) of high-level oxacillin-resistant MRSP strains carried type-III SCC*mec*. Hanselman, Kruth and Weese (2007) researched about methicillin-resistant staphylococcal colonization in dogs. They collected samples from nasal, axillary and rectal of dog. They found MRSP that isolated from nasal cavity in dogs but they did not found methicillin-resistant *S. intermedius*. None of the colonized dogs developed clinical infection during hospitalization.

In 2009, Epstein et al. reported 17 % of S(P)I that collected from nasal cavities of healthy dogs in Hong Kong were methicillin-resistant *S. (pseud)intermedius*

(MRS(P)I). In contrast, MRSA was not detected. *mecA* gene that were found in this report were described to be a mobile genetic element responsible for methicillin resistance and has been shown by others to be horizontally transmissible between dogs and humans. They called this MRSI pet acquired methicillin-resistant staphylococci (PA-MRS) that could play a role in zoonotic *mec*A gene spread (Epstein et al., 2008). In the same year, van Duijkeren et al. reported about transmission of MRS(P)I between animal and human. They studied from sick and healthy dogs as well as healthy human. MRS(P)I isolates were genotyped by PFGE and found pattern of animals' MRS(P)I as the same as healthy human (van Duijkeren et al., 2008). Analysis of 158 isolates from respiratory tract infections and skin/ear/mouth infections from dogs and cats in Germany revealed two canine MRSP infections (Schwarz et al., 2007). Both isolates were resistant to beta-lactams, gentamicin, tetracycline, sulfamethoxazole and one of the isolates was also resistant to chloramphenicol. In Switzerland, MRSP carrying the leukocidin gene *lukS-I* were cultured from three cats with urinary tract infections (Wettstein et al., 2008).

2.7 Treatment of MRS

Treatment of MRSA infection in animals is even more difficult than in humans, since certain antimicrobial compounds employed in human medicine (e.g. vancomycin, linezolid, stretogramins, tigecycline) are expensive and all except for linezolid must be administered intravenously. For the other species of staphylococci, theatment should depend on drug susceptibility test (Guardabassi et al., 2008).

2.8 Control of MRS in animals

Numerous documents on the control of MRSA in people have been published (e.g. CDC MRSA guidelines, available at www.cdc.gov/ncidod/dhap/ar_mrsa.html) and many of the principles may be applied to control in animals also. A summary of control measures for MRSA infection in animals is presented in Table 2-5.

Table 2-5 Summary of control measures for MRSA infection and colonization in animals (Leonard and Markey, 2008)

Control principle	Control measure	Comment
Prevent introduction of infection	Screen all incoming cases for infection and/or nasal carriage at admission to veterinary hospital and isolate until negative status established	May be possible in referral practices but only screening of suspect cases is practical in first opinion clinics
Prevent transmission from animal-to-human and human-to-animal	Hand hygiene and related measures • Correct hand washing • Alcohol-based hand sanitizers • Cover wounds and skin lesions • Use gloves, masks, eye protection, disposable aprons for contact with wounds, body fluids or other contaminated materials Strict asepsis during surgery Screening staff for MRSA colonization	These measures are among the most important as transmission via human hands is highly significant based on findings in human medicine This may be required where clusters of infection in animals are identified but should be carried out with sensitivity and in collaboration with the medical profession
Prevent transmission from animal to animal	Isolation of all suspect cases of MRSA infection • No entry to waiting room • Hospitalize in isolation area as far as possible Elimination of carriage may be a possibility but limited data available	
Prevent indirect transmission	High standards of cleaning and disinfection • Hand touch surfaces including doors, pens, stethoscopes, keyboards • Consulting rooms/isolation kennels/stables or other areas following boarding of known infected animals Dedicated thermometers, leads, head collars and other handling equipment for known positive or suspect cases	Environmental transmission may be of greater importance than in human medicine, especially in horses

2.9 Antimicrobial susceptibility test

The Kirby-Bauer test for antibiotic susceptibility, called the disc diffusion test, is a standard that has been used for years. It has been superceded in clinical laboratories by automated tests. Considerations about antimicrobial usage and application of antimicrobial susceptibility tests (ASTs) in veterinary medicine should include points as shown below. (Watts and Yancey, 1994)

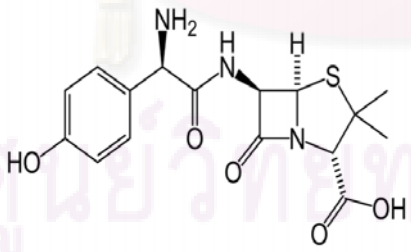
(i) The type of animal to which an antimicrobial compound is to be administered

(ii) Residue avoidance in food animals

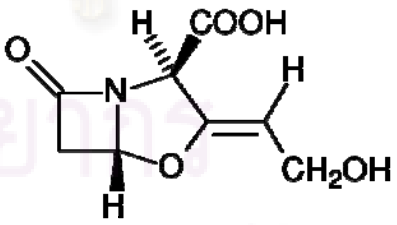
(iii) Possible extra-label usage of antimicrobial agents

In United Kingdom, antibiotic disks were used in testing multidrug resistance bacteria followed by British Society for Antimicrobial Chemotherapy (Andrews, 2001). The following antimicrobial discs were included: amoxicillin/clavulanic acid, methicillin, clindamycin, fusidic acid, ciprofloxacin, tetracycline, co-trimoxazole, cefalexin, ampicillin and mupirocin (Loeffler, 2005).

Amoxicillin/clavulanic acid



Amoxicillin



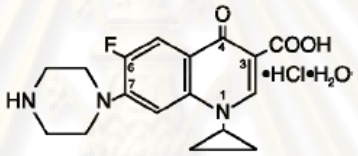
Clavulanic acid

Amoxicillin are aminopenicillins that are sensitive to hydrolysis by Gram-positive or Gram-negative beta-lactamases from penicillinase-producing *Staphylococcus* or Gram-negative bacteria.

Clavulanic acid is a synthetic beta-lactam drug that lacks significant intrinsic antibacterial activity but irreversibly binds and inactivates many different classA beta-lactamases. It is a natural product of *Streptomyces clavuligerus*. It readily penetrates both Gram-positive and Gram-negative bacteria. The potassium salt is used in drug formulation.

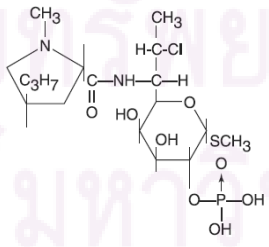
Amoxicillin-clavulanate, in contrast to amoxicillin alone, has activity against penicillinase-producing *Staphylococcus* and has enhanced activity against Gram-negative pathogens (Maddison, Page and Church, 2002).

Ciprofloxacin



Ciprofloxacin was first patented in 1983 by Bayer A.G. and subsequently approved by the United States Food and Drug Administration (FDA) in 1987. Ciprofloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV enzymes necessary to separate bacterial DNA, thereby inhibiting cell division (Drlica and Zhao, 1997).

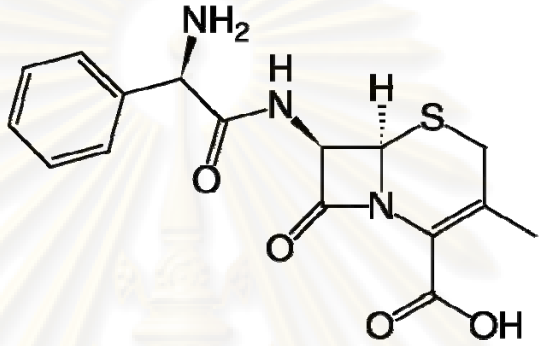
Clindamycin



Clindamycin is used primarily to treat infections caused by susceptible anaerobic bacteria, including infections of the respiratory tract, skin and soft tissue infections, and peritonitis. In patients with hypersensitivity to penicillins, clindamycin may be used to treat infections caused by susceptible aerobic bacteria as well. It is also

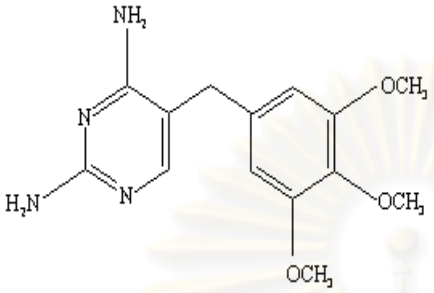
used to treat bone and joint infections, particularly those caused by *S. aureus* (Darley and MacGowan., 2004). The mechanism of action is similar to that of chloramphenicol, i.e. the site of action is the 50S ribosomal subunit (Maddison, Page and Church, 2002).

Cefalexin

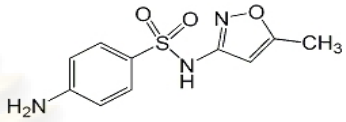


Cefalexin works by interfering with the ability of bacteria to form cell walls. The cell walls of bacteria are vital for their survival. They keep unwanted substances from entering their cells and stop the contents of their cells from leaking out. Cefalexin impairs the bonds that hold the bacterial cell wall together. This allows holes to appear in the cell walls and kills the bacteria. Cefalexin is a broad-spectrum antibiotic that kills a wide variety of bacteria that cause a wide variety of commonly-occuring infections. Cefalexin is given by mouth to treat infections of the upper and lower airways, ears, skin and soft tissue. It may also be used to treat urinary tract infections, certain sexually-transmitted infections, bone and joint infections and dental infections (Maddison, Page and Church, 2002).

Co-trimoxazole



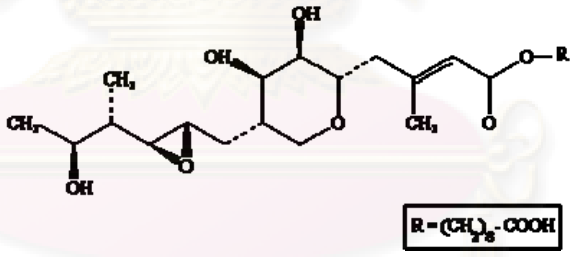
Trimethoprim



Sulfamethoxazole

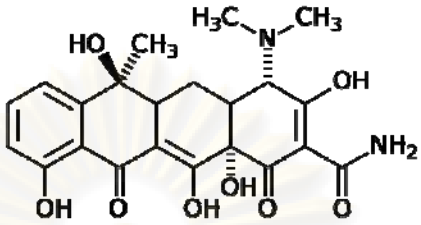
Trimethoprim and sulfamethoxazole both block the production of folic acid, a necessary metabolic nutrient for both bacteria and humans; each is an effective antibiotic when used alone (Maddison, Page and Church, 2002).

Mupirocin



This recently derived antibiotic is obtained from *Pseudomanas fluorescens* by fermentation. It is a strong inhibitor of bacterial isoleucyl transfer RNA synthetase and, thus, stop protein synthesis. It is bactericidal and most active against staphylococci and streptococci. Although it may have limited veterinary application, it has been used effectively in the topical treatment of feline acne (Carter and Wise, 2004).

Tetracycline



Tetracyclines have the broadest spectrum of antimicrobial activity. Tetracyclines inhibit bacterial protein synthesis by blocking the attachment of the transfer RNA-amino acid to the ribosome. More precisely they are inhibitors of the codon-anticodon interaction. Tetracyclines can also inhibit protein synthesis in the host, but are less likely to reach the concentration required because eukaryotic cells do not have a tetracycline uptake mechanism (Carter and Wise, 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

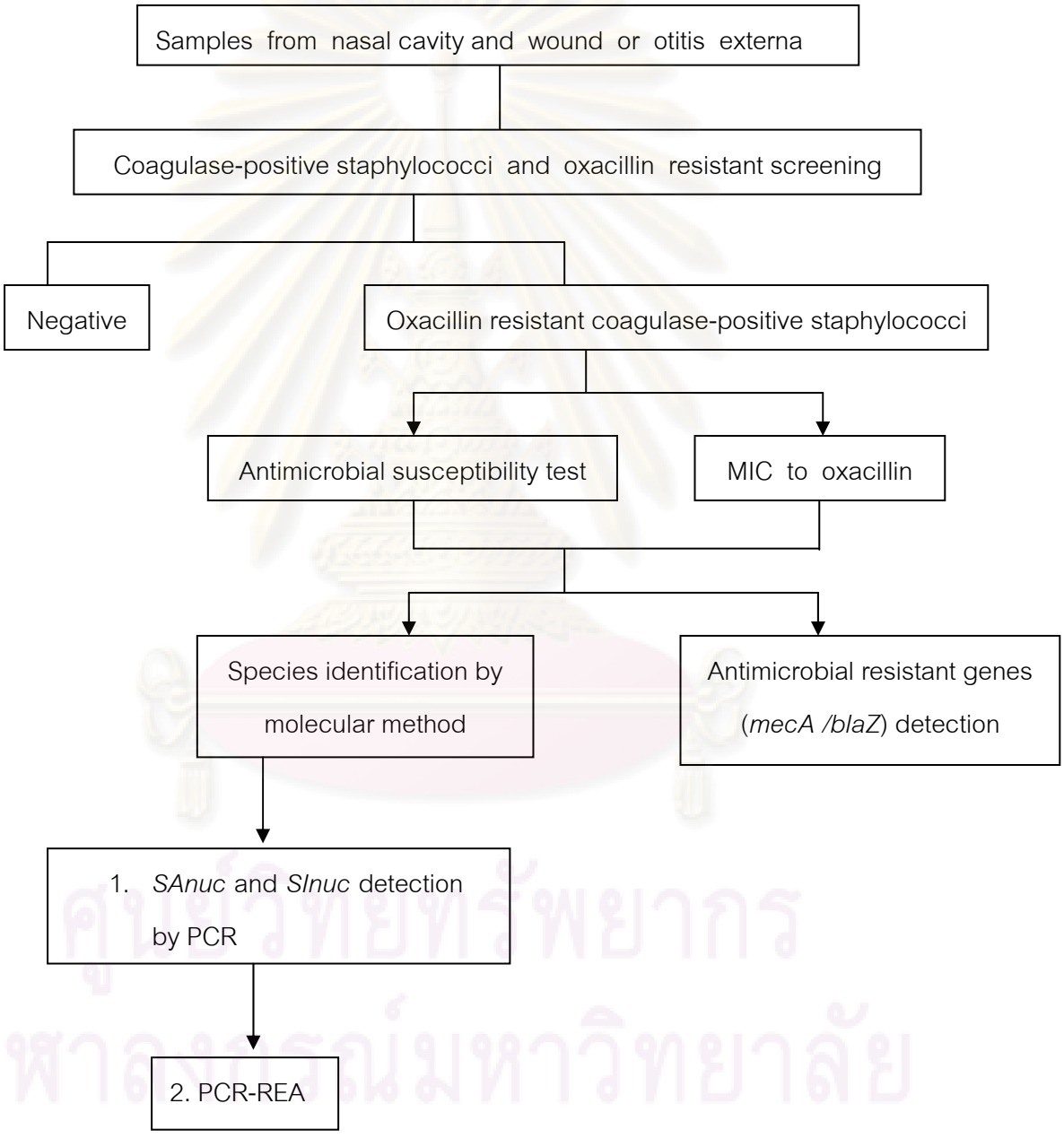
Samples were collected from 100 dogs attending the Small Animal Hospital of the faculty of Veterinary Science, Chulalongkorn University during the period of August to December 2008. The sampling procedure had been approved by the Chulalongkorn University Animal Care and Use Committee. Informed consent of the owners and veterinary staffs were obtained prior to sampling. Each dog was first inspected for signs of wound infection and otitis externa. Single use sterile cotton swabs on wooden applicators were moistened with sterile water and rolled over the sample area and streaked out on selective agar within 2 hour. The total of 60 samples from both wound and nasal cavity of each dog and 40 samples from both the otitis external and nasal cavity of each dog were collected.

3.2 Isolation and phenotypic characterization of staphylococci

Specimens were immediately inoculated on blood agar, mannitol salt agar, blood agar with 4 µg/ml oxacillin and mannitol salt agar with 4 µg/ml oxacillin. Plates were incubated at 37ºc for 24 hours for selective isolation of staphylococci. All colony types from each type of media were subculture. Each pure culture was gram strained and tested for catalase activity. Catalase-positive, gram positive cocci were identified as staphylococci. Coagulase test was used to exclude coagulase negative staphylococci. Differentiation of positive coagulase staphylococci was tested by Voges-Proskauer reaction (VP test) (Kloos and Bannerman, 1997; Sasaki et al.,2007). All coagulase-positive *Staphylococci spp*. (CPS) isolates were confirmed for species identification by

PCR methods described previously by El Zubeir et al. (2007) and Bannochr et al. (2009).

EXPERIMENTAL DESIGN



3.3 Antimicrobial Susceptibility Testing

All coagulase positive *staphylococcus spp.* (CPS) were investigated for their *in vitro* susceptibility to antimicrobial agents by agar diffusion method and agar dilution method were determined minimum inhibitory concentration (MIC) of oxacillin.

3.3.1 Agar diffusion method

Paper disk susceptibility test was performed according to the Kirby-Bauer method by recommended CLSI (CLSI, 2007). *S. aureus* ATCC 25923 was also included in this study as the control isolate. The susceptibility patterns of all CPS isolates against all the test antimicrobial agents were determined as following steps.

1. Mueller-Hinton agar (MHA) was prepared from a commercially available dehydrated base according to the manufacturer's instructions. After autoclaving, the agar was allowed to cool in a 45–50^{o}C water bath and poured to petri dishes (25 ml per dish) and left to cool at room temperature. Unless the plates were used within the same day, they were stored in a refrigerator (2 to 8 ^{o}C) and should be used within 7 days after preparation.

2. The loopful of overnight culture of CPS tested isolates were inoculated into 5 ml of sterile water. The turbidity of the inoculums was adjusted equivalent to 0.5 McFarland (approx 10^{8} colony-forming units [CFU/ml]). The tested isolates were inoculated on the MHA agar surface using sterile cotton swabs. The plates were left to dry for 3–5 min prior to applying the antibiotic disks.

3. The antimicrobial disks were placed on the surface of the inoculated agar plate with sterile forcep. Each disk was pressed down to ensure complete contact with the agar surface. They must be placed evenly so that they are not closer than 24 mm

from center to center. The plate were inverted and incubated at 33–35°C for 24 h before measuring the inhibition zone.

In this research, the following 8 antimicrobials were selected as representatives of important antimicrobial classes and used commonly in veterinary clinic. Antimicrobial disks were included: ciprofloxacin (CIP, 1 µg), clindamycin (DA, 2 µg), cefalexin (CL, 30 µg), amoxicillin/clavulanic acid (AMC, 3 µg), co-trimoxazole (SXT, 25 µg), mupirocin (MUP, 5 µg) and tetracycline (TE, 10 µg). For testing of methicillin-resistant staphylococci, oxacillin (0X, 1 µg) and cefoxitin (CEF, 30 µg) discs were used according to the followed CLSI (2007).

The diameter of each inhibition zone was measured with digital sliding venier caliper. The ranges of inhibition zone diameters were interpreted as either susceptibility, intermediate or resistant to the antimicrobial according to the guidelines of the CLSI.

3.3.2 Determination of beta-lactamase production in coagulase positive staphylococci (CPS) by Disk diffusion method (CLSI, 2007)

Disk diffusion test for beta-lactamase detection was constructed for detection of beta-lactamase producing staphylococci. The antibiotic disks containing oxacillin (OX, 1 µg), co-amoxiclav (AMC, 3 µg), and ampicillin (AMP, 2 µg) were placed on MHA plates. The AMC disk was placed between OX and AMP disk within a center-to-center distance of 2 cm. The plate was inverted and incubated at 37 °C for 24 hour.

A positive result, beta-lactamase production, will cause the growth-inhibition zone between AMC and AMP disks to expand, with or without changing the inhibition zone around OX disk. Any discernible growth within the zone of inhibition is methicillin resistance.

3.3.3 Determination of minimum inhibitory concentration (MIC) for oxacillin by agar dilution method (Kelly, Jacobs and Appelbaum, 1999).

Agar dilution method was performed according to CLSI, 2007 in order to determine minimal inhibitory concentration (MICs) of oxacillin against all tested isolates.

Preparation of antimicrobial solution

The two-fold dilution of oxacillin solution (0.03-256 µg/ml) were prepared by diluting the drug in MHA. One ml of each antimicrobial dilution in malting agar (45-55 ºc) was poured to sterile plates.

Preparation of inoculums

The well-isolates colony of each 18 hours coagulase positive *Staphylococcus ssp.* and *S. aureus* ATCC 29213 were selected from MHA plates and transferred to a tube containing 7 ml normal saline solution (NSS). The suspension was adjusted to match the turbidity of the 0.5 McFarland standard solution. This resulted in a bacterial suspension contained approximately 1 to 2 x 10^8 CFU/ml. Final inocula contained 10^4 organisms/spot. Plates were inoculated with a Steers replicator with 3-mm inoculating pins and incubated overnight at 35°C in air.

MIC Interpretive standard breakpoints (µg/ml)

The lowest concentration of antibiotic showing no growth was read as the MIC. CLSI breakpoints were used for MIC interpretation. Methicillin resistance is defined as MIC of oxacillin MIC was $\geq$ 4 µg/ml and the strains were categorized as methicillin resistant strains. MIC $\leq$ 2 µg/ml were interpreted as indicating susceptibility.

3.4 Molecular identification

Species identification of *S. aureus* (SA) and *S. intermedius* group (SIG) were confirmed by PCR mediated amplification of a species-specific sequence of thermonuclease encoding gene *nuc* (*SAnuc* and *SInuc*) (Baron *et al*., 2004). In order to separate *Staphylococcus pseudintermedius* from *Staphylococcus intermedius* groups, the PCR-REA was determined as described by Bannochr et al. (2009).

The methicillin resistant gene (*mecA*) and betalactamas gene (*blaZ*) were also detected using PCR methods according to Strommenger et al. (2003) and Charpentier et al. (2004), accordingly.

The oligonucleotide primer sequences and PCR programs are summarized in Table 3-1.

3.4.1 DNA extraction (Gentra Systems, Inc., Minneapolis MN)

a) One colony of MRCPS sample was inoculated into 5 ml LB broth, incubated in orbital incubator shaker (Gyromax™ 707R) at 37ºC 24 h. The 0.5 ml of bacterial culture was pipette into a 1.5 ml microfuge tube on ice and vortex at 13,500 rpm for 3 min at room temperature. The supernatant was discarded. Three hundred microliter of 1x TE buffer (10 mM Tris-l) and 2 µl of Lysis Enzyme Solution (20 mg/ml) were added and mixed together with bacterial cells. The suspension was then incubated at 37ºC for 30 min.

b) Cell walls digestion : Three hundred microliter Cell Lysis Solution was added to the cell pellet and gently pipetted up and down to lyse the cells. After that, the solution was centrifuged at 13,000 x g for 1 min. The supernatant was discarded and 2 µl RNase A solution was added into the cell lysate. The sample was then mixed by inverting the tube 25 times and incubated at 37 ºc for 15 min.

c) Sample was cooled down to room temperature. Two microliter of Protein Precipitation Solution was added to the cell lysate, vortexed vigorously at high speed for 20 sec and then centrifuged at 13,000 x g for 3 min. The precipitated proteins were

formed a tight white pellet. After that, the supernatant containing the DNA was poured into a clean 1.5 ml microfuge tube containing 300 µl of 100% isopropanol (2-propanol) and mixed. The tube was centrifuged at 13,000 x g for 1 min, the DNA was visible as a small white pellet. The supernatant was poured off and the tube was drained briefly on clean absorbent paper.

Finally, 300 µl 70% Ethanol was added. The tube was inverted several times and centrifuged at 13,000 x g for 1 min to clean the DNA pellet. The ethanol was carefully poured off. The 50 µl of DNA hydration solution was added. The DNA samples were stored at -20ºC before using as the template in all PCRs described below.

3.4.2 multiplex PCR detection of 16S rRNA, *SAnuc* and *SInuc* (Lautz et al., 2006)

A molecular identification was performed by amplifying the *nuc* genes of *S. intermedius* and *S. aureus* using species-specific oligonucleotide primers, respectively. The oligonucleotide primers were used together with an internal positive control that targeted a highly conserved region of 16S rDNA in a multiplex PCR as described by Baron et al. (2004). *Staphylococcus schleiferi* subsp. coagulans was identified by using the oligonucleotide primers SS16S-F and SS16S-R amplifying a species specific part of the 16S rRNA gene as described by Yamashita et al. (2005). The oligonucleotide primer sequences and PCR programs are summarized in Table 3-1.

The PCR reaction mixture (20 µl) contained 1.8 µl, 1.7 µl and 0.4 µl of *SInuc*, *SAnuc* and 16S primers (10 pmol/ µl), respectively, 0.8 µl dNTP (10 mmol; Milli-Q^{TM}), 2 µl of 10 X buffer, 6.4 mM $MgCl_2$, and 0.3 µl Taq DNA polymerase (Milli-Q^{TM}) and 5.6 µl dH_2O. Finally 5 µl DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycling.

3.4.3 PCR detection of *blaZ* (Charpentier et al., 2004)

The PCR reaction mixture (50 µl) contained 1 *blaZ* gene primers (10 pmol/ µl), 1 µl dNTP (10 mmol; Milli-Q^{TM}), 5 µl of 10 X buffer, 0.5 µl Taq DNA polymerase (Milli-Q^{TM}) and 42 µl dH_2O. Finally 5 µl DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycling.

3.4.4 PCR detection of *mecA* (Strommenger et al., 2003)

The PCR reaction mixture (50 µl) contained 1 *mecA* gene primers (10 pmol/ µl), 1 µl dNTP (10 mmol; Milli-Q^{TM}), 5 µl of 10 X buffer, 0.5 µl Taq DNA polymerase (Milli-Q^{TM}) and 42 µl dH_2O. Finally 5 µl DNA preparation was added to each reaction tube. The tubes were then subjected to thermal cycling.

3.4.5 PCR-REA detection of SIG (Bannochr et al., 2009)

In the current study, sequence analysis of one of the loci, *pta*, which encodes the enzyme phosphoacetyltransferase, revealed the presence of an *MboI* restriction site in all *S. pseudintermedius* isolates, which was absent in all *S. intermedius* and *S. delphini* isolates examined. Based on this discovery we have designed a simple, robust, and inexpensive PCR-REA diagnostic test for the identification of *S. pseudintermedius*. Staphylococcal genomic DNA isolation was carried out as previously described (Bannochr et al., 2007). PCR amplification of a 320-bp fragment of the *pta* gene was carried out in a 50-µl volume with a 0.2 µM concentration of each oligonucleotide primer (pta_f1, AAA GAC AAA CTT TCA GGT AA, and pta_r1, GCA TAA ACA AGC ATT GTA CCG), a 0.2 mM concentration of the deoxynucleoside triphosphates, 1.5 mM MgCl2, 0.5 U *Taq* DNA polymerase, and 50 ng DNA template, in a 1 x reaction buffer.

Twenty-five-microliter samples of the PCR mixtures were incubated with 5 U of *MboI* and 5 µl of 5 x digestion buffer for 2 h, and the digestion products were resolved in

3.5% (wt/vol) agarose by electrophoresis and comfirm by sending *pta* gene of random samples to sequencing at 1st BASE DNA Sequencing, Singapore.

Table 3-1 Oligonucleotide primer sequences and PCR conditions used in the present study

oligonucleotide primer	Sequence	Program	Size of PCR product (bp)	References
SAnuc primer1	5'-TGCTATGATTGTGGTAGCCATC-3'	1	420	Baron et al. (2004)
SAnuc primer2	5'-TCTCTAGCAAGTCCCTTTTCCA-3'			
SInuc primer1	5'-CAATGGAGATGGCCCTTTTA-3'	1	125	Baron et al. (2004)
SInuc primer2	5'-AGCGTACACGTTCATCTTG-3'			
16S primer1	5'-GGACGGGTGAGTAACACGTGG-3'	1	252	Baron et al. (2004)
16S primer2	5'-TCCCGTAGGAGTCTGGACCGT-3'			
blaZ Primer1	5'-TACAACTGTAATATCGGAGGG-3'	2	774	Charpentier et al. (2004)
blaZ Primer2	5'-AGGTTCAGATTGGCCCTTAGG-3'			
mecA Primer1	5'- AAAATCGATGGTAAAGGTTGGC -3'	3	532	Strommenger et al. (2003)
mecA Primer2	5'- AGTTCTGCAGTACCGGATTTGC -3'			
pta Primer1	5'- AAAGACAAACTTTCAGGTAA -3'	4	320	Bannochr et al. (2009)
pta Primer2	5'- GCATAAACAAGCATTGTACCG -3'			

* PCR program 1: 1x (95°C, 240s), 30x (95°C, 30s; 55°C, 30s; 72°C 30s), and 1x(72 °C, 420s). 2: 1 x (94°C, 300s), 30x (94°C, 60s; 55°C, 60s; 72°C 60s), and 1x (72°C, 420s). 3: 1 x (94°C, 240s), 40x (94°C, 30s; 55°C, 30s; 72°C 60s), and 1x (72°C, 300s). 4: 1x(95 °C , 120s), 30(95 °C , 60s; 53 °C , 60s; 72 °C, 60s), and 1x(72 °C, 420s)

3.4.6 PCR Interpretation

Increasing of 125 bp, 252 bp and 420 bp DNA on agarose gel of samples were identified to be *staphylococcus intermedius group* (SIG), *16S rRNA*, *Staphylococcus aureus*, respectively. MRSA with *SCCmec* type 3 was used as positive control.

Increasing of 320 bp, 532 bp ,and 774 bp on agarose gel of samples were identified as *pta, mecA,* and *blaZ* positive, respectively and *Staphylococcus aureus* ATCC 29213 and MRSA with *SCCmec* type 3 were used as positive control.

3.4.7 PCR-REA Interpretation

A *pta* PCR product of 320 bp was successfully amplified from all strains examined. *S. pseudintermedius* PCR products all contained a single MboI site, resulting in two restriction fragments of 213 bp and 107 bp, respectively. In contrast, SIG species *S. delphini* and *S. intermedius, S. schleiferi* and most of *S. aureus* strains did not contain an *Mbo*I restriction site (Bannoehr et al., 2009).

CHAPTER 4

RESULTS

4.1 Prevalence of coagulase positive staphylococci

A total of 200 clinical specimens derived from 100 dogs, 72 (36%) isolates were identified as *Staphylococcus spp.* by conventional methods (Gram-positive, catalase-positive and oxidase-negative). Most of the Staphylococcus isolates were obtained from nasal cavity (42%), followed by wound infection (30%) and otitis externa (30%) (Table 4-1).

Table 4-1 *Staphylococcus spp.* from 200 clinical specimens, 100 dogs

Samples	*Staphylococcus spp.* (*Staphylococcus spp.*/site)
Nasal (n=100)	42 (42%)
Wound (n= 60)	18 (30%)
Otitis externa (n=40)	12 (30%)
Total (n=200)	72 (36%)

Of the 72 isolates, 49 isolates (68.05%) were coagulase-positive *Staphylococcus spp*. (CPS). Most of them (85.71%) were identified to be *Staphylocccus intermedius* group (SIG) by negative to Voges-Proskaur (VP) test. Most of *Staphylococcus intermedius* group (SIG) gave very weak coagulase-positive results. They could take more than two days for clotting result (Table 4-2). The VP-positive and VP-variable isolates were identified as *Staphylococcus aureus* (SA) (6.12%) and other CPS (8.16%), respectively (Table 4-3). All of them were confirmed their species by Polymerase Chain Reaction (PCR) method.

Table 4-2 Time required for coagulase results

Species (number)	more than 72 h	within 72 h	within 24 h	within 4 h
SIG	5	23	2	12
S. aureus	1	0	1	1
Other coagulase positive	1	0	0	3

Table 4-3 VP-test related with PCR testing of Coagulase Positive *Staphylococcus spp.*

Clinical condition	Specimen	Number of Species (%)					
		Staphylo-coccus spp.	CPS	SIG[a] (SIG/CPS)	SA[b] (SA/CPS)	other CPS[c] (other CPS/CPS)	SPI[d]
Wound infection (n=60)	Pus. (n=60)	18 (30%)	15 (83.33 %)	14 (93.33%)	0 (0%)	1 (6.67%)	14
	Nasal (n=60)	24 (40%)	11 (45.83 %)	9 (81.82%)	0 (0%)	2 (18.18%)	9
Otitis externa (n=40)	Ear exudate (n=40)	12 (30%)	9 (75%)	7 (77.78%)	1 (11.11%)	1 (11.11%)	7
	Nasal (n=40)	18 (45%)	14 (77.78 %)	12 (85.71%)	2 (14.28%)	0 (0%)	12
Total No.	N = 200	N = 76 (38%)	N = 49 (64.47 %)	N = 42 (85.71%)	N = 3 (6.12%)	N = 4 (8.16%)	42
Dog no.	N =100	N = 54	N =39	N =32	N = 3	N = 4	32

a = VP-negative, SI*nuc*-positive

b = VP-positive, SA*nuc*-positive

c = VP-variable, SI*nuc*-negative, SA*nuc*-negtive

d = positive single *Mbo*I restriction site on (320 bp) *pta* gene

4.2 Identification of CPS by PCR method using 16S rRNA, *SAnuc* and *SInuc* gene

All coagulase positive *Staphylococcus spp.* that were isolated from 39 dogs were typeable by Identification of CPS by PCR method using *16S rRNA*, *SAnuc* and *SInuc* gene, providing three different patterns as shown in Figure 4-1 and Figure 4-2. Of the 49 CPS, 42 (85.71%) were identified as SIG by the present of the *SInuc* gene (125 bp) and 16S rRNA (252 bp), whereas 3 isolates (6.12%) were identified as SA by the presence of *SAnuc* gene (420 bp). The other CPS (4 isolates, 8.16%) were positive for only 16S rRNA (252 bp).

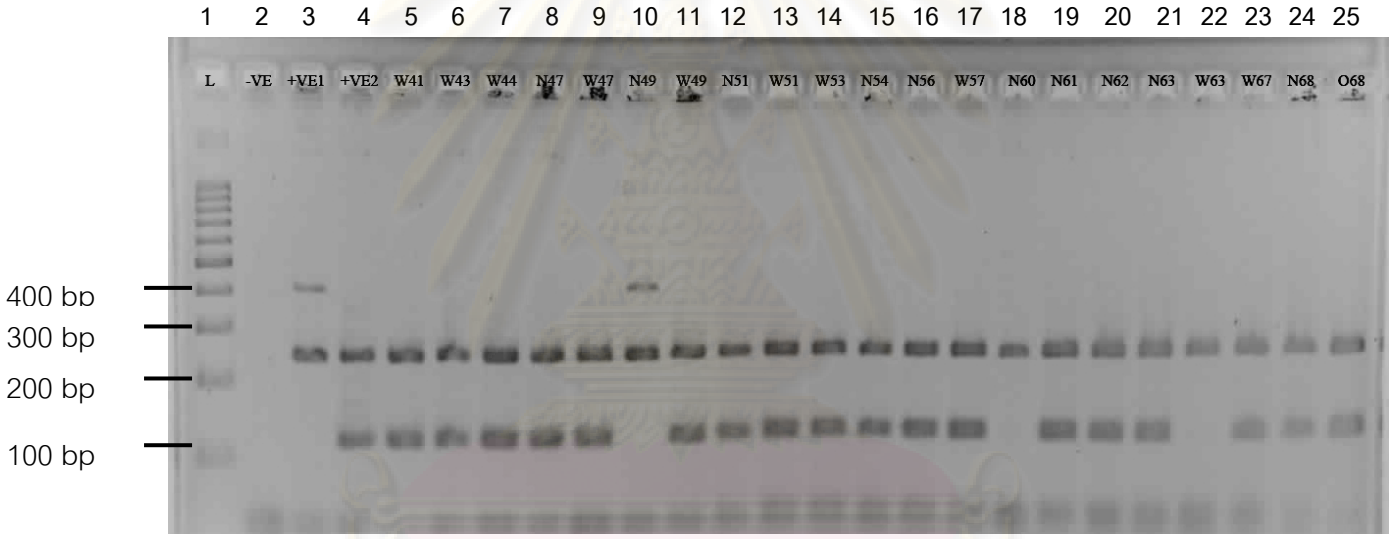


Figure 4-1 Pattern of PCR products of coagulase positive *Staphylococcus spp.*

Lane	Samples	16S rRNA	*SAnuc*	*SInuc*
1	100 bp DNA ladder	N/A	N/A	N/A
2	negative control	N/A	N/A	N/A
3	*SAnuc* positive control	positive	positive	negative
4	*SInuc* positive control	positive	negative	positive
5-25	Tested isolates			

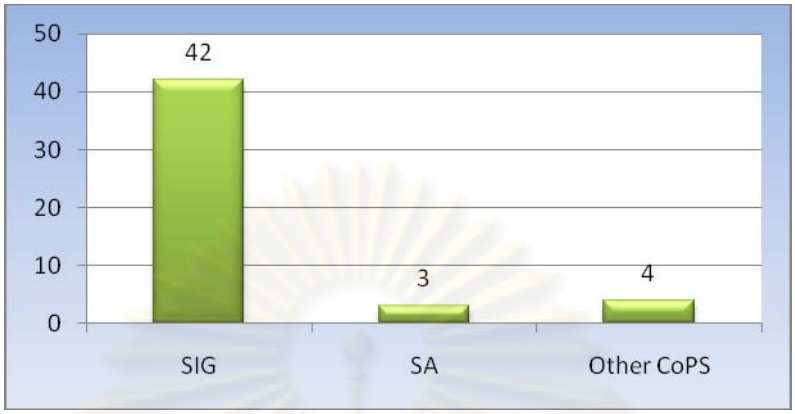


Figure 4-2 Total number of coagulase-positive *staphylococcus spp.*

SIG = *Staphylococcus intermedius* group; SA = *Staphylococcus aureus*; Other CoPS = Other coagulase-positive *staphylococcus spp.*

4.3 Identification of *S. pseudintermedius* by PCR-REA

Forty-two CPS that were positive with *16S rRNA* and *SInuc* genes were typed by PCR-REA technique. The pattern of agarose gel electrophoresis of *Mbo*I digested *pta* product is shown in Figure 4-3. The results of digested *pta* gene and their identification is summarized in Table 4-4. A *pta* PCR product of 320 bp of *S. pseudintermedius* contain a single *Mbo*I site resulting in 2 restriction fragments of 213 bp and 107 bp, respectively. In contrast, an *Mbo*I restriction site was absent in all *S. intermedius* and *S. delphini*. In this study, all SIG were identified as *S. pseudintermedius*.

Figure 4-3 All *S. pseudintermedius* PCR products contained a single *Mbo*I site.

Lane	Isolated	320 bp	216 bp	107 bp
1	100 bp DNA ladder	N/A	N/A	N/A
2	positive control (U)	positive	negative	negative
3	positive control (C)	positive	negative	negative
4-24	Tested isolates			

4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 = uncutted gene samples

5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 = cutted gene samples

Table 4-4 the results of PCR-REA of the *pta* gene

CPS	*S. pseudintermedius* (%)	*S. aureus* (%)	Other CPS (%)
Total	42 (100%)	0 (%)	0 (%)

Figure 4-4 Results of base sequencing

280740_W84_ptaR1 reverse complement (247bp)

ccagaaggagaagatgagcgtgtattaacagccgctgttgatttacaagcttctgactatgttgcgccgatcgtattagg
gaatgttgacaaaattaaagcacttgctgacagaaaaatctttaaatattgaaggtttaaatatcatccaacctgatacgg
cgacctaaaagcaacactagttgaacaatttgtagaacgtcgtaaagggaaagcgactgaagaacaagcacaatc
gttattaaatg

280739_W84_ptaF1 forward complement (247bp)

ccagaaggagaagatgagcgtgtattaacagccgctgttgatttacaagcttctgactatgttgcgccgatcgtattagg
gaatgttgacaaaattaaagcacttgctgcagaaaaatctttaaatattgaaggtttaaatatcatccaacctgatacgag
cgacctaaaagcaacactcgttgaacaatttgtagaacgtcgtaaagggaaagcgactgaagaacaagcacaatc
gttattaaatg

This was the *pta* gene from tested isolates. We random tested samples by binding technique. The results of *pta* gene's base sequencing showed sites of restriction enzyme (…gatc…) proving valid results (Figure 4-4).

GenBank: AM946758.1

Staphylococcus pseudintermedius partial *pta* gene for phosphate acetyltransferase, strain SD1071

Gtgcgtatcgtattaccagaaggagaagatgagcgtgtattaacagccgctgttgatttacaagcttctgactatgttgcgccaatcgtatt
agggaatgttgacaaaattaaagcacttgctgcagaaaaatctttaaatattgaaggtttaaatatcatccaacctgatacgagcgaccta
aaagcaacactcgttgaacaatttgtagaacgtcgtaaagggaaagcgactgaagaacaagcacaatcgttattaaatgatgtgaacta
cttcggtacaatgcttgtttatgcaggtgtttagtgagcggtgcagcccactcaacagcagacactgttcgtccagcgcttcaaattatcaa
aacaaaaccaggtgtttctaagacatcaggtattttcttcatgattaaagaagatcaacaatttattttcggtgactgtgcgattaaccctgaat
tagccgcatcagacttagctgaaattgcggttgaaagcgcgaaaactgcacaaagcttcggcatggacccacgtgtagcgatgttaag
cttctcaacaaaaggttctgc

GenBank: AM946757.1

Staphylococcus pseudintermedius partial *pta* gene for phosphate acetyltransferase, strain KM241

Gtgcgtatcgtattaccagaaggagaagatgagcgtgtattaacagccgctgttgatttacaagcttctgactatgttgcgccgatcgtatt
agggaatgttgacaaaattaaagcacttgctgcagaaaaatctttaaatattgaaggtttaaatatcgtccaacctgatacgagcgaccta
aaagcaacactcgttgaacaatttgtagaacgtcgtaaagggaaagcgactgaagaacaagcacaatcgttattaaatgatgtgaacta
cttcggtacaatgcttgtttatgcaggtcatgctgacggtttagtgagcggtgcagcccactcaacagcagacactgttcgtccagcactt
caaattatcaaaacaaaaccaggtgtttctaagacatcaggtattttcttcatgattaaagaagaccaacaatttattttcggtgactgtgcga
ttaaccctgaattagctgcatcagacttagctgaaattgcggttgaaagcgcgaaaactgcacaaagcttcggcatggacccacgtgta
gcgatgttaagcttctcaacaaaaggttctgc

GenBank: FM213460.1

Staphylococcus pseudintermedius partial *pta* gene for phosphate acetyltransferase, strain SD81

acagccgctgttgatttacaagcttctgactatgttgcgccgatcgtattagggaatgttgacaaaattaaagcacttgctgcagaaaaatc
tttaaatattgaaggtttaaatatcatccaacctgatacgagcgacctaaaagcaacactcgttgaacaatttgtagaacgtcgtaaaggg
aaagcgactgaagaacaagcacaatcgttattaaatgatgtgaagtacttcggtacaatgcttgtttatgcaggtcatgctgacggtttagt
gagcggtgcagcccactcaacagcagacactgttcgtccagcgcttcaaattatcaaaacaaaaccaggtgtttctaagacatcaggt
attttcttcatgattaaagaagatcaacaatttattttcggtgactgtgcgattaaccctgaattagccgcatcagacttagctgaaattgcggtt
gaaagcgcgaaaactgcacaaagcttcggcatg

Figure 4-5 GenBank datas of *S. pseudintermedius* strains including SD1071, KM241 and SD81.

By using PCR-REA, *S. pseudintermedius* identification confirmed the results from the reports in the past few years. According to the study by Bannoehr et al. (2009), this method is more specific than other methods which were used previously. As shown in figure 4-4 and 4-5, sequencing of PCR product results showed the restriction site (…GATC…) on DNA sequencing data obtained from forward and reward primers. By comparing with the *pta* gene of 3 *S. pseudintermedius* isolates including SD1071, KM241 and SD81 (see in Figure 4-5). The closest isolates was found to be KM241 with one point of gene mutation (99%).

4.4 Antimicrobial drug susceptibility

Susceptibility rates of 49 CPS isolates non-betalactam antibiotics were 22.45% for ciprofloxacin, 18.37% for clindamycin, 93.88% for mupirocin, 59.18% for co-trimoxazole and 10.2% for tetracycline. More than 75% of isolates were susceptible to amoxicillin/clavulanic acid whereas $\geq$ 73% were resistant to oxacillin, ciprofloxacin and tetracycline. Nearly all isolates were resistant to cefalexin (98%). Out of 49 isolates, 43 isolates (88%) were multidrug-resistant (Table 4-5; Figure 4-6 and 4-7).

Table 4-5 In vitro activity of 8 antimicrobial agents against 49 isolates of CPS as tested by disk diffusion method.

Antimicrobial agents	Susceptible(S)		Intermediate resistant (I)		Resistant (R)	
	No. of Isolate	%	No. of Isolate	%	No. of Isolate	%
Amoxicillin/clavulanic (AMC)	38	77.55	9	18.4	2	4
Cefalexin (CL)	1	2.041	0	0	48	98
Oxacillin (OX)	8	16.33	5	10.2	36	73
Ciprofloxaxin (CIP)	11	22.45	1	2.04	37	76
Clindamycin (DA)	9	18.37	11	22.4	29	59
Mupirocin (MUP)	46	93.88	0	0	3	6
Co-trimoxazole (SXT)	29	59.18	1	2.04	19	39
Tetracycline(TE)	5	10.2	1	2.04	43	88
Multidrug resistant[a]					43	88

a = Intermediate resistant or resistant to at least three classes of antimicrobial drugs.

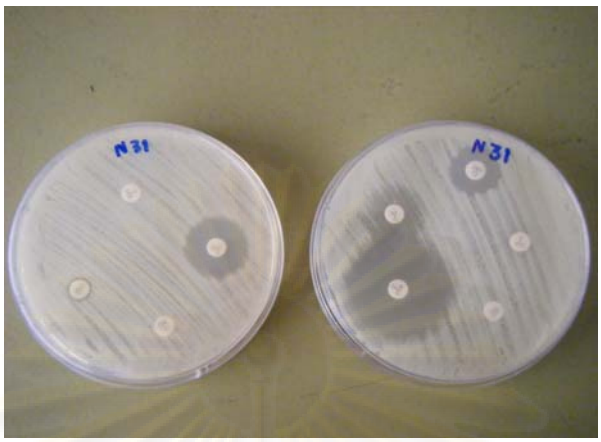


Figure 4-6 Drug susceptibility test of coagulase positive *Staphylococcus spp*.

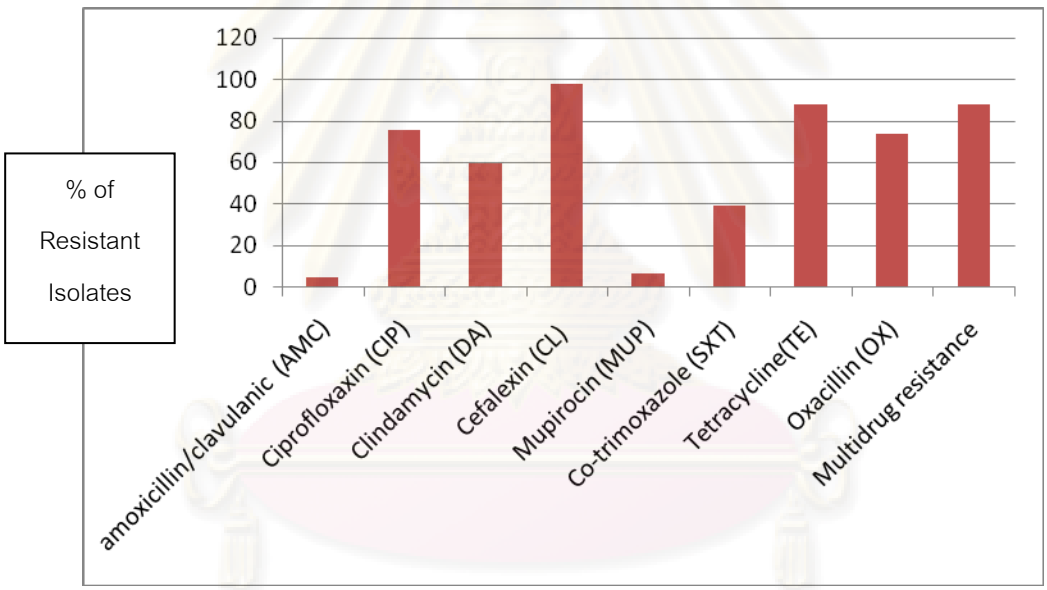


Figure 4-7 Percent of resistant isolates to 8 antimicrobial agents by disk diffusion method

The antimicrobial resistance patterns are shown in Table 4-6. Coresistance between fluoroquinolone group (FQL) (ciprofloxacin), beta-lactam group (ß-LT) (oxacillin, cefalaxin), Clindamycin and tetracycline were common (15 of 49, 30.61%). Coresistance to ß-LT (oxacillin and cefalexin), clindamycin, co-trimoxazole and mupirocin was uncommon. All of *S. pseudintermedius* isolates were multidrug resistant, whereas only one other coagulase positive were multidrug resistant. None of

Staphylococcus aureus were multidrug resistant (Table 4-6; Figure 4-8). Accordingly, their medicinal records as well as their previous uses of antimicrobials and other drugs are summarized in Table 4-7.

Table 4-6 Coresistance pattern of 43 multidrug resistant isolates of CPS

Coresistance pattern	No. of anti-microbial class	No. of multidrug-resistant isolates	%
FQL+ß-LT+TE	3	9	20.93
SXT+ß-LT+TE	3	1	2.32
FQL+DA+ß-LT+TE	4	15[a]	34.88
FQL+ß-LT+SXT+TE	4	5	11.62
DA+ß-LT+SXT+TE	4	3	6.97
DA+ß-LT+SXT+MUP	4	1	2.32
DA+ß-LT+SXT+MUP+TE	5	1	2.32
FQL+DA+ß-LT+SXT+TE	5	7	16.28
FQL+DA+ß-LT +SXT+MUP+TE	6	1	2.32

*multidrug-resistant isolates = isolated intermediate or resistance to at least three classes of antibiotic.

[a] = SP 14 isolates and other CPS 1 isolates.

FQL = fluoroquinolone group (Ciprofloxacin (CIP))

ß-LT = beta-lactam group (Oxacillin(OX), Cefalexin(CL))

TE = tetracycline

SXT = co-trimoxazole

DA = clindamycin

MUP = mupirocin

Table 4-7 The records of antimicrobials uses in multidrugs resistant *S. pseudintermedius* isolated from dogs

No.	Duration of treatment	Prescribed Antimicrobials	Other Drugs	Pre- diseases	Resisted antimicrobials
3	1 day	no	no	no	CIP,DA,CL,MET,TE,OX
6	1 day	no	no	no	CIP,DA,CL,MET,TE,OX
11	long term ≥ 1 month	gentamicin, SXT, fluoroquinolone,	metronidazole	Recurrent otitis externa	CIP, CL,MET,TE,OX
12	long term ≥ 1 month	AMC, DA, norfloxacin	prednisolone	Skin disease	DA, MUP,MET,SXT,TE,OX
19	long term ≥ 1 month	doxycycline	prednisolone	Skin disease	CIP,DA,CL,MET,TE,OX
22	1 day	no	no	no	CL,MET,SXT,TE,OX
24	long term ≥ 1 month	AMC, doxycycline, Bacitracin®, amoxycilin	prednisolone	Skin disease	CIP,DA,CL,MET,TE,OX
25	30 days	doxycycline	no	no	CIP,DA,CL,MET,TE,OX
27	30 days	gentamicin	metronidazole	no	DA,CL,MET,SXT,TE,OX
29	1 day	no	no	no	CIP,DA,CL,MET,TE,OX, SXT
30	1 day	no	no	no	CIP, CL,SXT,TE,OX
31	30 days	SXT	no	no	CIP,DA,CL,MET,TE,OX
32	1 day	no	no	no	CIP,DA,CL,MET,TE,OX
39	1 day	AMC	no	no	CIP,DA,CL,MET,TE,OX
40	12 days	Bactrim®, SXT	no	no	CIP,DA,CL,MET,SXT,TE
41	1 day	no	no	Skin disease	CIP,DA,CL,MET,SXT,TE
43	2 days	no	no	no	AMP,CIP,DA,CL,MET,TE,OX
44	2 days	no	no	no	CIP ,CL,MET,TE,OX
47	20 days	doxycycline, norfloxacin	no	no	CIP,DA,CL,MET,SXT,TE,OX
49	1 day	no	no	no	CIP ,CL,MET,TE,OX
51	6 days	no	no	cancer	CIP ,CL,MET,STX,TE,OX
53	30 days	SXT, enrofloxacin	no	pyoderma	CIP ,CL,MET,STX,TE,OX
54	3 days	AMC	no	no	CIP ,DA,CL, STX,TE
56	1 day	no	no	no	CIP ,CL,MET,STX,TE,OX
57	8 days	SXT, Bactacin®	no	no	DA,CL.MUP,MET,STX,OX
61	10 days	Bacitracin®	no	no	CIP,DA,CL,MET,TE,OX

ciprofloxacin (CIP, 1 µg), clindamycin (DA, 2 µg), cefalexin (CL, 30 µg), co-amoxiclav (AMC, 3 µg), co-trimoxazole (SXT, 25 µg), mupirocin (MUP, 5 µg) and tetracycline (TE, 10 µg), oxacillin (0X, 5 µg) and cefoxitin (CEF, 30 µ)

Bacitracin® = Bactoprenol phosphate, Bactrim® = sufamethoxazole+trimethoprim

Table4-7 The records of antimicrobials uses in multidrugs resistant *S. pseudintermedius* isolated from dogs (continued)

No.	Duration of treatment	Antimicrobials	Other Drugs	Pre- disease	Resistance to antimicrobials
62	17 days	no	no	cancer	CIP ,CL,MET,TE,OX
63	long term ≥ 1 month	AMC	prednisolone	no	CIP ,CL,MET,TE,OX
67	1 day	no	no	no	AMC,CIP,DA,CL,MUP,MET,SXT ,TE,OX
68	1 day	no	no	no	CIP,DA,CL,MET, TE,OX, SXT
74	long term ≥ 1 month	enrofloxacin, CL	no	no	CIP,DA,CL, MET,SXT,TE,OX
82	long term ≥ 1 month	CL	no	Skin disease	CIP,DA,CL, SXT,TE
83	1 day	no	no	no	CIP,CL,MET,TE,OX
88	1 day	no	no	no	CIP,DA,CL,MET, TE,OX

ciprofloxacin (CIP, 1 µg), clindamycin (DA, 2 µg), cefalexin (CL, 30 µg), co-amoxiclav (AMC, 3 µg), co-trimoxazole (SXT, 25 µg), mupirocin (MUP, 5 µg) and tetracycline (TE, 10 µg), oxacillin (0X, 5 µg) and cefoxitin (CEF, 30 µ)
Bacitracin®= Bactoprenol phosphate, Bactrim® = sufamethoxazole+trimethoprim

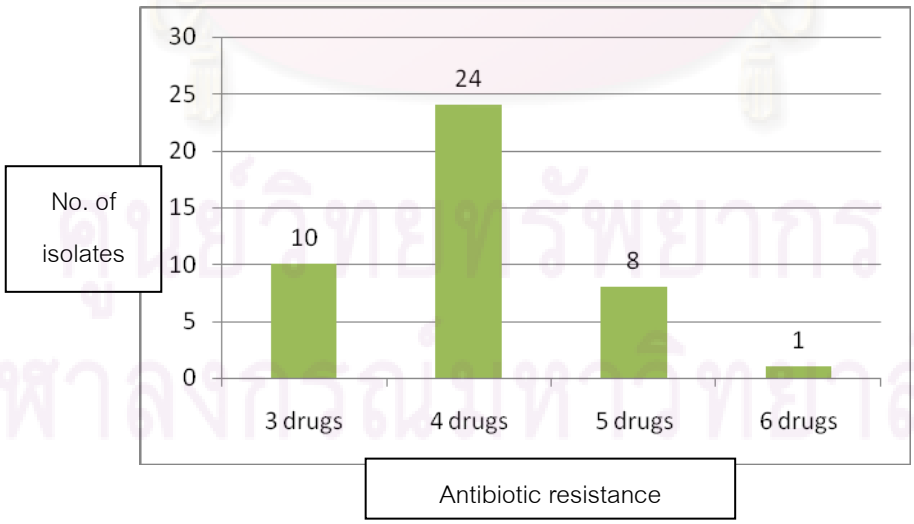


Figure 4-8 Distribution of the multidrug-resistant isolates of CPS according to the number of antibiotics to which they showed resistance

4.5 Determination of beta-lactamase production in coagulase positive *Staphylococcus spp.* (CPS) by Disk diffusion method (CLSI, 2007)

By interpete the result from phenotype, isolates gave synergistic zone of AMC and AMP (6 isolates; 12.24%) produced beta-lactamase enzyme. For isolates gave synergistic zone of OX-AMC-AMP were interpete to be sensitive with all drugs and may be produce enzyme bata-lactamase and isolates did not give inhibition zone were resist to all drug. (Table 4-8, Figure 4-10).

Table 4-8 Percent of beta-lactamase production screening test in 49 CPS isolates.

Synergistic zone	No. of isolates (%)
OX-AMC-AMP	11 (22.91%)
AMC-AMP	6 (12.24%)
Resistant to all (no inhibition zone)	32 (65.30%)

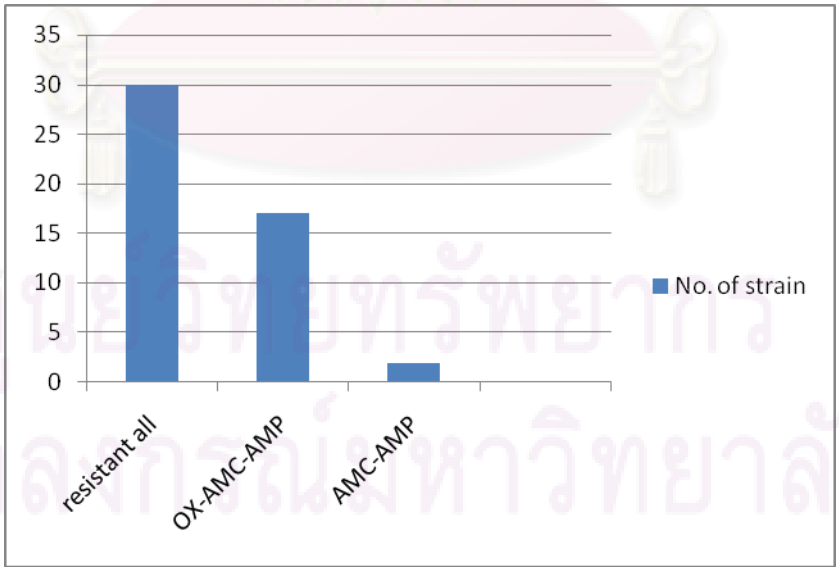


Figure 4-9 Beta-lactamase production screening test in 49 CPS isolates.

OX = oxacillin, AMC = amoxicillin/clavulanic acid and AMP = ampicillin

Figure 4-10 Appearance of growth-inhibitory zone in beta-lactamase producing CPS isolates by using of ampicillin, amoxicillin/clavulanic acid and oxacillin

4.6 Determination of minimum inhibitory concentration (MIC) for oxacillin by agar dilution method.

The range of MICs were observed, as well as the MIC_{50} and MIC_{90} of oxacillin among the 49 isolates of CPS were shown in Table 4-9 and 4-10 and Figure 4-11. Sixteen (32.65%) of CPS isolates were susceptible to oxacillin with MIC range from 0.5-2 µg/ml (susceptibility breakpoint $\leq$ 2 µg/ml). While 33 isolates (67.34%) were resistant to oxacillin with MIC range from 4 µg/ml to greater than 256 µg/ml. The MIC_{50} and MIC_{90} were 256 µg/ml. The result of MIC to oxcaillin correlated with disk diffusion method were same resistant result.

Table 4-9 MIC distribution with MIC_{50}, MIC_{90} of oxacillin among 49 isolates of coagulase positive staphylococci by agar dilution method

	Oxacillin	
	MIC range*	No. of isolates (%)
	0.03	0
	0.06	0
	0.12	0
	0.25	0
	0.5	1 (2.04)
	1	11 (22.44)
	2	4 (8.16)
	4	2 (4.08)
	8	0
	16	0
	32	1 (2.04)
	64	2 (4.08)
	128	4 (8.16)
MIC_{50}, MIC_{90}	256	19 (38.77)
	>256	5 (10.20)

* unit of MIC µg/ml

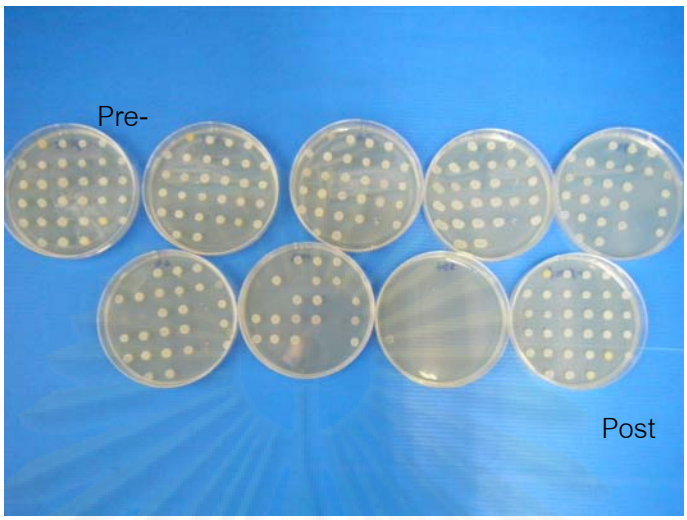


Figure 4-11 MIC of oxacillin against 32 isolates of coagulase positive *Staphylococcus spp* was determined by agar dilution method

Table 4-10 Number of oxacillin susceptibility and resistant CPS

isolates (no.)	oxacillin-susceptibility		oxacillin-resistant	
	no. of isolates	MIC range	no. of isolates	MIC rang
SP (42)	9	1-2	33	4->256
SA (3)	3	1	-	-
Other CPS (4)	4	0.5-2	-	-

Thirty-three of 42 SP isolates were resistant to oxacillin in the MIC range of 4->256, whereas all SA and other CPS were not resistant to oxacillin.

4.7 PCR detection of *blaZ* gene

All coagulase positive *Staphylococcus spp.* isolated from 39 dogs were typeable by PCR technique as shown in Figure 4-12. Of the 49 CPS, 31 isolates (63.26%) were positive for *blaZ* gene (774 bp) which identified that these isolates were beta-lactamase producers.

Figure 4-12 Agarose gel electrophoresis of all coagulase positive *Staphylococcus spp*'s PCR products using specific primers to *blaZ*. The amplicon size of *blaZ* are 774 bp.

Lane	Isolated	*blaZ* (774 bp)
1	1 kb DNA ladder	N/A
2	Positive control	positive
3-24	samples	
25	negative control	negative

4.8 PCR detection of *mecA* gene

All coagulase positive *Staphylococcus spp.* isolated from 39 dogs were typeable by this technique as shown in Figure 4-13. Of the 49 CPS, 30 (61.22%) were positive for *mecA* gene (532 bp) which could confirm that these isolates were methicillin-resistant isolates.

Figure 4-13 PCR production of coagulase positive *Staphylococcus spp* that isolated from dogs using type 2 MRSA for positive control.

Lane	Isolated	*mecA* (532 bp)
1	1 kb DNA ladder	N/A
2	negative control	negative
3	positive control	positive
4-25	samples	

Table 4-11 Correlation between oxacillin susceptiblility and the presence of the resistant gene *blaZ* and *mecA*

Species (No. of isolates)	No. of isolates (oxacillin MIC rang)	No. of isolates positive by PCR			No. of PCR-negative isolates
		blaZ[a]	*mecA*[b]	*blaZ+mecA*	
S. pseudintermedius (42)	33 OXA-resistant	4	6	22	1
	9 OXA-susceptible	3	2	-	4
S. aureus (3)	3 OXA-susceptible	2	-	-	1
Other CPS(4)	4 OXA-susceptible	-	-	-	4
Total no.	49	9	8	22	10

[a] = Isolates were considered to be beta-lactamase hyperproducer when oxacillin MIC ≥ 4 µg/ml and *blaZ*-positive.

[b] = Isolates were considered to be methicillin resistant when oxacillin MIC ≥ 4 µg/ml and *mecA*-positive (gold standard method).

The correlation between oxacillin susceptibility and the presence of the *blaZ* and *mecA* gene is summarized in Table 4-11. Of 49 CPS isolates, 33 (67.34%) were OXA-resistant (MIC ranging from 4 to > 256 µg/ml) and 16 (32.65%) were OXA-susceptible (MIC ranging from 0.5 to 2 µg/ml). All of OXA-resistant were SP and 22/33 (66.66%) were haboring both *mecA* and *blaZ* genes. There were 6/33 (18.18%) OXA-resistant isolates positive for only *mecA* and only 4 (12.12%) isolates were positive for only *blaZ* and considered to be beta-lactamase hyperproducers.

Of 16 OXA-susceptible CPS isolates, 3 SP isolates (18.75%) and 2 SA isolates (12.5%) were harboring *blaZ* gene. Only 2 OXA-susceptible SP (12.5%) were positive for *mecA* gene. Nine of ten OXA-susceptible CPS isolates were both negative for *mecA* and *blaZ* gene.

Table 4-12 Correlation between beta-lactamase screening test and the presence of the resistant gene *blaZ* and *mecA*

Synergistic zone	*blaZ* only	*mecA* only	*blaZ* and *mecA*	negative *blaZ* and *mecA*
OX-AMC-AMP (12 isolates)	2	1	1	7
AMC-AMP (6 isolates)	1	1	3	1
Resistant to all (32 isolates)	6	6	18	2

As shown in Table 4-12, there was no good correlation between oxacillin susceptibility and the presence of the resistant gene *blaZ* and *mecA*.

The result obtained from phenotypic and genotypic studies showed most of all isolated giving synergistic zone of AMC-AMP and OX-AMC-AMP were *blaZ* and *mecA* gene negative. Howevre, some of them carried either *blaZ* and *mecA* gene and four of them carried both. 7 isolates with synergistic zone of OX-AMC-AMP carried neither *blaZ* nor *mecA* but only 3 of them carried *blaZ* gene. Instantly, 4 isolates that synergistic zone of AMC-AMP carried *blaZ* and only 1 isolated carried neither *blaZ* nor *mecA*. Some of the resistant isolates carried either *blaZ* gene or *mecA* gene. Eventually, most of resistant isolates carried both of *blaZ* and *mecA* gene. whereas 2 of them carried neither mecA nor blaZ. 6 of them carried either *mecA* and *blaZ* gene.

CHAPTER 5

DISCUSSION AND CONCLUSION

Staphylococcus spp. are considered to be opportunistic pathogens in human and animals. Some of them are commonly found in healthy people and animals and can cause infections when people or animals get injured or sick. Typically, these organisms intrinsically cause no problem at all, but transmission of antimicrobial resistance within their groups can be a major problem in community. Some of them can continue to be dangerous pathogens causing infections which are difficult to treat because of the organism's intrinsic resistance to many antimicrobials and its propensity to develop resistance during therapy (Hill et at., 2006). The methicillin-resistant staphylococci have been considered to be a challenge for the veterinary profession. Recently, it was proposed that the methicillin-resistant staphylococcal infections are important causes of morbidity and mortality in companion animals, and may be involved in zoonotic transmission (Weese and van Duijkeren, 2009).

Coagulase-positive staphylococci (CPS), especially members of the *Staphylococcus intermedius* group (*S. intermedius*, *S. pseudintermedius* and *S. delphini*), are opportunistic pathogens in various animal species, particularly in dogs and cats (Biberstein et al., 1984; Cox et al.,1988). Moreover, the occurrence of MRSA in companion animals have been widely reported in many countries in Europe. Thus, this study was aimed to investigate prevalence of methicillin resistant coagulase positive staphylococci (MRCPS) and their antimicrobial susceptibilities in outpatient dogs from our veterinary teaching hospital. Using of molecular identification, mechanisms of methicillin resistance could be also determined.

Isolation and phenotypic characterization of staphylococci

The result from this research showed that nasal cavity of dogs was the most common site of *Staphylococcus spp. S. pseudintermedius* was found to be the most common coagulase positive staphylococci. Our finding correlated with the recent reports of Abraham et al., 2007 and Griffeth et al., 2008. Recently, many reports showed that prevalence of MRS(P)I in the community and upon admission to veterinary hospitals were variable. The population rates of MRS(P)I in dogs were 1.5-2% (Griffeth et al., 2008; Hanselman et al., 2007; Vengust et al., 2006) and higher frequency of MRS(P)I (up to 7%) was found at skin infection of dogs (Griffeth et al., 2008). Previously, *S. intermedius* was considered to be the most important CPS in dogs and its methicillin resistance traits have emerged in bacteria isolated from companion animals, especially dogs. Recently, a phylogenic study has shown that *S. pseudintermedius*, not *S. intermedius*, is the common CPS found in dogs (Sasaki et al., 2007a). Accordingly, the canine pathogen is likely to be reclassified as *S. pseudintermedius*. Since it is commonly confused with *S. intermedius* in routine diagnostic veterinary bacteriology. It has been described that what has been identified as *S. intermedius* in the past may be truly *S. pseudintermedius*. The study of Sasaki et al. (2007a) found that 30% of veterinary staff, 57.7% of inpatient dogs and 48.4% outpatient dogs in Japan were infected with CPS. Among these, 31.6% were MRCPS. In this research, I also found that MRCPS was the most common isolates among the CPS (61.12%). However, lower rate of MRSP was reported by Hanselman, Kruth, and Weese (2008). They found only 4 isolates from nasal swab of 193 dogs (2.1%) were MRSP. As same as Geoghegan (2009) found that ability of colonization in nasal cavity of *S. pseudintermedius* were found closely out of *S. aurerus*. Recently, Ruscher et al. (2009), found the rate of MRSP isolated from wounds was significantly higher than those from all other infection sites and most of them were found in dogs.

According to present knowledge, biochemical assay for screening of CPS could not separate *S. pseudintermedius* from *S. intermedius*. It was discussed recently that *S. (pseud)intermedius* may be not only misidentified as *S. intermedius* but also *S. aureus* in medical laboratories as well. Therefore its real incidence in humans in the past may be underestimated. The report of Talan et al. (1989a) who analyzed 14 isolates from human dog-bite wounds. They were originally identified as *S. aureus* and three of them (22%) were found to be *S. intermedius*. Accordingly, more specific diagnostic tools including genotyping, should be done in order to avoid misidentification (Weese and van Duijkeren, 2009).

Antimicrobial Susceptibility Testing

All of *S. pseudintermedius* in this study were multidrug-resistant isolates and nasal cavity was the best place to accumulate multidrug-resistant *Staphylococcus spp*. (MDRS). Most staphylococcal cassette chromosome *mec* (SCC*mec*) elements collected antibiotic resistance genes and passed them by to other *Staphylococcus* group (Machado et al., 2007). They can exchange gene between Staphylococci group or the other bacteria nearby. From the medicinal records, there was a correlation between long term treatment of antimicrobial drugs and the occurrence of multidrug resistance but not all of them. The isolates had similar antibiotic resistance profiles, characterized by resistance to most beta-lactams, tetracyclines, fluoroquinolones and co-trimoxazole. Some dogs (No. 12 and 82) were treated with various antibiotics and showed drug resistance pattern to all previous used antibiotics. A similar multidrugs resistance was also found in isolates obtained from a dog (No.41) visited for the first time in our clinic. However, this dog was suspected to be treated previously with many antibiotics.

Thus, long-term treatments of skin infections may be a major cause of multidrug-resistance in dogs. According to a previous report from Germany, thirty-three percent of all *S. (pseud)intermedius* isolates from one veterinary dermatology referral clinic in Germany was found to be multidrugs resistance (Loeffler et al., 2007). Moreover, there

was also a relationship between long-term treatment and multidrug-resistant bacteria found in animals. In this case, dogs could be carriers or reservoirs of MDRS and might cause dog-to-human transmission, especially veterinarians and pet owners or vice versa. In addition, these isolates are a significant hazard because of the antimicrobial resistance they possess to cefalexin, ciprofloxacin, amoxicillin and co-trimoxazole, drugs frequently used for the treatment of human *S. aureus*. Moreover, it was revealed recently that multidrug resistant opportunistic pathogens have become endemic to the veterinary hospital environment (Sasaki et al., 2007a; Griffeth et al., 2008; Hanselman et al., 2007; Vengust et al., 2006). Thus, studying of MDRS in veterinarians, hospital staff as well as hospital environment should be investigated further in Thailand.

The use of cefoxitin disk to determine methicillin resistant staphylococci as recommended by CLSI may be appropriate for only *S. aureus*. Because it was shown in this study that all oxacillin resistant staphylococci isolates tested were sensitive to cefoxitin by CLSI breakpoint. However, Skov et al. (2003) recommended that the diameter of inhibition zone of oxacillin up to 30 mm indicating the presence of *mecA* gene in *S. aureus* may be a good criteria for evaluation of MRCPS.

The MIC_{50} and MIC_{90} of oxacillin against all SP were 256 µg/ml indicating that they were MRSP. The MIC results of the four isolates interpreted with double disk diffusion test were absolutely oxacillin resistant by either over-production of beta-lactamase or modified intrinsic PBP with low affinity to the drugs belonging to the beta-lactam group (Tomasz et al., 1989; Hackbarth et al., 1995; Henze and Berger-Bachi, 1996). The screening test of beta-lactamase producing showed that most of oxacillin resistant isolates did not produce beta-lactamase, eventhough they were not susceptible to all beta-lactam drugs used in the susceptibility test. Thus, mechanism of methicillin resistant should not be due to beta-lactamase production by CPS. The existence of penicillin binding protein 2a (PBP2a) was suspected to be the cause of this resistance and molecular detection of *mecA* gene confirmed this hypothesis. Most isolates with MIC more than 4 µg/ml carried *mecA* gene. Four isolates with MIC more than 4 µg/ml carried *blaZ* gene but not *mecA* gene. Thus, it can be concluded that most

of MRSP identified in this study carried *mecA* gene and were highly resistant to many antimicrobials.

However, no good correlation between beta-lactamase screening test and the presence of the resistant gene *blaZ* and *mecA* was found. Some of isolates showed synergistic zone of OX-AMC-AMP carried neither *blaZ* nor *mecA* gene and some of isolates with synergistic zone of AMC-AMP carried *blaZ* gene.Thus, beta-lactamase screening test by antimicrobial disk diffusion method used in this study was not suitable for screening of beta-lactamase producing by *Staphylococcus spp.*

Previous reports in the past proposed that prevalence of MRS can be varied according to geographic location (Aarestrup and Jensen, 1998; Vintov et al., 2003). More than 90% of staphylococcal isolates now produce penicillinase, regardless of the clinical setting. The gene for beta-lactamase is part of a transposable element located on a large plasmid, often with additional antimicrobial resistance genes (e.g., gentamicin and erythromycin). Spread of penicillin resistance primarily occurs by spread of resistant strains (Lowy, 2003). In this study, two *S. aureus* isolates were haboring only the *blaZ* gene. Transferring of the *blaZ* gene between *Staphylococcus* group can be a problem in veterinary practice. Moreover, transferring of resistance genes between CNS and *S. aureus* has been reported indicating that CNS may act as a resistance gene reservoir for *S. aureus* too. (Jaffe et al.,1980; Forbes and Schaberg, 1983; Naidoo, 1984; Archer, 1996; Udo, Jacob and Mokadas, 1997).

Most CPS isolates (67.34%) were OXA-resistant (MIC ranging from 4 to > 256 µg/ml) and most of them were haboring both *mecA* and *blaZ* genes. Because of carrying of *mecA* and *blaZ* genes, MIC of these isolates were very high. Accordingly, animal-to-human *mecA* gene transmission can be a concern problem of human medicine since *mecA* gene can transfer by horizontal gene transfer (Wielder et al., 2001; Guardabassi et al., 2004; Morris et al., 2006; Vengust et al., 2006). In addition, healthy animals can carry also MRSP. Over 90% of healthy domestic dogs have been reported to carry coagulase-positive staphylococci such as *S. (pseud)intermedius* (Lloyd, 2007), with a small proportion of such isolates proving *mecA*-positive (Gortel et al., 1999;

Griffeth et al., 2008). Eventhough, MRSP infections in this study appear to be higher than previous report, MRSP in people is not a serious problem as MRSA. Typically, *S. pseudintermedius* cause no problem at all. It is commonly found on skin, nostils or intestinal tract of healthy dogs. It can be an opportunistic pathogen when animals get ill or injured.

Many studies reported about prevalence of this organism colonization in human by *S. (pseud)intermedius*. Talan et al. (1989), investigated the among 144 healthy veterinary college staff members, only one person was colonized with *S. (pseud)intermedius*. In hospital, two *S. (pseud)intermedius* isolates were found in patients : one from a healthy carrier and one from pleural fluid, which was thought to be contamination rather than infection (Mahoudeau et al., 1997). Therefore, its importance as a zoonotic pathogen is less than that of MRSA. However, several cases of zoonotic transmission of methicillin-susceptible and methicillin resistant *S. (pseud)intermedius* between dogs and humans have been published (Weese and van Duijkeren., 2009). In 2004, Guardabassi et al. investigated *S. (pseud)intermedius* in 13 dogs affected by deep pyoderma, their owners and 13 individuals without daily contact with dogs. The occurrence of *S. (pseud)intermedius* in the owners of the infected dogs was significantly higher (6/13) than in the control group (1/13) and they often carried the same *S. (pseud)intermedius* strain as their dogs. However, all persons were sampled a second time and were found to be no longer carriers and the dogs no longer had purulent lesions. Thus, direct contact with the lesions is a probably risk factor for the transmission of the organism to humans.

MRSP is the most commonly cause of skin and ear infections. The prevalence of MRSP in dogs in this study was found to be higher than MRSA. Eventhough, the emergence of MRSA in animals in Small Animal Hospital of Chulalongkorn University was not found, large population of animals in Thailand should be further investigated. Transmission of resistance genes from MRSP and MRSA between people and animals in Thailand should also be determined. Furthermore, study of SCC*mec* and the other drug-resistance gene in *S. pseudintermedius* should also be identified.

REFERENCES

Aarestrup, F.M. and Jensen, N.E. 1998. Development of penicillin resistance among *Staphylococcus aureus* isolated from bovine mastitis in Denmark and other countries. Microbiol Drug Resist. 4(3):247–56.

Andrews, J.M. 2001. BSACstandardized disc susceptibility testing method. Antimicrob Chemother. 48(1):43–57.

Anzai, T., Kamada, M., Kanemaru, T., Sugita, S., Shimizu, A. and Higuchi, T. 1996. Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from mares with metritis and its zooepidemiology. J Equi Sci. 7(1):7–11.

Archer, G.L., Thanassi, J.A., Niemeyer, D.M.K. and Pucci., M.J. 1996. Characterization of IS1272, an insertion sequence-like element from *Staphylococcus haemolyticus*. Antimicrob Agents Chemother. 40(4):924–9.

Bannoehr, J., Ben Zakour, N.L., Waller, A.S., Guardabassi, L., Thoday, K.L., van den Broek, A.H., and Fitzgerald, J.R. 2007. Population genetic structure of the *Staphylococcus intermedius* group: insights into *agr* diversification and the emergence of methicillin-resistant strains. J Bacteriol. 189(23):8685–8692.

Bannoehr, J., Franco, A., Iurescia, M., Battisti, A. and Fitzgerald J.R. 2009. Molecular Diagnostic Identification of *Staphylococcus pseudintermedius*. Clin Microbio. 47(2):469-471.

Baron, F., Cochet, M., Pellerini, J.L., Zakour, N.B., Lebon, A., Navarro, A., Proudy, I., Loir, Y.L. and Gautier, M. 2004. Development of a PCR test to differentiate between *Staphylococcus aureus* and *Staphylococcus intermedius*. J Food Prot. 67(10):2302–2305.

Beck, W., Berger-Bachi, B. and Kayser, F. 1986. Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of mec-specific DNA. J Bacteriol. 165(2):373-8.

Bender, J.B., Torres, S.M.F., Gilbert, S.M., Olsen, K.E. and LeDell, K.H., 2005. Isolation of methicillin-resistant *Staphylococcus aureus* from a non-healing abscess in a cat. Vet Rec. 157(13):388–389.

Biberstein, E.L., Jang, S.S., Hirsh, D.C., 1984. Species distribution of coagulase-positive staphylococci in animals. J Clin Microbiol. 19(5):610–615.

Bottger, E.C. 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol Lett. 539(1-2):171–176.

Bush, K., Jacoby, G. A. and Medeiros, A.A. 1995. A functional classification scheme for beta-lactamase and its correlation with molecular structure. Antimicrob Agents Chemother. 39(6):1211-1233.

Carter, G.R. and Wise, D.J. 2004. Staphylococcus. Essentials of veterinary bacteriology and mycology. 6th ed. Iowa : Blackwell Publishing Company. 193-198.

Clarridge, J.E. 2004. Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clin Microbiol Reviews.17(4):840–862.

Charpentier, E., Anton, A.I., Barry, P., Alfonso, B., Fang, Y. and Novick, R.P. 2004. Novel Cassette-Based Shuttle Vector System for Gram-Positive Bacteria. Appli and Env Microbiol. 70(10):6076-6085.

Cox, H.U., Hoskins, J.D., Newman, S.S., Foil, C.S., Turnwald, G.H. and Roy, A.F.1988. Temporal study of staphylococcal species on healthy dogs. Am J Vet Res. 49(6):747–751.

Darley, E.S. and MacGowan, A.P. 2004. Antibiotic treatment of gram-positive bone and joint infections. J Antimicrob Chemother. 53(6):928–35.

Devriese, L.A., Vandamme, L.R., and Fameree, L. 1972. Methicillin (cloxacillin)- resistant *Staphylococcus aureus* strains isolated from bovine mastitis cases. Zentralblatt fur Veterinarmedizin B. 19(7):598–605.

Devriese, L., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A. and Haesebrouck, F. 2005.

Staphylococcus pseudintermedius sp. nov., a coagulase-positive species from animals. Int J Syst Evol Microbiol. 55(2005):1569–1573.

Diederen B.M.W. and Kluytmans J.A.J.W. 2006. The emergence of infections with community-associated methicillin resistant *Staphylococcus aureus*. J Infect. 52(3):157–168.

Drlica, K. and Zhao, X. 1997. DNA gyrase, topoisomerase IV and the 4-quinolones. Microbiol Mol Biol Rev. 61(3):377–92.

Epstein, C.R., Yam, W.C., Peiris, J.S.M., and Epstein, R.J. 2009. Methicillin-resistant commensal staphylococci in healthy dogs as a potential zoonotic reservoir for community-acquired antibiotic resistance. Infect Genet and Evol. 9(2):283-285.

Forbes, B.A. and Schaberg, D.R. 1983. Transfer of resistance plasmids from Staphylococcus epidermidis to Staphylococcus aureus: evidence for conjugative exchange of resistance. J Bacteriol.153(2):627–34.

Garrity, G.M., and Holt, J.G. 2001. The road map to the manual, *In* G. M. Garrity (ed), Bergey's manual of systematic bacteriology. New York:Springer-Verlag, 119–166.

Geoghegan, J.A., Smith, E.J., Speziale, P. and Foster, T.J. 2009. *S. pseudintermedius* expresses surface proteins that closely resemble those from *S. aureus*. Vet Microbi. xxx (2009) : xxx–xxx.

Gerberding, J, Miick, C, Liu, H, and Chamber H. 1991. Comparison of conventional susceptibility tests with direct detection of penicillin-binding protein 2a in borderline oxacillin-resistant strains of *Staphylococcus aureus*. Antimicrob Agents Chemother. 35(2):2574-9.

Goni, P., Vergara, Y., Ruiz, J., Albizu, I., Vila, J. and Gomez-Lus, R. 2004. Antibiotic resistance and epidemiological typing of *Staphylococcus aureus* strains from ovine and rabbit mastitis. Inter J Antimicrob Agent. 23(2004):268–272.

Gortel, K., Campbell, K.L., Kakoma, I., Whittem, T., Schaeffer, D.J. and Weisiger, R.M., 1999. Methicillin resistance among staphylococci isolated from dogs. Am J Vet Res. 60(12):1526–1530.

Griffeth, G.C., Morris, D.O., Abraham, J.L., Shofer, F.S. and Rankin, S.C. 2008. Screening for skin carriage of methicillin-resistant coagulase-positive staphylococci and *Staphylococcus schleiferi* in dogs with healthy and inflamed skin. Vet Dermatol. 19(3):142–149.

Guardabassi, L., Loeber, M., Jacobson, A. 2004. Transmission of multiple antimicrobial-resistant Staphylococcus intermedius between dogs affected by deep pyoderma and their owners. Vet. Microbiol. 98(1):23–27.

Guardabassi, L., Houser, G.A., Frank L.A. and Papich, M.G. 2008. Guidelines for antimicrobial use in dogs and cats. Guidelines for Antimicrobial Use in Animals. L. Guardabassi, L.B. Jensen,and H. Kruse (eds.). Oxford, UK: Wiley-Blackwell. 183-206.

Hackbarth C, Kocagoz T, Kocagoz S, Chambers H. Point mutations in *Staphylococcus aureus* PBP 2 gene affect penicillin-binding kinetics and are associated with resistance. Antimicrob Agents Chemother. 39(1995):103-6.

Hájek, V. 1976. *Staphylococcus intermedius*, a new species isolated from animals. Int. J. Syst. Bacteriol. 26(1976):401–408.

Hanselman, B.A., Kruth, S. and Weese, J.S. 2007. Short communication : Methicillin-resistant staphylococcal colonization in dogs entering a veterinary teaching hospital. Vet Microb. 126(2008):277–281.

Harmsen, D., and Karch, H. 2004. 16S rDNA for diagnosing pathogens: a living tree. ASM News. 70:19–24.

Hartmann, F., Trostle, S.S., and Klohnen, A.A.O. 1997. Isolation of a methicillin-resistant *Staphylococcus aureus* from a postoperative wound infection in a horse. J Amer Vet Med Assoc. 211(5):590–592.

Henze, U. and Berger-Bachi, B. 1996. Penicillin-binding protein 4 overproduction increase beta-lactam resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 40(1996):2121-5.

Henze, U., Sidow, T., Wecke, J., Labischinski, H. and Berger-Bächi, B. 1993. Influence of *femB* on methicillin resistance and peptidoglycan metabolism in *Staphylococcus aureus*. J Bacterio. 175(6):1612-1620.

Hill, P.B., Lo, A., Eden, C. A., Huntley, S., Morey, V., Ramsey, S., Richardson, C., Smith, D.J., Sutton, C., Taylor, M. D., Thorpe, E., Tidmarsh, R., and Williams, V. 2006. Survey of the prevalence, diagnosis and treatment of dermatological conditions in small animals in general practice. Vet Rec. 158(16):533–539.

Hiramatsu, K.1995. Molecular evolution of MRSA. Microbiol Immunol. 39(8):531-43.

Hiramatsu, K., Cui, L., Kuroda, M. and Ito, T. 2001. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 9(10):486-93.

Igimi, S., Takahashi, E. and Mitsuoka T. 1990. *Staphylococcus schleiferi* subsp. *coagulans* subsp. nov., Isolated from the External Auditory Meatus of Dogs with External Ear Otitis. Int J Syst Bacteriol. 40(4):409-411.

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsuminoto, K., Tiensasitorn, C. and Hiramatsu, K. 2001. Structural comparison of three types of staphylococcal cassette chromosome mec intergrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chromother. 45(5):1323-36.

Ito, T., Okuma, K., Ma, XX., Yuzawa, H. and Hiramatsu, K. 2003. Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. Microb Drug Resist. 6(1):41-52.

Maddison, J., Page, S. and Church, D. 2002. Small Animal clinical Pharmacology. London:Harcourt Publishers.115-148.

Jaffe, W., Sweeney, H.M. and Nathan, C. 1980. Identity and interspecific transfer of gentamicin-resistance plasmids in *Staphylococcus aureus* and *Staphylococcus epidermidis*. J Infect Dis. 141(6):738–47.

Jones, R.D., Kania, S.A., Rohrbach, B.W., Frank, L.A. and Bemis, D.A. 2007. Prevalence of oxacillin- and multidrug-resistant staphylocci in clinical samples from dogs: 1,772 samples(2001-2005). J Am Vet Med Assoc. 230(2):221-7.

Kania, S., Williamson, N., Frank, L., Wilkes, R., Jones, R. and Bemis, D. 2004. Methicillin resistance of staphylococci isolated from the skin of dogs with pyoderma. Am J Vet Res. 65(9):1265–1268.

Kelly, L.M., Jacobs, M.R. and Appelbaum, P.C. 1999. Comparison of Agar Dilution, Microdilution, E-Test, and Disk Diffusion Methods for Testing Activity of Cefditoren against *Streptococcus pneumonia*. J Clin Microbiol. 37(10):3296–3299.

Kernodle, D.S. 2000. Mechanisms of resistance to beta-lactam antibiotics. In Gram-positive pathogens. V.A. Fischetti, R.P. Novick, J.J. Ferretti, D.A. Portnoy, and J.I. Rood, editors. American Society for Microbiology. Washington, DC, USA. 609–620.

Kloos, W.E. and Bannerman, T.L. 1997. Staphylococcus and Micrococcus. Manual of Clinical Microbiology. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (eds.). 7th ed. Washington DC, USA : Society for Microbiology. 264-282.

Kolbert, C.P. and Persing. D.H. 1999. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Curr Opin Microbiol. 2(3):299–305.

Kovacevic, S., Veal, L.E., Hsiung, H.M. and Miller, J.R. 1985. Secretion of staphylococcal nuclease by Bacillus subtilis. J Bacteriol. 162(2):521-528.

Kuwahara-Arai, K., Kondo, N., Hori, S., Tateda-Suzuki, E. and Hiramatsu, K. 1996. Suppression of methicillin resistance in a *mecA*-containing pre-methcillin-resistant *Staphylococcus aureus* strain is caused by the *mecI*-mediated repression of PBP 2' production. Antimicrob Agents Chemother. 40(12):2680-5.

Lee, J. 1994. *Staphylococcus intermedius* isolated from dog-bite wounds. J Infect. 29(1):105.

Lee, J.H. 2003. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appli and Env Microbiol. 69(11):6489–6494.

Leonard F. C. and Markey B. K. 2008. Meticillin-resistant *Staphylococcus aureus* in animals: A review. Vet J. 175(1) : 27–36.

Loeffler, A., Boag, A., Sung, J., Lindsay, J., Guardabassi, L., Dalsgaard, A., Smith, H., Stevens, K. and Lloyd, D. 2005. Prevalence of methicillinresistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. J Antimicrob Chemother. 56(4):692–697.

Lowy, F.D. 2003. Antimicrobial resistance: the example of *Staphylococcus aureus*. J Clin Invest. 111(9):1265–1273.

Lu, W., Sun, Y., Bauer, M., Paule, S., Koenigs, P. and Kraft, W. 1999. Penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus*: kinetic characterization of its interactions with beta-lactams using electrospray mass spectrometry. Biochem. 38(20):6537-46.

Naas, T., Coignard, B., Carbonne, A., Blanckaert, K., Bajolet, O., Bernet, C., Verdeil, X., Astagneau, P., Desenclos, J.-C. and Nordmann, P. 2006. VEB-1 Extended-Spectrum beta-lactamase–producing *Acinetobacter baumannii*, France. Emerg Infect Disease. 12(8):1214-1222.

Naidoo, J. 1984. Interspecific co-transfer of antibiotic resistance plasmids in staphylococci in vivo. J Hyg. 93(1):59–66.

Machado, A.B.M.P., Reiter, K.C., Paiva, R.M. and Barth, A.L. 2007. Distribution of staphylococcal cassette chromosome *mec* (SCC*mec*) types I, II, III and IV in coagulase-negative staphylococci from patients attending a tertiary hospital in southern Brazil. J Med Microbiol. 56(2007):1328-1333.

Mahoudeau, I., Delabranche, X., Prevost, G., Monteil, H., Piemont, Y., 1997. Frequency of isolation of Staphylococcus intermedius from humans. J Clin Microbiol. 35(8) : 2153–2154.

Mascaretti, O.A. 2003. Bacteria versus antibacterial agents. 1st ed. Washington DC, USA:American Society for Microbiology. 97-137.

Medleau, L., Long, R.E., Brown, J., Miller, W.H., 1986. Frequency and antimicrobial susceptibility of Staphylococcus species isolated from canine pyodermas. Am J Vet Res. 47(2):229–231.

McDougal, L. and Thornsberry, C. 1986. The role of beta-lactamase in staphylococcal resistance to penicillin-resistant penicillin and cephalosporins. J Clin Microbiol. 23(5):823-9.

Morris, D., Rook, K., Shofer, F. and Rankin, S. 2006. Screening of *Staphylococcus aureus*, *Staphylococcus intermedius*, and *Staphylococcus schleiferi* isolates obtained from small companion animals for antimicrobial resistance: a retrospective review of 749 isolates (2003–04). Vet Dermatol. 17(5): 332–337.

Palys, T., L. K. Nakamura and F.M. Cohan. 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int J Syst Bacteriol. 47(4):1145–1156.

Pak, S.I., Han, H.R. and Shimizu, A., 1999. Characterization of methicillin resistant *Staphylococcus aureus* isolated from dogs in Korea. J Vet Med Sci. 61(9):1013–1018.

Penny, R.H.C. and Muirhead, M.R. 1981. Skin. In: Diseases of swine,ed. Leman AD, Glock RD, Mengeling WL, et al., 5th ed., Iowa State University Press, Ames, IA. 81.

Piriz, S., Valle, J., Mateos, E.M., de la Fuente, R., Cid, D., Ruiz-Santaquiteria, J.A. and Vadillo, S. 1996. In vitro activity of fifteen antimicrobial agents against methicillin-resistant and methicillin-susceptible Staphylococcus intermedius. J Vet Pharmacol Ther. 19(2):118–123.

Rowland, S.J. and Dyke, K.G. 1989. Characterization of staphylococcal beta-lactamase transposon Tn*552*. EMBO J. 8(9):2761–2773.

Ruscher, C., Lübke-Becker, A., Wleklinski, C.G., Soba, A., Wieler, L.H. and Walther, B. 2009. Short communication : Prevalence of Methicillin-resistant *S. pseudintermedius* isolated from clinical samples of companion animals and equidaes. Vet Microb. 136(2009):197–201.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S. and Hiramatsu, K. 2007a. Methicillin-resistant *Staphylococcus pseudintermedius* in a veterinary teaching hospital. J Clin Microbiol. 45(4):1118–1125.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S. and Hiramatsu, K. 2007b. Reclassification of phenotypically-identified Staphylococcus intermedius strains. J Clin Microbiol. 45(9):2770–2778.

Schwarz, S., Kadlec, K. and Strommenger, B. 2008. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* detected in the BfT-GermVet monitoring programme 2004–2006 in Germany. J Antimicrob Chemother. 61(2): 282–285.

Skinner, S., Inglis, B., Mattews, P. and Stewart, P. 1988. Mercury and tetracycline resistance gene and flanking repeats assocociated with methicillin resistance determinant in *Staphylococcus aureus*. Mol Microbiol. 2(2):289-92.

Skov R., Smyth, R., Clausen, M., Larsen, A.R., Frimodt-Møller, N., Olsson-Liljequist , B., and Kahlmeter, G. 2003. Evaluation of a cefoxitin 30 µg disc on Iso-Sensitest agar for detection of methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother. 52(2):204-7.

Songer, J.G. and Post, K.W. 2004. Veterinary Microbiology Bacterial and Fungal Agents of Animals Disease. China: Elsevier Saunders. 35-42.

Stewart ,P., Dubin, D., Chikramane, S., Inglis, B., Mathews, P. and Poston, S. 1994. IS257 and small plasmid insertion in the mec region of the chromosome of *Staphylococcus aureus*. Plasmid. 31(1):12-20.

Strommenger, B., Kettlitz, C., Werner, G., Witte, W. 2003. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. J Clin Microbiol. 41(9):2480–2482.

Talan, D.A., Goldstein, E.J., Staatz, D. and Overturf, G.D. 1989a. *Staphylococcus intermedius*: clinical presentation of a new human dog bite pathogen. Ann Emerg Med. 18(4):410–413.

Talan, D.A., Staatz, D., Staatz, A. and Overturf, G.D.,1989b. Frequency of *S. intermedius* as human nasopharyngeal flora. J Clin Microbiol. 27(10):2393.

Tomasz, A., Drugeon, H., de Lencastre, H., Jabes, D., McDougall, L. and Bille, J. 1989. New mechanism for methicillin resistance in Staphylococcus aureus:clinical

isolates that lack the PBP2a gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob Agents Chemother. 33(1989):1869-74.

Tortoli, E. 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev. 16(2):319–354.

Tucker, P. W., Hazen, E.E. and Cotton, F. A. 1978. Staphylococcal nuclease reviewed: a prototypic study in contemporary enzymology. I. Isolation, physical and enzymatic properties. Mol Cell Biochem. 22(2-3):67-77.

Ubukata, K., Nonoguchi, M., Song, M., Matsuhashi, M. and Konno, M. 1990. Homology of *mecA* gene in methicillin-resistant *Staphylococcus haemolyticus* and *Staphylococcus simulans* to that of *Staphylococcus aureus*. Antimicrob Agents Chemother. 34(1):170-2.

Udo, E.E., Jacob, L.E. and Mokadas, E.M. 1997. Conjugative transfer of high-level mupirocin resistance from *Staphylococcus haemolyticus* to other staphylococci. Antimicrob Agents Chemother. 41(3): 693–5.

van Duijkeren, E., Box, A.T.A., Heck, M.E., Wannet, W.J. and Fluit, A.C. 2004. Methicillin-resistant staphylococci isolated from animals. Vet Microbiol. 103(1-2):91–97.

van Duijkeren, E. and Houwers, D.J. 2008. Transmission of methicillin-resistant *Staphylococcus intermedius* between humans and animals. Vet Microbiol. 128 (1–2):213–215.

van Hoovels, L., Vankeerberghen, A., Boel, A., Van Vaerenbergh, K. and De Beenhouwer, H., 2006. First case of *Staphylococcus pseudintermedius* infection in a human. J Clin Microbiol. 44(12):4609–4612.

Vengust, M., Anderson, M., Rousseau, J., Weese, J., 2006. Methicillin-resistant staphylococcal colonization in clinically normal dogs and horses in the community. Lett Appl Microbiol. 43(6):602–606.

Vintov, J., Aarestrup, F.M., Zinn, C.E. et al. 2003. Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. Vet Microbiol. 95(1-2): 133–47.

Voss, A., Loeffen, F., Bakker, J., Klaassen, C., Wulf, M., 2005. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg Infect Dis. 11(12):1965–1966.

Watts, J.L. and Yancey, R.J. 1994. Identification of veterinary pathogens by use of commercial identification systems and new trends in antimicrobial susceptibility testing of veterinary pathogen. Clin Microbiol Rev. 7(3):346-356.

Weese, J.S., Dick, H., Willey, B.M., McGeer, A., Kreiswirth, B.N., Innis, B. and Low, D.E. 2006. Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. Vet Microb. 115(1-3):148–155.

Weese, J.S. and van Duijkeren, E. 2009. Methicillin-resistant *S. aureus* and *S. pseudintermedius* in veterinary medicine. Vet Microbiol. (2009).

Werckenthin, C., Cardoso, M., Martel, J.L. and Schwarz, S. 2001. Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus*, and canine *Staphylococcus intermedius.* Vet Res. 32(3-4):341–362.

Wettstein, K., Descloux, S., Rossano, A., Perreten, V., 2008. Emergence of methicillin-resistant Staphylococcus pseudintermedius in Switzerland: three cases of urinary tract infections in cats. Schweiz Arch Tierheilkd. 150(7): 339–343.

Wielders, C.L., Vriens, M.R., Brisse, S., de Graaf-Miltenburg, L.A., Troelstra, A., Fleer, A., Schmitz, F.J., Verhoef, J. and Fluit, A.C. 2001. In-vivo transfer of mecA DNA to *Staphylococcus aureus*. 26(357):1674-5.

Wu, C., Hoskins, J., Blaszczak, L., Preston, D. and Skatrud, P. 1992. Construction of a water soluble form of penicillin-binding protein 2a from a methcillin-resistant *Staphylococcus aureus* isolate. Antimicrob Agents Chemother. 36(3):533-9.

Wu, S., Pisciteli, C., de Lencastre, H. and Tomasz, A. Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*. Microb Drug Resist. 2(4):435-41.

Yamashita, K., Shimizu, S., Kawano, J., Uchida, E., Haruna, A. and Igimi, S. 2005. Isolation and characterization of Staphylococci from external auditory meatus of dogs with or without otitis externa with special reference to *Staphylococcus schleiferi* subsp*. coagulans* isolates. J Vet Med Sci. 67(3): 263–268.

Zhu, L.X., Zhang, Z.W., Wang, C., Yang, H.W., Jiang, D., Zhang, Q., Mitchelson, K., and Cheng, J. 2007. Simultaneous Detection of Antibiotic Resistance Genes among Staphylococcal Clinical Isolates with a DNA Microarray. J Clin Microbiol. 45(11):3514-21.

Zubeir, I., Kanbar, T., Alber, J., Lammler, C., Akineden, O., Weiss, R., Zschöck, M., 2007. Phenotypic and genotypic characteristics of methicillin/oxacillin-resistant *Staphylococcus intermedius* isolated from clinical specimens during routine veterinary microbiological examinations. Vet Microbiol. 121(1-2):170–176.

Appendices

Appendix A

Table A-1 100 dog history and hospital number

No.	HN.	Date	Kind of wound
1	5112624	4/8/2008	Otitis externa
2	5008486	4/8/2008	Infectious wond
3	5016607	4/8/2008	Otitis externa
4	5009041	4/8/2008	Otitis externa
5	5110206	5/8/2008	Abcess
6	5109127	6/8/2008	Otitis externa
7	5112779	6/8/2008	Abcess
8	4919990	7/8/2008	Abcess
9	5110020	7/8/2008	Otitis externa
10	5112209	7/8/2008	Abcess
11	4903956	13/8/2008	Otitis externa
12	4915372	25/8/2008	Otitis externa
13	5113262	27/8/2008	Otitis externa
14	5110273	27/8/2008	Infectious wound
15	5113903	28/8/2008	Otitis externa
16	5106890	1/9/2008	Otitis externa
17	5110108	2/9/2008	Otitis externa
18	4907077	3/9/2008	Otitis externa
19	5010124	4/9/2008	Otitis externa
20	5111128	7/9/2008	Otitis externa
21	5114367	7/9/2008	Otitis externa
22	5114394	9/9/2008	Otitis externa
23	5014908	11/9/2008	Infectious wound
24	4901953	12/9/2008	Infectious wound in ear
25	5111066	15/9/2008	Otitis externa
26	5106146	15/9/2008	Infectious wound
27	5113408	16/9/2008	Pyoderma

No.	HN.	Date	Kind of wound
28	5114817	16/9/2008	Infectious wound
29	4912096	16/9/2008	Otitis externa
30	5115010	18/9/2008	Infectious wound
31	5011638	19/9/2008	Otitis externa with skin infection
32	4911953	19/9/2008	Infectious wound
33	4908363	19/9/2008	Infectious wound
34	5115102	19/9/2008	Infectious wound
35	5114446	22/9/2008	Otitis externa
36	5115236	22/9/2008	Otitis externa
37	5115250	23/9/2008	Otitis externa
38	5115309	23/9/2008	Otitis externa
39	5115413	25/9/2008	Otitis externa
40	5111504	25/9/2008	Infectious wound
41	5115519	26/9/2008	Pyoderma
42	5115659	29/9/2008	Infectious wound
43	5020168	2/10/2008	Infectious wound
44	5115871	2/10/2008	Infectious wound
45	4901538	6/10/2008	Infectious wound
46	4914122	6/10/2008	Infectious wound
47	5115054	9/10/2008	Infectious wound
48	5115944	9/10/2008	Infectious wound
49	5116243	9/10/2008	Otitis externa
50	5116251	9/10/2008	Infectious wound
51	5115935	9/10/2008	Infectious wound
52	5114406	10/10/2008	Infectious wound
53	5113994	10/10/2008	Infectious wound
54	5116235	10/10/2008	Otitis externa
55	4916703	15/10/2008	Infectious wound

No.	HN.	Date	Kind of wound
56	5110600	15/10/2008	Infectious wound
57	5116171	15/10/2008	Infectious wound
58	5116610	17/10/2008	Infectious wound
59	5116550	17/10/2008	Infectious wound
60	5116962	22/10/2008	Abcess
61	5116579	22/10/2008	Infectious wound
62	5116240	26/10/2008	Otitis externa
63	5112727	26/10/2008	Infectious wound
64	5018504	28/10/2008	Infectious wound
65	5117233	29/10/2008	Infectious wound
66	4915284	29/10/2008	Infectious wound
67	5117384	30/10/2008	Infectious wound
68	5111774	30/10/2008	Otitis externa
69	5117652	3/11/2008	Infectious wound
70	5117665	3/11/2008	Infectious wound
71	4900094	4/11/2008	Infectious wound
72	5117788	6/11/2008	Infectious wound
73	5001914	7/11/2008	Otitis externa
74	5105564	7/11/2008	Otitis externa
75	5114622	10/11/2008	Infectious wound
76	5105082	12/11/2008	Infectious wound
77	5118184	12/11/2008	Otitis externa
78	5118177	12/11/2008	Otitis externa
79	5116202	13/11/2008	Infectious wound
80	5116699	14/11/2008	Infectious wound
81	5110111	17/11/1951	Otitis externa
82	5114622	17/11/1951	Infectious wound
83	5116203	17/11/1951	Infectious wound

No.	HN.	Date	Kind of wound
84	4918153	18/11/1951	Infectious wound
85	4917304	18/11/1951	Infectious wound
86	5102677	19/11/1951	Abcess
87	5118483	19/11/1951	Infectious wound
88	5021912	19/11/1951	Otitis externa
89	4912148	20/11/1951	Otitis externa
90	5117293	20/11/1951	pyoderma
91	4906247	24/11/1951	Otitis externa
92	5104734	25/11/1951	Otitis externa
93	4901460	26/11/1951	Infectious wound
94	4903956	25/11/1951	Otitis externa
95	5118825	27/11/1951	Infectious wound
96	4907431	27/11/1951	Infectious wound
97	5107749	27/11/1951	Infectious wound
98	5110170	27/11/1951	Infectious wound
99	5119032	27/11/1951	Otitis externa
100	5119026	28/11/1951	Infectious wound

Table A-2 Coagulase positive Staphylococci drug history and disease

No.	SEX	breed	duration	Antibiotic drug	Steriod Drug	Pre- disease
3	M	GR	1 day	no	no	no.
6	M	mixed	1 day	no	no	no.
11	M	Bangkeaw	long term	gentamicin, SXT, fluoroquinolone,	metronidazole	Recurrent otitis externa
12	M	GR	long term	AMC, DA, norfloxacin	prednisolone	Skin disease
19	F	LR	long term	doxycycline	prednisolone	Skin disease
22	M	mixed	1 day	no	No	no.
24	M	mixed	long term	AMC, doxycycline, bactacin®, amoxycilin	prednisolone	Skin disease
25	M	mixed	30 day	doxycycline	no	no.
27	M	mixed	30 day	gentamicin,	metronidazole	no.
29	F	mixed	1 day	no	no	no.
30	F	mixed	1 day	no	no	no.
31	F	Cocker	30 day	SXT	no	no.
32	M	mixed	1 day	no	no	no.
39	M	Poodle	1 day	AMC	no	no.
40	F	mixed	12 day	bactrim®, SXT	no	no
41	M	Pug	1 day	no	no	Skin disease
43	M	mixed	2 day	no	no	no
44	M	mixed	2 day	no	no	no
47	M	Chizu	20 day	doxycycline, norfloxacin	no	no
49	M	Poodle	1 day	no	no	no
51	M	M.P.	6 day	no	no	cancer
53	F	Poodle	30 day	SXT, enrofloxacin	no	pyoderm
54	M	GR	3 day	AMC	no	no
56	M	mixed	1 day	no	no	no
57	F	LR	8 day	SXT, bactacin®	no	no
61	F	Poodle	10 day	bactacin®	no	no
62	F	Poodle	17 day	no	no	cancer

No.	SEX	breed	duration	antibiotic drug	Steriod Drug	Pre- disease
63	M	Pug	60 day	AMC	prednisolone,	no
67	M	mixed	1 day	no	no	no
68	M	Poodle	1 day	no	no	no
74	M	Chizu	long term	enrofloxacin, CL	no	no
82	M	Chizu	long term	CL	no	Skin disease
83	M	Chiwawa	1 day	no	no	no
88	M	mixed	1 day	no	no	no
92	M	Poodle	1 day	no	no	no
100	M	mixed	2 day	no	no	no

SXT = Co-trimoxazole

CL = Cefalexcin

AMC = Co-amoxiclav

DA = Clindamycin

Table A-3 result of all coagulase positive staphylococcus test

Dogs No.	isolate No.	Gram.	mannital+	catalase	coagulase test	V.P.	16S rRNA	SA	SI
1	N1	+ cocci	pos.	pos.	pos.	pos.	pos.	pos.	neg.
2	N3	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
3	N6	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
4	N11	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
5	N12	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
6	O19	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
7	N22	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
8	N24	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
9	W24	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
10	O25	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
11	N27	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
12	W27	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
13	N29	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
14	O29	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
15	N30	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
16	N31	+ cocci	pos.	pos.	pos.	pos.	pos.	neg.	pos.
17	N32	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
18	W32	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
19	N39	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
20	O39	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
21	W40	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
22	W41	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
23	W43	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
24	W44	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
25	N47	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
26	W47	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
27	N49	+ cocci	pos.	pos.	pos.	pos.	pos.	pos.	neg.
28	W49	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
29	N51	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
30	W51	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
31	W53	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
32	N54	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
33	N56	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
34	W57	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
35	N60	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	neg.
36	N61	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
37	N62	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
38	N63	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.

Dogs No.	isolate No.	Gram.	mannital+	catalase	coagulase test	V.P.	16S rRNA	SA	SI
39	W63	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	neg.
40	W67	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
41	N68	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
42	O68	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
43	N74	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
44	W82	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
45	W83	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
46	O88	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	pos.
47	O89	+ cocci	pos.	pos.	pos.	neg.	pos.	neg.	neg.
48	O92	+ cocci	pos.	pos.	pos.	pos.	pos.	pos.	neg.
49	N100	+ cocci	pos.	pos.	pos.	pos.	pos.	neg.	neg.

pos. = positive

neg. = negative

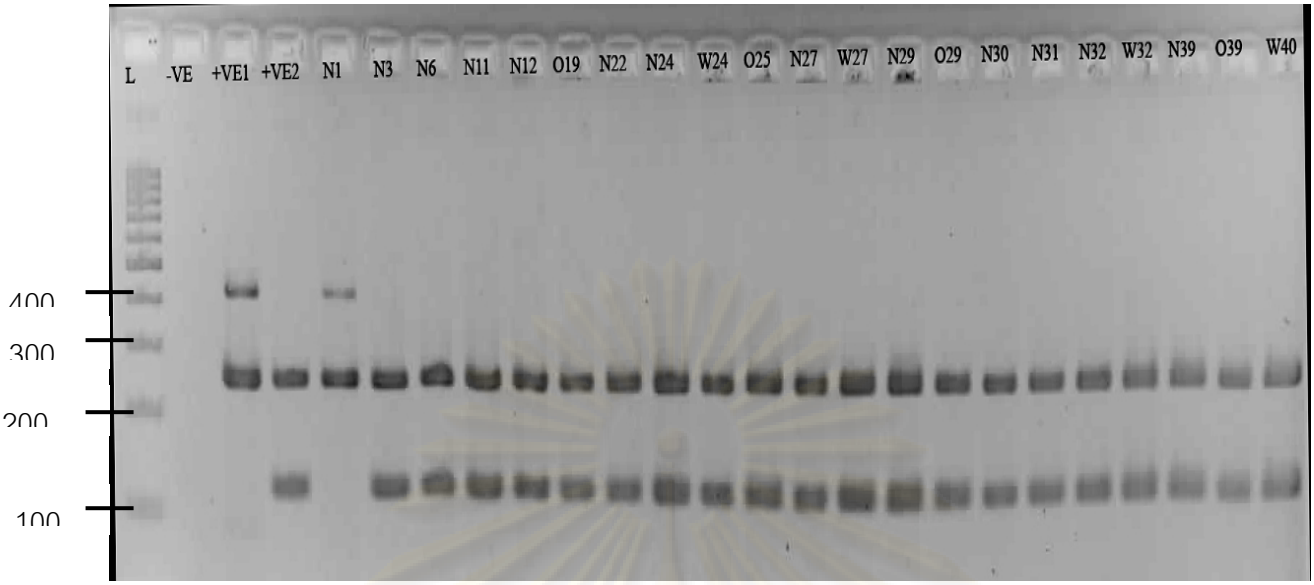


Figure A-1 PCR production of coagulase positive *Staphylococcus spp* .

Lane	Isolated	*16s rRNA*	*SAnuc*	*SInuc*	Lane	Isolated	*16s rRNA*	*SAnuc*	*SInuc*
1	100 bp DNA ladder	N/A	N/A	N/A	13	W24	positive	negative	positive
2	negative control	N/A	N/A	N/A	14	O25	positive	negative	positive
3	*SAnuc* positive control	positive	positive	negative	15	N27	positive	negative	positive
4	*SInuc* positive control	positive	negative	positive	16	W27	positive	negative	positive
5	N1	positive	positive	negative	17	N29	positive	negative	positive
6	N3	positive	negative	positive	18	O29	positive	negative	positive
7	N6	positive	negative	positive	19	N30	positive	negative	positive
8	N11	positive	negative	positive	20	N31	positive	negative	positive
9	N12	positive	negative	positive	21	N32	positive	negative	positive
10	O19	positive	negative	positive	22	W32	positive	negative	positive
11	N22	positive	negative	positive	23	N39	positive	negative	positive
12	N24	positive	negative	positive	24	O39	positive	negative	positive
					25	W40	positive	negative	positive

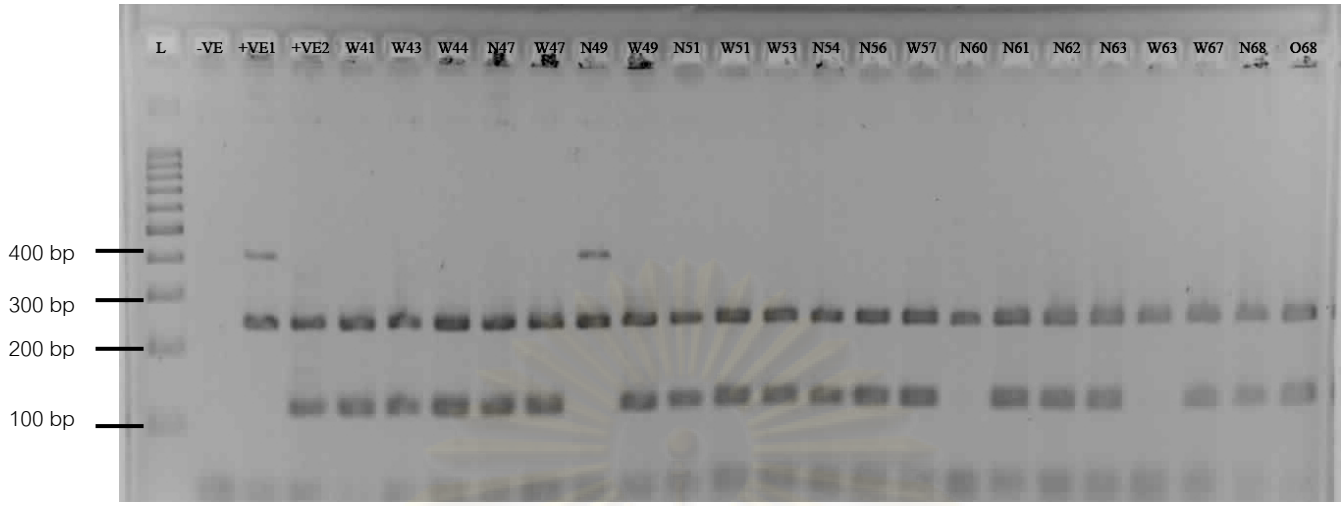


Figure A-2 PCR production of coagulase positive *Staphylococcus spp.* (continue)

Lane	Isolated	*16s rRNA*	*SAnuc*	*SInuc*	Lane	Isolated	*16s rRNA*	*SAnuc*	*SInuc*
1	1 kb DNA ladder plus	N/A	N/A	N/A	13	W53	positive	negative	positive
2	negative control	N/A	N/A	N/A	14	N54	positive	negative	positive
3	*SAnuc* positive control	positive	positive	negative	15	N56	positive	negative	positive
4	*SInuc* positive control	positive	negative	positive	16	W57	positive	negative	positive
5	W41	positive	negative	positive	17	N60	positive	negative	negative
6	W43	positive	negative	positive	18	N61	positive	negative	positive
7	W44	positive	negative	positive	19	N62	positive	negative	positive
8	N47	positive	negative	positive	20	N63	positive	negative	positive
9	W47	positive	negative	positive	21	W63	positive	negative	negative
10	N49	positive	positive	negative	22	W32	positive	negative	positive
11	W49	positive	negative	positive	23	W67	positive	negative	positive
12	N51	positive	negative	positive	24	N68	positive	negative	positive
					25	W68	positive	negative	positive

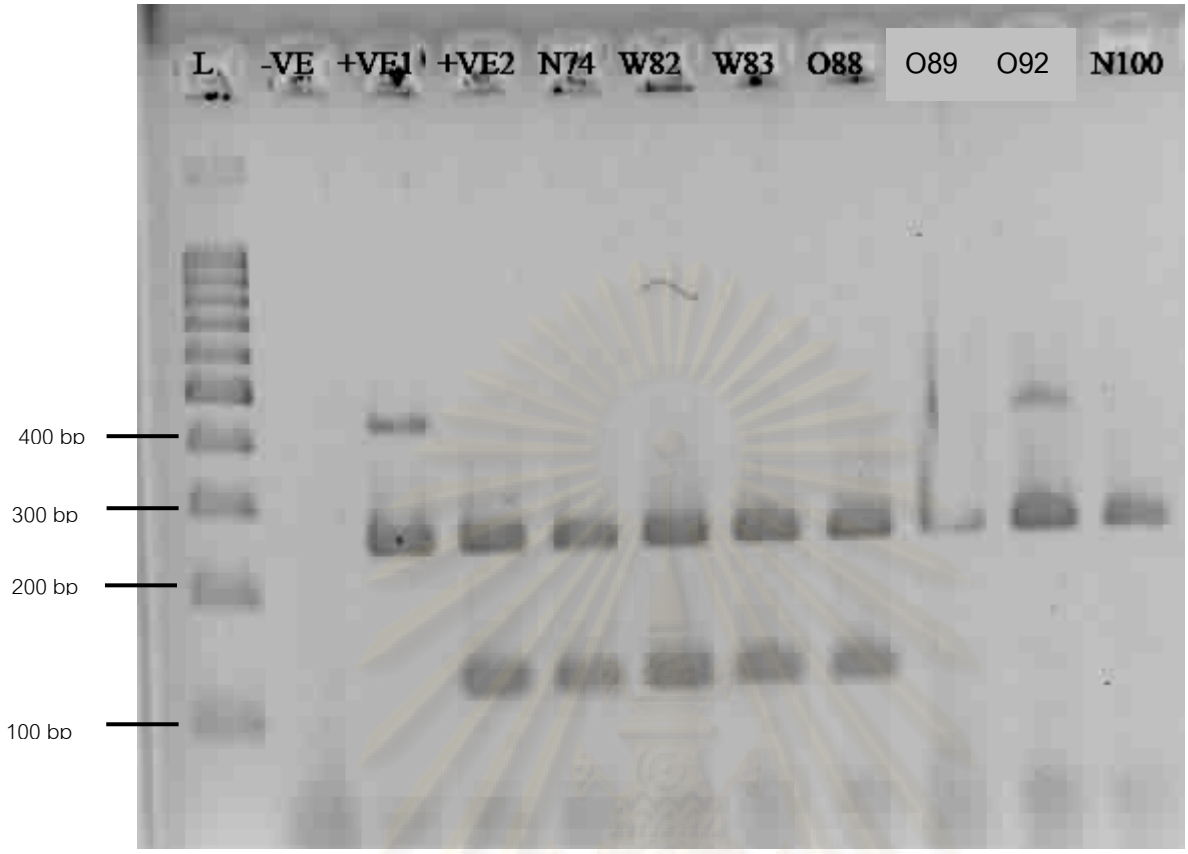


Figure A-3 PCR production of coagulase positive *Staphylococcus spp.* (continue)

Lane	Isolated	*16s rRNA*	*SAnuc*	*SInuc*
1	100 bp DNA ladder	N/A	N/A	N/A
2	negative control	N/A	N/A	N/A
3	*SAnuc* positive control	positive	positive	negative
4	*SInuc* positive control	positive	negative	positive
5	N74	positive	positive	negative
6	W82	positive	negative	positive
7	W83	positive	negative	positive
8	O88	positive	negative	positive
9	O89	positive	negative	positive
10	O92	positive	positive	negative
11	N100	positive	negative	positive

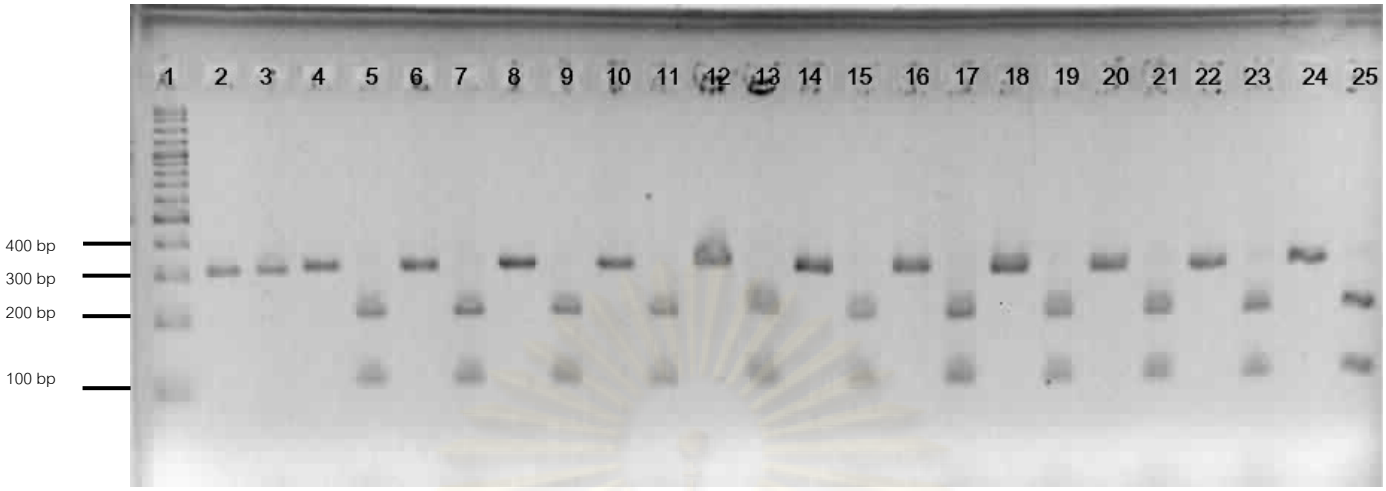


Figure A-4 All positive *SInuc* gene were confirmed by *pta* gene and cut by restriction enzyme *Mbo*I.

Lane	Isolated	320 bp	216 bp	107 bp	Lane	Isolated	320 bp	216 bp	107 bp
1	100 bp DNA ladder	N/A	N/A	N/A	13	O19 (C)	negative	positive	positive
2	positive control (U)	positive	negative	negative	14	N22 (U)	positive	negative	negative
3	positive control (C)	positive	negative	negative	15	N22 (C)	negative	positive	positive
4	N3 (U)	positive	negative	negative	16	N24 (U)	positive	negative	negative
5	N3 (C)	negative	positive	positive	17	N24 (C)	negative	positive	positive
6	N6 (U)	positive	negative	negative	18	W24 (U)	positive	negative	negative
7	N6 (C)	negative	positive	positive	19	W24 (C)	negative	positive	positive
8	N11 (U)	positive	negative	negative	20	O25 (U)	positive	negative	negative
9	N11 (C)	negative	positive	positive	21	O25 (C)	negative	positive	positive
10	N12 (U)	positive	negative	negative	22	N27 (U)	positive	negative	negative
11	N12 (C)	negative	positive	positive	23	N27 (C)	negative	positive	positive
12	O19 (U)	positive	negative	negative	24	W27 (U)	positive	negative	negative
					25	W27 (C)	negative	positive	positive

U = uncut PCR product

C = cut PCR product

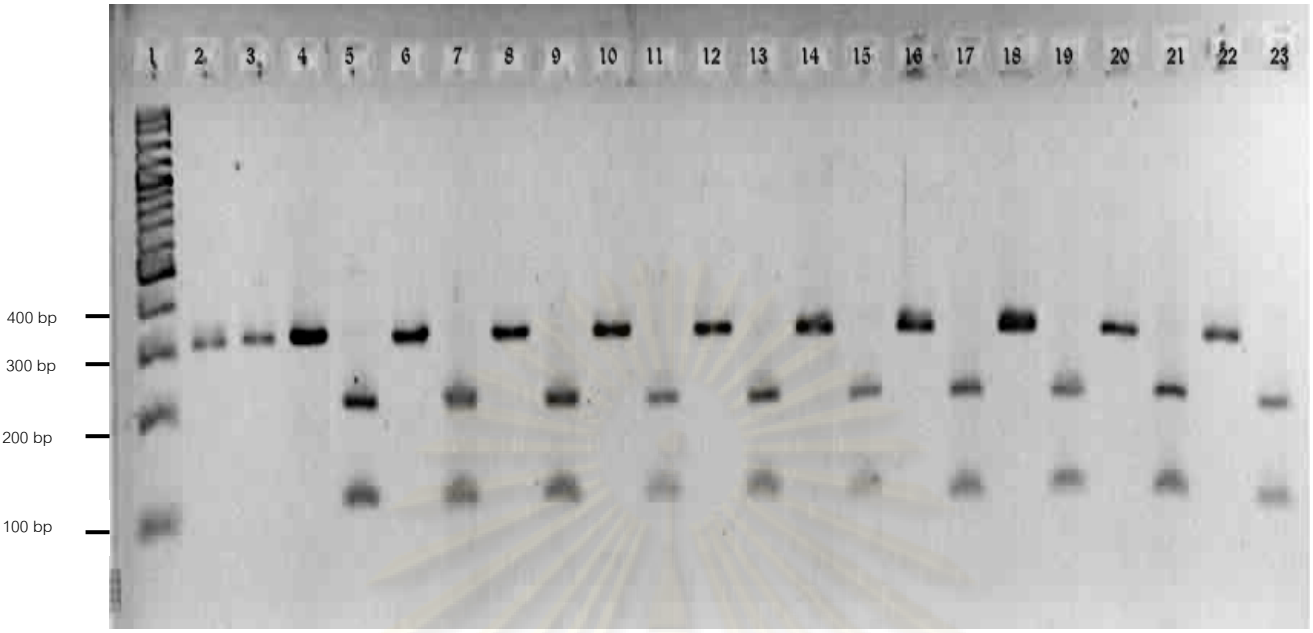


Figure A-5 All positive *SInuc* gene were confirm by pta gene and cut by restriction enzyme *Mbo*I (continued).

Lane	Isolated	320 bp	216 bp	107 bp	Lane	Isolated	320 bp	216 bp	107 bp
1	100 bp DNA ladder	N/A	N/A	N/A	13	N32 (C)	negative	positive	positive
2	positive control (U)	positive	negative	negative	14	W32 (U)	positive	negative	negative
3	positive control (C)	positive	negative	negative	15	W32 (C)	negative	positive	positive
4	N29 (U)	positive	negative	negative	16	N39 (U)	positive	negative	negative
5	N29 (C)	negative	positive	positive	17	N39 (C)	negative	positive	positive
6	O29 (U)	positive	negative	negative	18	O39 (U)	positive	negative	negative
7	O29 (C)	negative	positive	positive	19	O39 (C)	negative	positive	positive
8	N30 (U)	positive	negative	negative	20	W40 (U)	positive	negative	negative
9	N30 (C)	negative	positive	positive	21	W40 (C)	negative	positive	positive
10	N31 (U)	positive	negative	negative	22	W41 (U)	positive	negative	negative
11	N31 (C)	negative	positive	positive	23	W41 (C)	negative	positive	positive
12	N32 (U)	positive	negative	negative					

U = uncut PCR product

C = cut PCR product

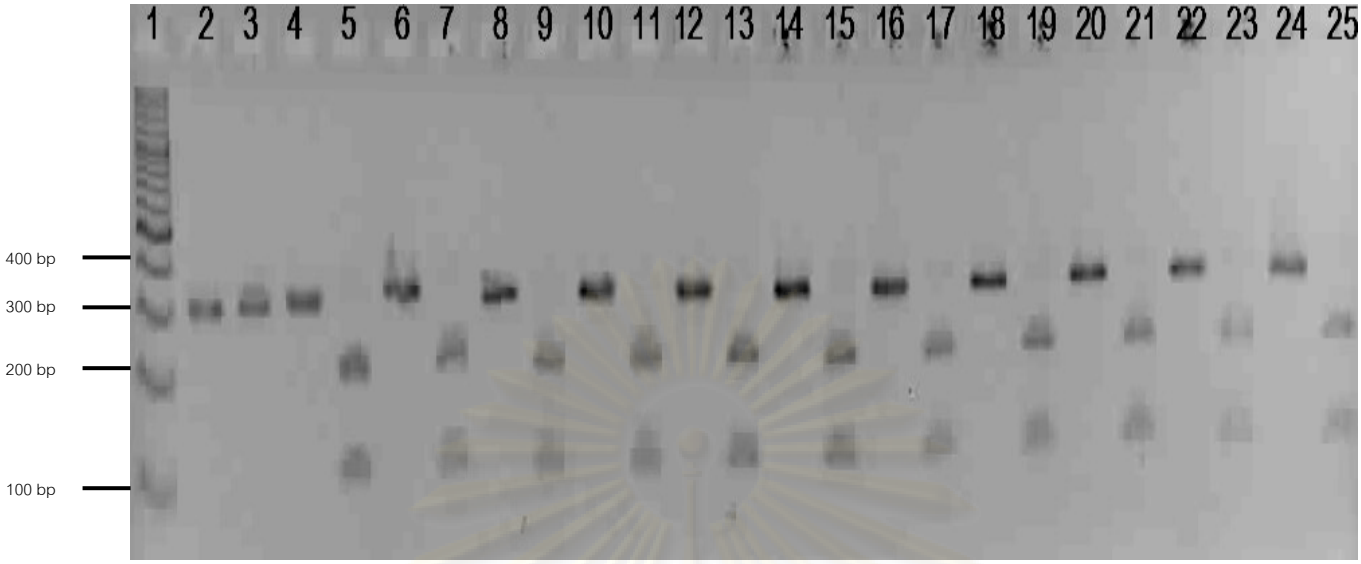


Figure A-6 All positive *SInuc* gene were confirm by pta gene and cut by restriction enzyme *Mbo*I (continued).

Lane	Isolated	320 bp	216 bp	107 bp	Lane	Isolated	320 bp	216 bp	107 bp
1	100 bp DNA ladder.	N/A	N/A	N/A	13	W49 (C)	negative	positive	positive
2	positive control (U)	positive	negative	negative	14	N51 (U)	positive	negative	negative
3	positive control (C)	positive	negative	negative	15	N51 (C)	negative	positive	positive
4	W43 (U)	positive	negative	negative	16	W51 (U)	positive	negative	negative
5	W43 (C)	negative	positive	positive	17	W51 (C)	negative	positive	positive
6	W44 (U)	positive	negative	negative	18	W53 (U)	positive	negative	negative
7	W44 (C)	negative	positive	positive	19	W53 (C)	negative	positive	positive
8	N47 (U)	positive	negative	negative	20	N54 (U)	positive	negative	negative
9	N47 (C)	negative	positive	positive	21	N54 (C)	negative	positive	positive
10	W47 (U)	positive	negative	negative	22	N56 (U)	positive	negative	negative
11	W47 (C)	negative	positive	positive	23	N56 (C)	negative	positive	positive
12	W49 (U)	positive	negative	negative	24	W57 (U)	positive	negative	negative
					25	W57 (C)	negative	positive	positive

U = uncut PCR product

C = cut PCR product

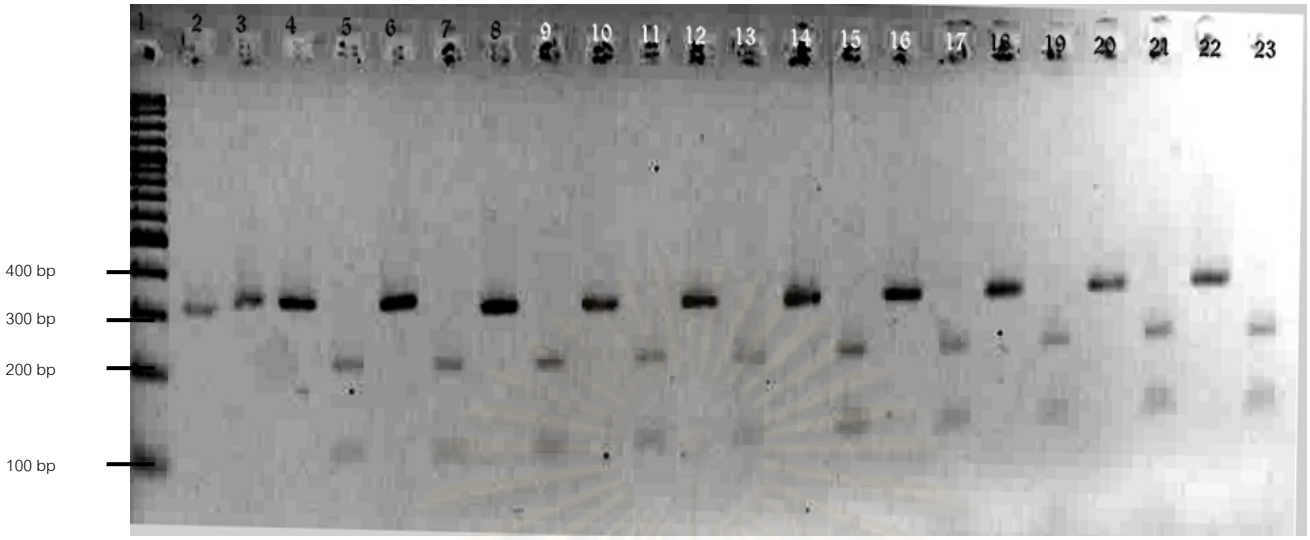


Figure A-7 All positive *SInuc* gene were confirm by pta gene and cut by restriction enzyme *Mbo*I (continued).

Lane	Isolated	320 bp	216 bp	107 bp	Lane	Isolated	320 bp	216 bp	107 bp
1	100 bp DNA ladder	N/A	N/A	N/A	13	N68 (C)	negative	positive	positive
2	positive control (U)	positive	negative	negative	14	O68 (U)	positive	negative	negative
3	positive control (C)	positive	negative	negative	15	O68 (C)	negative	positive	positive
4	N61 (U)	positive	negative	negative	16	N74 (U)	positive	negative	negative
5	N61 (C)	negative	positive	positive	17	N74 (C)	negative	positive	positive
6	N62 (U)	positive	negative	negative	18	W82 (U)	positive	negative	negative
7	N62 (C)	negative	positive	positive	19	W82 (C)	negative	positive	positive
8	N63 (U)	positive	negative	negative	20	W83 (U)	positive	negative	negative
9	N63 (C)	negative	positive	positive	21	W83 (C)	negative	positive	positive
10	W67 (U)	positive	negative	negative	22	O88 (U)	positive	negative	negative
11	W67 (C)	negative	positive	positive	23	O88 (C)	negative	positive	positive
12	N68 (U)	positive	negative	negative					

U = uncut PCR product

C = cut PCR product

Table A-4 time relation with coagulase positive test

isolate No.	coagulase test
N1	+4
N3	+2
N6	+2
N11	+2
N12	+2
N17	0
O19	+2
N22	+2
O22	0
N24	0
W24	+1
O25	+2
N27	+2
W27	+2
N29	+2
O29	+2
N30	+2
N31	+2
N32	+1
W32	+2
N33	0
O35	0
N39	+4
O39	+4
W40	+4
N41	0
W41	+4
W43	+4
W44	+2
N47	+2

isolate No.	coagulase test
W47	+3
N49	+3
O49	+3
N51	+2
W51	+2
W53	+2
N54	+2
O54	0
N55	0
N57	+1
W57	0
N60	0
W60	+1
N62	0
N63	+1
W67	+2
N68	+2
O68	+2
W69	0
N73	0
N74	0
N75	0
N80	0
N82	0
W82	0
N83	0
W83	+1
N84	0
W84	0
N86	0

isolate No.	coagulase test
O88	+4
O89	+4
O92	+1
N93	0
N94	0
N96	0
N97	0
N100	+4

+4 = clot within 4 hour (be hard solid)

+3 = clot within 24 hour (be hard solid)

+2 = clot within 72 hour (be hard solid)

+1 = clot over72 hour (be soft solid)

0 = not clotting

Table A-5 drug sensitivity test

isolate No.	AMC		CIP		DA		CL		MUP		SXT		TE		0X		Cefoxitin	
N1	27.56	S	27.31	S	33.94	S	22.68	R	28.46	S	32.2	S	7.36	R	16.4	S	28.2	S
N3	13.76	S	4	R	8.87	R	4	R	34.79	S	18.03	S	5	R	4	R	24.03	S
N6	14.02	S	4	R	8.02	R	4	R	30.17	S	19.01	S	5.19	R	4	R	23.33	S
N11	15.25	S	4	R	16.43	I	4	R	32.56	S	19.24	S	4.93	R	4	R	25.49	S
N12	13.65	S	33.41	S	4	R	33.82	S	4	R	4	R	5.29	R	4	R	29.29	S
O19	12.38	I	4	R	9.01	R	4	R	31.69	S	19.47	S	4.91	R	4	R	25.28	S
N22	14.17	S	29.93	S	30.32	S	4	R	32.17	S	4	R	4	R	4	R	25.51	S
N24	13.94	S	4	R	17.6	I	4	R	33.22	S	18.85	S	4.56	R	4	R	24.49	S
W24	11.76	I	4	R	14.01	R	4	R	34.69	S	12.14	I	5.6	R	4	R	25.16	S
O25	12.39	I	4	R	12.53	R	4	R	32.74	S	18.97	S	6.12	R	4	R	23.83	S
N27	15.76	S	31.08	S	4	R	4	R	31.32	S	4	R	5.86	R	4	R	28.55	S
W27	14.09	S	32.22	S	4	R	4	R	30.83	S	4	R	4.58	R	4	R	28.43	S
N29	14.22	S	4	R	13.12	R	4	R	34.87	S	19.65	S	5.03	R	4	R	25.42	S
O29	11.45	I	17.07	I	4	R	4	R	29.19	S	19.75	S	4	R	4	R	25.05	S
N30	26.39	S	4	R	15.92	I	18.26	R	33.23	S	4	R	4	R	10.68	I	26.73	S
N31	13.88	S	4	R	13.27	R	4	R	32.04	S	18.03	S	4.16	R	4	R	23.81	S

R = resistance, I = intermiated, S = Sensitive

isolate No.	AMC		CIP		DA		CL		MUP		SXT		TE		0X		Cefoxitin	
N32	11.92	I	4	R	12.87	R	4	R	30.87	S	18.35	S	4.33	R	4	R	24.87	S
W32	12.74	I	4	R	17.59	I	4	R	31.39	S	18.57	S	4.84	R	4	R	23.65	S
N39	14.81	S	4	R	14.08	R	4	R	33.92	S	18.39	S	4.7	R	4	R	25.17	S
O39	13.31	S	4	R	4	R	4	R	31.85	S	18.95	S	5.77	R	4	R	24.1	S
W40	25.94	S	4	R	12.94	R	16.64	R	33.28	S	4	R	5.22	R	11.65	I	26.04	S
W41	25.84	S	4	R	13.25	R	14.42	R	30.19	S	4	R	5.24	R	11.03	I	28.47	S
W43	9.15	R	4	R	13.4	R	4	R	31.88	S	19.17	S	5.37	R	4	R	21.97	I
W44	13.91	S	4	R	16.19	I	4	R	32.96	S	17.96	S	6.07	R	4	R	24.41	S
N47	14.24	S	4	R	15.49	I	4	R	33.69	S	19.69	S	6.07	R	4	R	26.54	S
W47	16.19	S	4	R	10.18	R	4	R	31.92	S	4	R	6.34	R	4	R	25.07	S
N49	22.52	S	25.72	S	31.42	S	21.14	R	24.32	S	30.4	S	28.79	S	16.14	S	28.38	S
W49	14.93	S	4	R	18.82	I	4	R	32.38	S	19.29	S	4.63	R	4	R	24.47	S
N51	16.11	S	13.83	R	30.27	S	4	R	31.24	S	4	R	5.23	R	4	R	23.93	S
W51	17.07	S	13.64	R	29.05	S	4	R	29.73	S	4	R	5.11	R	4	R	25.11	S
W53	14.34	S	4	R	17.25	I	4	R	32.97	S	4	R	6.53	R	4	R	24.53	S
N54	23.9	S	4	R	4	R	15.52	R	29.77	S	4	R	4.87	R	10.42	I	26.46	S
N56	16.14	S	13.3	R	27.6	S	4	R	29.39	S	4	R	5.95	R	4	R	25.26	S

R = resistance, I = intermiated, S = Sensitive

isolate No.	AMC		CIP		DA		CL		MUP		SXT		TE		0X		Cefoxitin	
N57	30.23	S	35.91	S	5.42	R	21.74	R	4	R	9.24	R	22.54	S	4	R	23.88	S
W60	11.69	I	4	R	14.89	R	4	R	33	S	17.56	S	4.76	R	4	R	24.17	S
N61	13.33	S	4	R	14.17	R	4	R	29.94	S	17.79	S	5.4	R	4	R	23.82	S
N62	15.17	S	4	R	15.22	I	4	R	27.62	S	17.04	S	4.84	R	4	R	22.96	S
N63	12.07	I	4	R	15.61	I	4	R	31.57	S	17.03	S	4.85	R	4	R	22.47	S
W63	28.24	S	29.73	S	28.83	S	21.75	R	29.71	S	31.41	S	10.36	I	17.21	S	27.86	S
W67	10.43	R	4	R	14.97	R	4	R	10.7	R	4	R	5.34	R	4	R	24.82	S
N68	15.07	S	4	R	14.55	R	4	R	29.56	S	17.72	S	5.85	R	4	R	24.72	S
O68	24.35	S	4	R	14.08	R	16.25	R	27.9	S	4	R	4.89	R	13.27	S	28.45	S
N74	22.93	S	4	R	14.26	R	4	R	29.49	S	4	R	4.57	R	9.66	R	27.6	S
W82	24.57	S	4	R	14.6	R	18.3	R	31.09	S	4	R	4.74	R	12.93	I	27.66	S
W83	12.8	I	4	R	16.51	I	4	R	29.4	S	18.17	S	5.74	R	4	R	24.12	S
O88	25.74	S	4	R	12.89	R	18.3	R	29.2	S	4	R	5.31	R	13.27	S	28.86	S
O89	29.46	S	32.42	S	29.69	S	14.4	R	29.42	S	25.42	S	27.6	S	13.08	S	33.48	S
O92	29.8	S	24.68	S	10.68	R	28.05	R	28.68	S	24	S	9.18	S	19.7	S	37.14	S
N100	23.51	S	25.88	S	26.19	S	22.94	R	23.01	S	29.7	S	26.54	S	17.6	S	27.2	S
S. ATCC	38.97	S	22.33	S	4	S	23.78	S	25.06	S	29.72	S	28.65	S	18.67	S	30.72	S

ciprofloxacin (CIP, 1 µg), clindamycin (DA, 2 µg), cefalexin (CL, 30 µg), co-amoxiclav (AMC, 3 µg), co-trimoxazole (SXT, 25 µg), mupirocin (MUP, 5 µg) and tetracycline (TE, 10 µg), oxacillin (0X, 5 µg) and cefoxitin (CEF, 30 µg

R = resistance, I = intermiated, S = Sensitive

Table A-6 double disk diffusion test

isolate No.	OX		AMC		AMP		Co.
N1	18	S	27	S	16.5	S	OX-AMC-AMP
N3	n/a	R	15	R	n/a	R	n/a
N6	n/a	R	15	R	n/a	R	n/a
N11	n/a	R	19	R	8	R	n/a
N12	n/a	R	14	R	10	R	n/a
W19	n/a	R	15	R	n/a	R	n/a
N22.	n/a	R	17	R	11	R	n/a
N24	n/a	R	15	R	9	R	n/a
W24	n/a	R	15	R	10	R	n/a
W25	16	S	29	S	9	R	OX-AMC-AMP
N27	n/a	R	18	R	10	R	n/a
W27	n/a	R	18	R	12	R	n/a
N29	n/a	R	15	R	n/a	R	n/a
W29	n/a	R	16	R	n/a	R	n/a
N30	16	S	29	S	20	R	OX-AMC-AMP
N31	n/a	R	14	R	n/a	R	n/a
N32	n/a	R	12	R	n/a	R	n/a
W32	n/a	R	14	R	n/a	R	n/a
N39	n/a	R	15	R	9	R	n/a
W39	n/a	R	16	R	8	R	n/a
W40	13	S	25	S	18	R	AMC-AMP
W41	n/a	R	12	R	n/a	R	n/a
W43	n/a	R	11	R	n/a	R	n/a
W44	n/a	R	13	R	8	R	n/a
N47	n/a	R	13	R	n/a	R	n/a
W47	n/a	R	20	S	14	R	AMC-AMP
N49	20	S	25	S	18	R	OX-AMC-AMP

isolate No.	OX		AMC		AMP		Co.
W49	n/a	R	19	R	7	R	n/a
N51	n/a	R	18	R	8	R	n/a
W51	n/a	R	20	S	9	R	n/a
W53	n/a	R	25	S	22	R	AMC-AMP
N54	15	S	27	S	16	R	AMC-AMP
N56	n/a	R	18	R	n/a	R	n/a
W57	n/a	R	12	R	n/a	R	n/a
N60	18	S	31	S	28	S	OX-AMC-AMP
N61	n/a	R	13	R	7	R	n/a
N62	n/a	R	16	R	10	R	n/a
N63	n/a	R	12	R	n/a	R	n/a
W63	17	S	29	S	19	R	OX-AMC-AMP
W67	n/a	R	13	R	10	R	n/a
N68	n/a	R	13	R	11	R	n/a
W68	14	S	26	S	18	R	AMC-AMP
N74	12	I	24	S	14	R	n/a
W82	15	S	26	S	18	R	OX-AMC-AMP
W83	n/a	R	14	R	9	R	n/a
O88	15	S	30	S	18	R	OX-AMC-AMP
O89	15	S	27	S	30	S	OX-AMC-AMP
O92	22	S	25	S	18	R	AMC-AMP
N100	20	S	25	S	16	R	OX-AMC-AMP
S.25923	21	S	35	S	34	S	OX-AMC-AMP

co-amoxiclav (AMC, 3 µg), oxacillin (0X, 5 µg) and amplicilin (AMP, 2 µg), N/A = No clear zone

Table A-7 MIC of Oxacillin

isolate No.	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	MIC
N1	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
N3	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N6	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N11	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N12	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	>256
W19	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	>256
N22	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	4
N24	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W24	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
O25	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	128
N27	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	64
W27	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	64
N29	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W29	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N30	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
N31	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256

pos. = positive, neg. = negative

isolate No.	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	MIC
N32	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W32	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N39	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W39	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W40	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	32
W41	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W43	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	>256
W44	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N47	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W47	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	128
N49	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
W49	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N51	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	128
W51	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	128
W53	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	128
N54	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	4
N56	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256

pos. = positive, neg. = negative

isolate No.	256	128	64	32	16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	MIC
N57	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W60	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	32
N61	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	>256
N62	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
N63	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	256
W63	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	4
W67	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	>256
N68	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	>256
W68	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
N74	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	2
W82	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
W83	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	pos.	>256
W88	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
W89	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
W92	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
N100	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	pos.	pos.	1
S. ATCCC 29123	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.	pos.	0.25

pos. = positive, neg. = negative

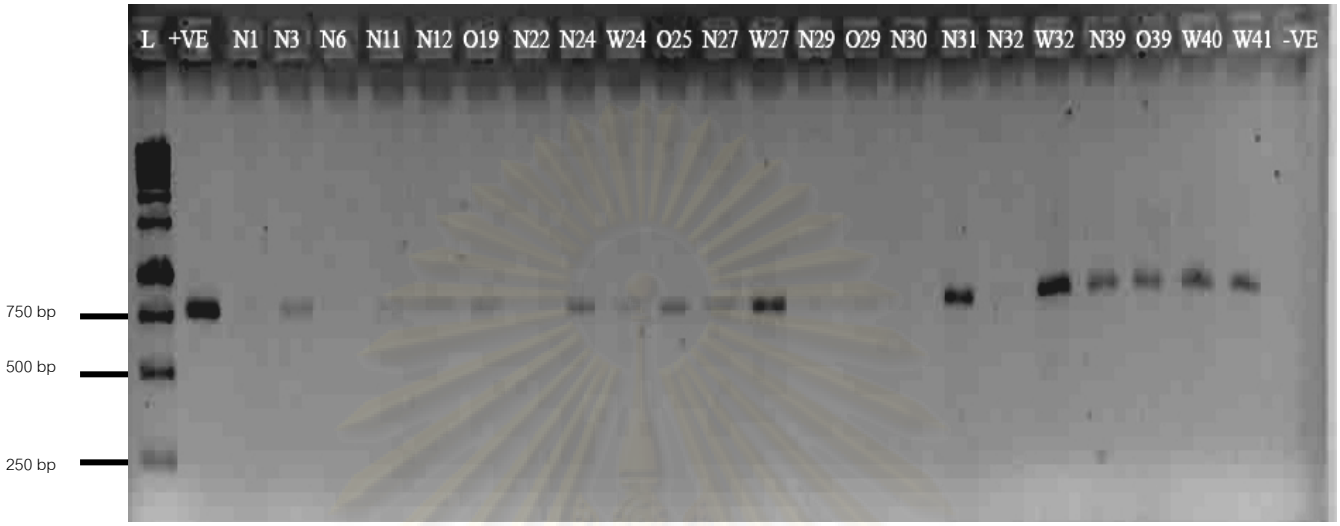


Figure A-8 Agarose gel electrophoresis of all coagulase positive *Staphylococcus spp.*'s PCR products using specific primers to *blaZ*

Lane	Isolated	*blaZ* (774 bp)	Lane	Isolated	*blaZ* (774 bp)
1	1 kb DNA ladder plus.	N/A	13	N27	positive
2	Positive control	positive	14	W27	positive
3	N1	negative	15	N29	negative
4	N3	positive	16	O29	negative
5	N6	negative	17	N30	negative
6	N11	positive (weak)	18	N31	positive
7	N12	positive (weak)	19	N32	negative
8	O19	Positive (weak)	20	W32	positive
9	N22	negative	21	N39	positive
10	N24	positive	22	O39	positive
11	W24	positive	23	W40	positive
12	O25	positive	24	W41	positive
			25	negative control	negative

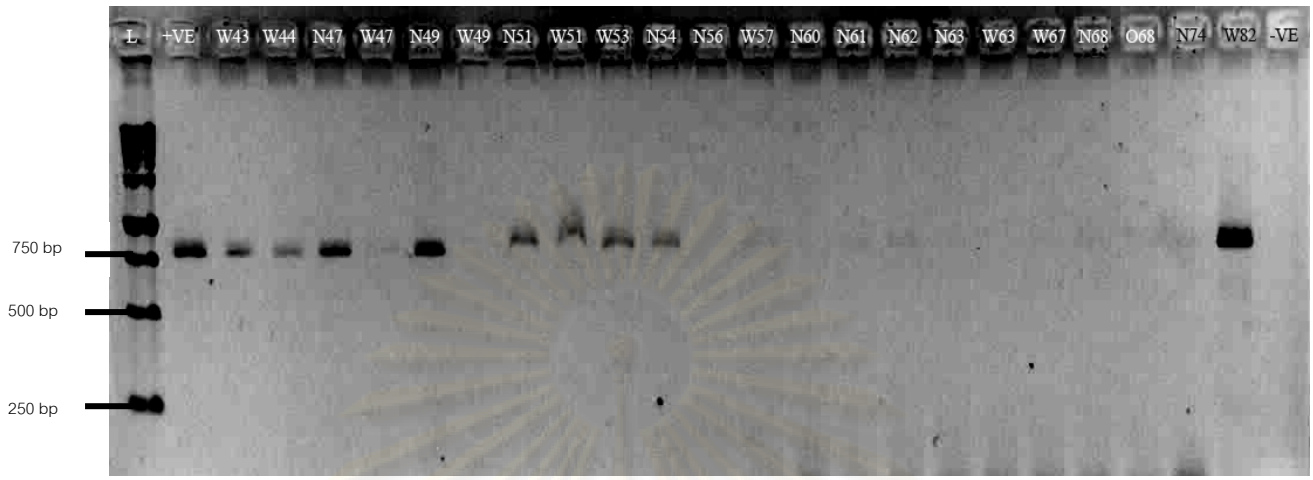


Figure A-9 Agarose gel electrophoresis of all coagulase positive *Staphylococcus spp.*'s PCR products using specific primers to *blaZ* (continued)

Lane	Isolated	*blaZ* (774 bp)	Lane	Isolated	*blaZ* (774 bp)
1	1 kb DNA ladder plus.	N/A	13	N56	negative
2	Positive control	positive	14	W57	positive (weak)
3	W43	positive	15	N60	negative
4	W44	positive	16	N61	positive (weak)
5	N47	positive	17	N62	positive (weak)
6	W47	negative	18	N63	negative
7	N49	positive	19	W63	negative
8	W49	negative	20	W67	negative
9	N51	positive	21	N68	positive (weak)
10	W51	positive	22	O68	negative
11	W53	positive	23	N74	positive (weak)
12	N54	positive	24	W82	positive
			25	negative control	negative

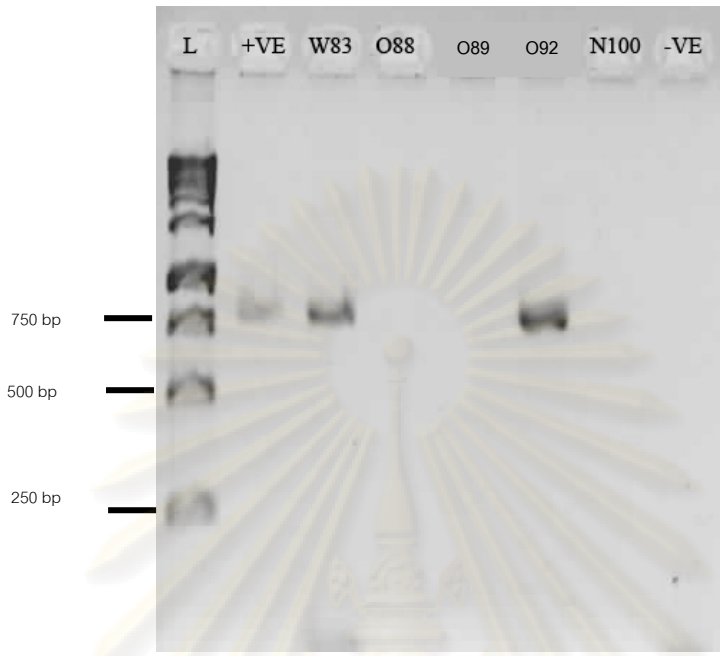


Figure A-10 Agarose gel electrophoresis of all coagulase positive *Staphylococcus spp.*'s PCR products using specific primers to *blaZ* (continued)

Lane	Isolated	*blaZ* (774 bp)
1	1 kb DNA ladder plus.	N/A
2	positive control	positive
3	W83	positive
4	O88	negative
5	O89	negative
6	O92	positive
7	N100	negative

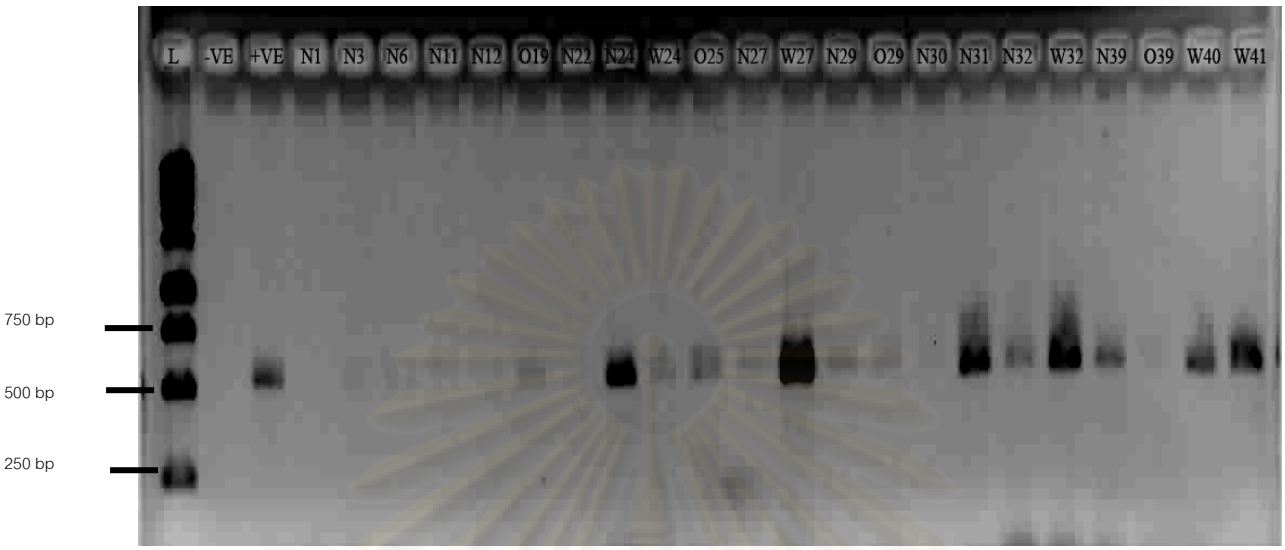


Figure A-11 PCR production of coagulase positive *Staphylococcus spp*. isolated from dogs using *SCCmec* type 2 MRSA for positive control

Lane	Isolated	*mecA* (532 bp)	Lane	Isolated	*mecA* (532 bp)
1	1 kb DNA ladder	N/A	13	O25	positive
2	negative control	negative	14	N27	positive
3	positive control	positive	15	W27	positive
4	N1	negative	16	N29	positive
5	N3	positive (weak)	17	O29	positive
6	N6	positive (weak)	18	N30	positive (weak)
7	N11	positive (weak)	19	N31	positive
8	N12	negative	20	N32	positive
9	O19	positive	21	W32	positive
10	N22	negative	22	N39	positive
11	N24	positive	23	O39	positive (weak)
12	W24	positive	24	W40	positive
			25	W41	positive

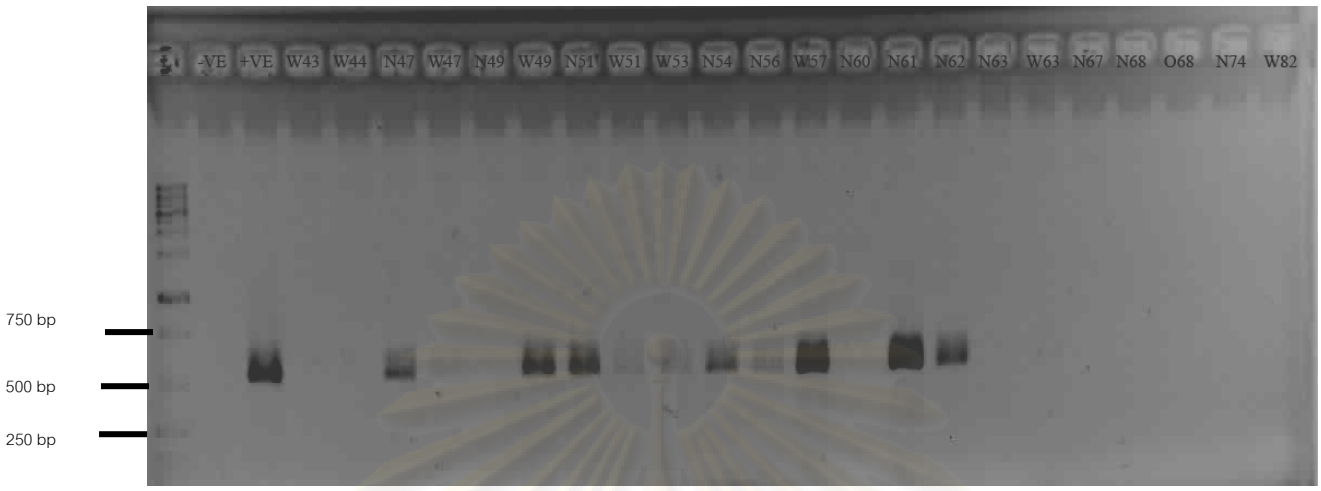


Figure A-12 PCR production of coagulase positive *Staphylococcus spp*. that isolated from dogs using *SCCmec* type 2 MRSA for positive control (continued)

Lane	Isolated	*mecA* (532 bp)	Lane	Isolated	*mecA* (532 bp)
1	1 kb DNA ladder	N/A	13	N54	positive
2	negative control	negative	14	N56	positive
3	positive control	positive	15	W57	positive
4	W43	negative	16	N60	negative
5	W44	negative	17	N61	positive
6	N47	positive	18	N62	positive
7	W47	positive (weak)	19	N63	negative
8	N49	negative	20	W63	negative
9	W49	positive	21	W67	negative
10	N51	positive	22	N68	negative
11	W51	positive	23	O68	negative
12	W53	positive	24	N74	negative
			25	W82	negative

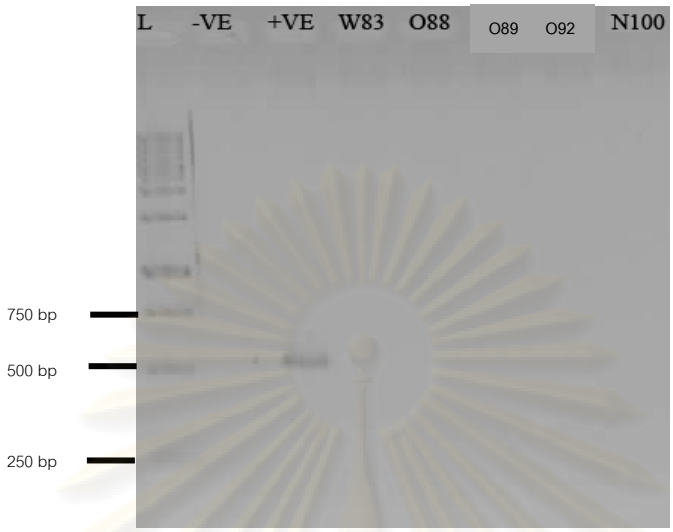


Figure A-13 PCR production of coagulase positive *Staphylococcus spp*. that isolated from dogs using *SCCmec* type 2 MRSA for positive control (continued)

Lane	Isolated	*mecA* (532 bp)
1	1 kb DNA ladder plus.	N/A
2	negative control	negative
3	positive control	positive
4	W83	negative
5	O88	negative
6	O89	negative
7	O92	negative
8	N100	negative

Appendix B

Alpha-naphthol solution

Alpha-naphthol	5	g.
Ethanol	100	ml.

Potassium hydroxide solution

Potassium hydroxide CP.	40	g.
Creatine	0.3	g.
Deionize water	100	ml.

Cell Suspension Solution (Puregene™ DNA Purification System)

- Tris [hydroxymethyl] aminomethane
- Ethylenediamine tetraacetic acid
- Sorbitol

Protein Precipitation Solution (Puregene™ DNA Purification System)

- Ammonium cetate

DNA Hydration Solution (Puregene™ DNA Purification System)

- Tris [hydroxymethyl] aminomethane
- Diaminetetraacetic acid

Cell Suspension Solution (Puregene™ DNA Purification System)

- Tris [hydroxymethyl] aminomethane
- Ethylenediamine tetraacetic dodecyl sulfate

NTP Preparation

1. **dATP** (2'-deoxysadenosine 5'-triphosphate sodium salt)
   concentration ; 100 mM aqueous solution (pH 7.0)
   quantity : 25 µmoles
   volume : 250 µl
2. **dCTP** (2'-deoxycytidine 5'-triphosphate sodium salt)
   concentration ; 100 mM aqueous solution (pH 7.0)
   quantity : 25 µmoles
   volume : 250 µl
3. **dGTP** (2'-deoxyguanosine 5'-triphosphate sodium salt)
   concentration ; 100 mMaqueous solution (pH 7.0)
   quantity : 25 µmoles
   volume : 250 µl
4. **dTTP** (2'-deoxythymidine 5'-triphosphate sodium salt)
   concentration ; 100 mMaqueous solution (pH 7.0)
   quantity : 25 µmoles
   volume : 250 µl

2mM dNTP

100 mM dATP	20	µl
100 mM dCTP	20	µl
100 mM dGTP	20	µl
100 mM dTTP	20	µl
MilliQ water	920	µl

The components were mixed in a microtube and stored at -20ºC.

Agar dilution preparation

Oxacillin dilution was prepared by adding 6 ml of oxacillin stock solution (256 µl/ml) to each 54 ml of Muller-Hinton molten agar (1:10 dilution). Pipetted 25 ml of

solution and poured in a sterile plates. The oxacillin stock solution was diluted two fold until reaching a concentration of 0.03 µg/ml so that after following the aforementioned procedure, the final concentrations of oxacillin in the media were 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06 and 0.03 µg/ml, accordingly. Sterile distilled water was used for preparing the oxacillin stock solutions and dilutions.

BIOGRAPHY

Miss Punpichaya Fungwitaya was born in January 5, 1983 in Bangkok, Thailand. My graduated with a Bachelor of Veterinary Science in 2006 from the Faculty of Veterinary Science, Mahidol University, Thailand. Now, I live at Phatum thani.