Livestock-associated methicillin-resistant coagulase-negative staphylococci in pig in Lamphun Province, Thailand, carrying Type-IX SCC*mec* element

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Abstract

Methicillin-resistant coagulase-negative staphylococci (MRCNS) are important opportunistic pathogens of nosocomial infections. An increase in reports of MRCNS in livestock, especially pigs and the evidences of transmission between domestic animals and humans pose a significant public health concern worldwide. The aims of this study were to determine the diversity of the staphylococcal cassette chromosome *mec* (SCC*mec*) elements among MRCNS isolates from pigs and to examine the species distribution and antibiotic resistance of these isolates. Sixteen isolates of pig MRCNS were recovered and identified at a species level and subsequently confirmed by PCR detection of staphylococcal 16S *rRNA, nuc* and *mecA* genes. These isolates consisted of six staphylococcal species. The most predominate species was *S. saprophyticus* (9 isolates), followed by *S. cohnii* (2 isolates) and *S. haemolyticus* (2 isolates). All isolates were resistant to at least three classes of 10 tested antibiotics – penicillin, erythromycin and clindamycin, but susceptible to vancomycin, gentamicin and tigecycline. However, some isolates were also resistant to other non-beta-lactam antibiotics. The most prevalent SCC*mec* type was type IX, which was detected in six isolates (37.5%). This is the first report of SCC*mec* type IX in MRCNS in pigs in Thailand. SCC*mec* type V was also detected in three isolates (18.8%). In addition, seven isolates (43.8%) were non-typeable and possibly possess a novel type of SCC*mec*. Therefore, MRCNS in pigs might be a potential source of diverse SCC*mec* elements that result to beta-lactam resistance among other staphylococci, especially *S. aureus*, an important human pathogen. ***Bull Chiang Mai Assoc Med Sci 2013; 46(3): 250-259***

Keywords: *SCCmec, methicillin resistance, coagulase-negative staphylococci, livestock-associated MRCNS*

Methicillin-resistant coagulase-negative staphylococci ที่สัมพันธ์กับปศุสัตว์ในสุกรในจังหวัดลำพูน ประเทศไทย มี SCC*mec* type IX

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บทคัดย่อ

เชื้อ Methicillin-resistant coagulase-negative staphylococci (MRCNS) เป็นเชื้อก่อโรคฉวยโอกาสสำคัญที่ทำให้เกิดการติดเชื้อในโรงพยาบาล รายงานการพบเชื้อ MRCNS ในปศุสัตว์โดยเฉพาะอย่างยิ่งในสุกรที่เพิ่มสูงขึ้นและหลักฐานยืนยันการแพร่เชื้อ MRCNS ระหว่างสัตว์เลี้ยงกับมนุษย์ก่อให้เกิดความวิตกกังวลทางการสาธารณสุขทั่วโลก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความหลากหลายทางพันธุกรรมของ Staphylococcal cassette chromosome *mec* (SCC*mec*) elements ของเชื้อ MRCNS ที่แยกได้จากสุกร และศึกษาการกระจายของสปีชีส์ตลอดจนการดื้อต่อยาปฏิชีวนะของเชื้อเหล่านี้ เชื้อ MRCNS ทั้งหมด 16 ไอโซเลทที่แยกได้จากสุกรได้รับการพิสูจน์สปีชีส์และยืนยันผลด้วยการตรวจหายีน staphylococcal 16S *rRNA, nuc* และ *mecA* ด้วยวิธี PCR เชื้อเหล่านี้ประกอบด้วย staphylococci ทั้งหมด 6 สปีชีส์ สปีชีส์ที่พบมากที่สุด คือ *S. saprophyticus* (9 ไอโซเลท) รองลงมาเป็น S. *cohnii* (2 ไอโซเลท) และ *S. haemolyticus* (2 ไอโซเลท) เชื้อทั้งหมดดื้อต่อยาปฏิชีวนะอย่างน้อยสามกลุ่มจากทั้งหมด 10 ชนิดที่ทำการทดสอบ ทุกไอโซเลทดื้อต่อยา penicillin, erythromycin และ clindamycin แต่ไวต่อยา vancomycin, gentamicin และ tigecycline อย่างไรก็ตาม ยังพบว่ามีเชื้อบางไอโซเลทดื้อต่อยาปฏิชีวนะอื่น ๆ ที่ไม่อยู่ในกลุ่ม beta-lactam อีกด้วย SCC*mec* ที่ตรวจพบมากที่สุดเป็น SCC*mec* type IX โดยพบในเชื้อ 6 ไอโซเลท (37.5%) ซึ่งเป็นรายงานการพบ SCC*mec* type IX ครั้งแรกในเชื้อ MRCNS ที่แยกได้จากสุกรในประเทศไทย พบ SCC*mec* type V ในเชื้อ 3 ไอโซเลท (18.8%) นอกจากนี้ มีเชื้อ 7 ไอโซเลท (43.8%) ที่ไม่สามารถจำแนกชนิด SCC*mec* ได้ ซึ่งเป็นไปได้ว่าอาจจะเป็น SCC*mec* ชนิดใหม่ ดังนั้น เชื้อ MRCNS จากสุกรอาจเป็นแหล่งของ SCC*mec* elements ที่มีความหลากหลาย ซึ่งทำให้เกิดการดื้อต่อยากลุ่ม beta-lactam ในเชื้อ staphylococci โดยเฉพาะเชื้อก่อโรคสำคัญในคนอย่าง *S. aureus* ***วารสารเทคนิคการแพทย์เชียงใหม่ 2556; 46(3): 250-259***

คำหัส: *SCCmec, methicillin resistance, coagulase-negative staphylococci, livestock-associated MRCNS*

Introduction

The *mecA* gene encoding penicillin-binding protein 2a (PBP 2a or PBP 2') is responsible for methicillin and beta-lactam antibiotics resistance. This gene is located on the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile genetic element that is a vehicle for exchanging the resistance gene between staphylococcal species.[1] Generally, SCC*mec* consists of mec complex, *mecA* and its regulatory genes (*mecRI*) *ccr* complex, conferring mobility of SCC by *ccr* gene-encoding recombinases and joining (J) regions, insertion sites of plasmid and transposons.[2] The origin of the *mecA* gene is unknown, but a recent study demonstrated that the *mecA* gene originated from certain livestock-associated coagulase-negative staphylococci (CNS) species, such as *S. sciuri*.[3] However, Tsubakishita *et al.,* (2010) found that the *mecA* homologue (99 to 100% sequence homology with the *mecA* gene in MRSA strain N315) in *S. fleuretti* is located in proximity to genes required for growth of staphylococci. This suggested that *S. fleuretti,* a closely related species to *S. sciuri*, might be the origin of the *mecA* gene in MRSA.[4] To date, 11 different SCC*mec* types (I-XI) have been classified according to combination of *ccr* types and *mec* classes. In contrast to SCC*mec* types I–III that are usually detected in hospital-associated methicillin-resistant staphylococci (MRS) strains, SCC*mec* types IV and V are generally detected in community- and livestock-associated MRS strains.[5]

Since 2005, livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) have been reported in several animal species. The clonal complex (CC) 398 belonging to SCC*mec* type IV is the most frequently detected LA-MRSA clone in pigs worldwide, especially in Europe.[6, 7] In contrast, CC9–SCC*mec*-V lineage predominates among LA-MRSA in Asia.[8-10] Recently, CC9-SCC*mec* -IX was detected in pigs and pork in Thailand.[11-13] Although methicillin-resistant coagulase-negative staphylococci (MRCNS) in healthy animals has been reported many times, few studies of LA-MRCNS in pigs have been carried out.[14] Previous studies showed that SCC*mec* types of LA-MRCNS were heterogeneous. Zhang *et al.,* (2009) characterized 60 MRCNS isolates from eight animal species, including pigs. Twenty-four isolates were classified into SCC*mec* types I, III, IV and V, with type III predominating.[15] Tulinski *et al.,* (2011) isolated 44 MRS isolates from pigs and the surrounding farm environment. All 65 isolates of 10 different MRS species belonged to either SCC*mec* types III, IV, V, VI and a novel subtype of type IV, or were non-typeable. Among these, SCC*mec* type V predominated.[16] Altogether, the high prevalence of MRCNS and the detection of non-typeable isolates suggested potential role of MRCNS as a reservoir of diverse SCC*mec*.

During the past few years, MRCNS have become a significant public health problem. The prevalence of methicillin resistance has been reported to be higher in CNS than *S. aureus*, with global rates ranging from 75-90% during the 1990s.[17] Mortality from septicemia due to MRCNS was 25.7% in an ICU ward.[18] Nosocomial infection caused by MRCNS has been increasing. In general, MRCNS represents approximately 80% of nosocomial infections and 30-40% of isolates obtained from healthy carriers or the community.[19, 20] The most frequently isolated CNS species associated with nosocomial infections have been identified in *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus*.[21] Among human isolates, SCC*mec* types have been described in several staphylococcal species, including type I, II and III.[14] Ruppe *et al.,* (2009) described a high diversity of SCC*mec* structures in MRCNS from outpatients, including types IV, V and III. Among these CA-MRCNS strains, type-IV and type-V SCC*mec* predominated and were associated with *S. epidermidis* and *S. haemolyticus,* respectively.[22] The capacity of MRCNS to cause human disease, together with their potential to spread from animals to humans through the food chain and direct contact, may pose public health risks. To date, however, epidemiological data of MRCNS in livestock is limited. The presence of MRCNS in pigs in Thailand is unknown. SCC*mec* typing can be useful for molecular characterization of MRS and enables understanding of the diversity and evolution of SCC*mec* in MRS. The aims of this study were to determine the diversity of SCC*mec* elements among

MRCNS isolated from pigs and to examine the species distribution and antibiotic resistance of these isolates.

Materials and methods

Sample collection and isolation of MRCNS

Nasal and rectal swabs were collected during January - February 2010 from 57 pigs among 4 smallholder farms (farm A, n=15 pigs; farm B, n=15 pigs; farm C, n=14 pigs and farm D, n=13 pigs – all farms are members of the Chiang Mai-Lamphun Pig Farmers Cooperative Ltd.) in Lamphun Province, Thailand. All 114 samples (57 nasal swabs and 57 rectal swabs) were transported in Cary-Blair medium (Oxoid) and processed in the laboratory within 24 h. The swabs were enriched in brain-heart infusion broth (BHI) containing 7% NaCl for 48 h and streaked on Mannitol salt agar (MSA) supplemented with 2, 4 µg/ml oxacillin. Presumptive MRCNS colonies were cultured on blood agar and incubated overnight at 35°C. They were subsequently identified by Gram's stain, catalase test, coagulase test and fermentation of glucose and mannitol. All isolates were subjected to the cefoxitin disk (30 µg) and oxacillin disk (1 µg) screening test for methicillin resistance recommended by the Clinical Laboratory Standards Institute (CLSI).

Detection of staphylococcal 16S *rRNA*, *nuc* and *mecA* genes

All MRCNS isolates were confirmed by PCR screening of staphylococcal ribosomal RNA gene (16S *rRNA*) and the gene conferring methicillin resistance (*mecA*). The absence of *Staphylococcus aureus*-specific thermonuclease gene (*nuc*) was used to identify CNS. The primers used in this study included 16S rRNA-F (5′-GCAAGCGTTATCCGGATTT-3′), 16S rRNA-F (5′-CTTAATGATGGCAACTAAGC-3′), NUC1 (5′-GCGATTGATGGTGATACGGTT-3′), NUC2 (5′-AGCCAAGCCTTGACGAACTAAAGC-3′), MECA1 (5′-GCAATCGCTAAAGAACTAAG-3′) and MECA2 (5′-GGGACCAACATAACCTAATA-3′).[23, 24] *S. aureus* ATCC 25923, *S. aureus* ATCC 43300 (MRSA) and *S. epidermidis* ATCC 14990 were used as quality control strains for all PCR performed in this study.

Species identification

MRCNS were identified at a species level based on their physiological characteristics: novobiocin resistance, anaerobic growth, esculin hydrolysis, enzyme production (urease, ornithine decarboxylase (ODC), pyrrollidonyl arylamidase (PYR), alkaline phosphatase and β-glalactosidase) and utilization of maltose, sucrose, raffinose, D-trehalose, D-mannitol, D-mannose, D-xylose and D-cellobiose.[25]

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed according to CLSI guidelines (Approved Standard M 100-S22, 2012). The antimicrobial disks used for antimicrobial resistance profiles of the MRCNS isolates included: penicillin (10 µg), clindamycin (2 µg), erythromycin (15 µg), fosfomycin (50 µg), trimethoprim-sulfamethoxazole (25 µg) vancomycin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), fusidic acid (10 µg) and tigecycline (15 µg). Minimal Inhibitory Concentration (MIC) of oxacillin was performed using an agar dilution method. *S. aureus* ATCC 25923, *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300 (MRSA) were used as quality control strains for the disk diffusion test and MIC assay.

SCC*mec* typing

The SCC*mec* types were determined by multiplex PCR as previously described by Kondo *et al.* (2007).[26] The method has two multiplex PCR (M-PCR) reactions: M-PCR 1 for amplification of *ccr* gene and M-PCR 2 for amplification *mec*. The combination of *ccr* types and mec classes were used to define the SCC*mec* types; the classification followed the guidelines proposed by the International Working Group on the Classification of SCC elements (http://www.sccmec.org). Control strains for SCC*mec* typing included four MRSA strains: epidemic MRSA (EMRSA)-8 (SCC*mec* type I), N315 (SCC*mec* type II), EMRSA-4 (SCC*mec* type III) and EMRSA-10 (SCC*mec* type IV).

Results

Species identification

A total of 16 MRCNS were isolated from the nasal and rectal swabs of pigs (13-15 pigs/farm) on three smallholder farms (Table 1). Most of the MRCNS isolates were recovered from the nasal swabs. Six staphylococcal species were identified: *S. saprophyticus, S. cohnii, S. haemolyticus, S. kloosii, S. xylosus* and *S. caprae.* The most frequently identified species was *S. saprophyticus* (9 isolates) followed by *S. cohnii* (2 isolates) and *S. haemolyticus* (2 isolates).

Table 1 Species distribution of MRCNS isolates in pig farms

Farms	No. of pigs	No. of MRCNS isolates	Species (no. of isolates)
A	15	2	*S. saprophyticus* (2)
B	15	-	-
C	14	6	*S. saprophyticus* (5), *S. kloosii* (1)
D	13	8	*S. saprophyticus* (2), *S. haemolyticus* (2), *S. cohnii* (2), *S. xylosus* (1), *S. caprae* (1)
Total	57	16	

Antimicrobial susceptibility testing

The MRCNS species showed similar antibiotic resistance patterns (Table 2). All isolates were susceptible to vancomycin, gentamicin and tigecycline and resistant to penicillin, erythromycin and clindamycin. Some of the MRCNS isolates were also resistant to fusidic acid (69%), trimethoprim-sulfamethoxazole (44%), chloramphenicol (31%) and fosfomycin (12%). A board range of oxacillin MIC was observed, from 8 to 512 µg/ml. More than half (56%) of the isolates had MIC higher than 128 µg/ml (512 fold higher than the CLSI breakpoint).

Table 2 Antibiotic resistance of different MRCNS species

Species	Percentage (isolate number) of antibiotic resistance									
	P	E	DA	FOS	SXT	VA	C	CN	FD	TGC
S. saprophyticus (9)	100 (9)	100 (9)	100 (9)	22 (2)	44 (4)	0 (0)	44 (4)	0 (0)	89 (8)	0 (0)
S. cohnii (2)	100 (2)	100 (2)	100 (2)	0 (0)	50 (1)	0 (0)	50 (1)	0 (0)	100 (2)	0 (0)
S. haemolyticus (2)	100 (2)	100 (2)	100 (2)	0 (0)	50 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. kloosii (1)	100 (1)	100 (1)	100 (1)	0 (0)	100 (1)	0 (0)	0 (0)	0 (0)	100 (1)	0 (0)
S. xylosus (1)	100 (1)	100 (1)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. caprae (1)	100 (1)	100 (1)	100 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	100 (16)	100 (16)	100 (16)	12 (2)	44 (7)	0 (0)	31 (5)	0 (0)	69 (11)	0 (0)

* P, penicillin; E, erythromycin; DA, clindamycin; FOS, fosfomycin; SXT, trimethoprim-sulfamethoxazole; VA, vancomycin; C, chloramphenicol; CN, gentamicin; FD, fusidic acid and TGC, tigecycline

SCC*mec* typing

In this study, we found a high diversity of SCC*mec* elements among MRCNS isolates (Table 3). The most frequently found SCC*mec* type was SCC*mec* type IX, detected in six isolates of four species: *S. saprophyticus* (3 isolates), *S. cohnii* (1 isolate), *S. caprae* (1 isolate) and *S. haemolyticus* (1 isolate). SCC*mec* type V was detected in three isolates of two species: *S. saprophyticus* (2 isolates) and *S. haemolyticus* (1 isolate). Seven out of 16 MRCNS isolates (43.8%) were non-typeable.

Table 3 Diversity of SCC*mec* elements among LA-MRCNS isolates

SCC*mec* type	*ccr* type	*mec* class	Species	Isolates code*
IX	1	C2	*S. saprophyticus*	C2, C5, D3
			S. cohnii	D7
			S. haemolyticus	D5
			S. caprae	D4
V	5	C2	*S. saprophyticus*	A2, D8
			S. haemolyticus	D6
Non-typable	1 & 5	A & C2	*S. cohnii*	D1
	-	C2	*S. saprophyticus*	A1, C3, C4
			S. kloosii	C1
		A	*S. saprophyticus*	C6
	5	A	*S. xylosus*	D2

* The code of MRCNS isolated from each farms (A, B, C, and D)

Discussion

Six staphylococcal species were identified from 16 MRCNS isolated from three of four smallholder pig farms in Lamphun Province. Prevalence of MRCNS in Thai pig farms located in different geographic locations may vary widely. *S. saprophyticus* was the most frequently identified species, followed by *S. cohnii* and *S. haemolyticus*. These predominant species can cause infections in humans, especially *S. saprophyticus* and *S. haemolyticus*, which are frequently involved in clinical cases. *S. saprophyticus* is a common cause of urinary tract infections along with more severe complications including septicemia, pyelonephritis and endocarditis in humans.[27] Moreover, it is an animal-originated food contaminant found in 7.3% of rectal swabs from pigs.[28] *S. cohnii* is an opportunistic pathogen. They have been found colonizing on human skin in only small numbers. Colonization of *S. cohnii* in hospital environments has been previously reported, with almost all isolates identified as methicillin-resistant staphylococci.[29] This species has also been found in healthy animals: cattle, fowl and pigs.[30] *S. haemolyticus* is the second most common cause of nosocomial CNS infections, including: blood stream, urinary tract, wound, bone, and joint infections. Most of these nosocomial pathogens exhibit multi-drugs resistance.[31] In addition to being found in several healthy animals: cattle, sheep, goats, pigs and turkeys, *S. haemolyticus* has been found in cows presenting with bovine mastitis.[15, 32] Tulinski *et al.,* found 10 staphylococcal species on seven pig farms in the Netherlands. Although the most common species found on these farms was *S. aureus,* the majority of isolates – *S. cohnii* (16%), *S.* epidermidis (11%) and *S. haemolyticus* (11%) – were CNS.[16]

Most of MRCNS isolates were resistant to at least three classes of antibiotics, suggesting MDR phenotypes. All 16 isolates were resistant to penicillin, erythromycin and clindamycin but susceptible to vancomycin, gentamicin

and tigecycline. Twelve isolates (75%) were also resistant to other non-beta-lactam antibiotics: fosfomycin, trimethoprim-sulfamethoxazole, chloramphenicol and fusidic acid. This study demostrated the high prevalence of MRCNS with MDR phenotypes in healthy pigs on the farms surveyed. In this study, a broad range of oxacillin MIC, raning from 8 to 512 µg/ml, was detected among the MRCNS isolates,with more than half higher than 128 µg/ml. Among the MRCNS isolates, different levels of expression of *mecA* may be the reason for the diverse MIC found. The homogeneous expression of *mecA* confers a high level of methicillin resistance among MRCNS, in cotrast to heterogeneous gene expression.[33, 34]

SCC*mec* type IV and V are common among MRCNS isolated from livestock.[15, 35] To our knowledge, this is the first report of MRCNS harboring SCC*mec* type IX in pigs in Thailand. However, this SCC*mec* type has been previously reported in LA-MRSA associated with CC9 and CC398 strains. So far, these strains isolated from pigs and humans associated with livestock have been documented only in Thailand. Five MRSA isolates were also detected previously in one of the four farms investigated in this study; they were characterized as ST9-t337-IX. Only two SCC*mec* types were identified in MRCNS isolates, including SCC*mec* type IX (6 isolates) and type V (3 isolates). SCC*mec* type IX predominated in a variety of CNS species, including: *S. saprophyticus, S. cohnii*, *S. caprae*, and *S. haemolyticus*. Nevertheless, SCC*mec* type V was also found in *S. saprophyticus* and *S. haemolyticus*. Therefore, to date, type-IX SCC*mec* elements are geographically restricted to LA-MRSA and -MRCNS in Thailand. The *ccr* gene was not detected in most of the non-typeable isolates. The *ccr* gene might be an unrecognized type, deleted or contain a mutation, thus making it undetectable by PCR technique. In contrast, the class C2 *mec* was commonly detected in these isolates. The class C2 *mec* has been identified in both SCC*mec* type V and IX. In addition, we found combinations of more than one SCC*mec* type in single MRCNS species which were found in *S. cohnii* containing type-1, type -5 *ccr*/class A, C2 *mec*. Thus, this isolate could be identified as SCC*mec* types V and IX. However, the class A *mec*, which was reported in SCC*mec* types II, III and VIII, was also found in this isolate. The co-existence of two SCC*mec* types has been found in clinical MRCNS isolates reported by Zong *et al.*[36] These included SCC*mec* types III+V, II+V, II+IV and IV+V.[36] Interestingly, we found the combination of *ccr* type and *mec* class, which has not been recognized before, type -5 *ccr*/class A *mec* in *S. xylosus*. These may be novel SCC*mec* types. However, they need to be characterized further.

Conclusion

MRCNS strains in pigs with MDR phenotypes were associated with opportunistic pathogens, such as *S. saprophyticus* and *S. haemolyticus.* This is of concern in view of the spread of MDR staphylococci via the food chain and transmission from pigs to humans and subsequently to hospitals. The type-IX SCC*mec* element was distributed among diverse CNS species, suggesting the possibility of horizontal transfer between staphylococcal species, including *S. aureus*, an important human pathogen. Molecular typing methods such as SCC*mec* typing are important for insight into the evolution and epidemiology of MRCNS. Pig farms may provide an environment for MRCNS strains as potential reservoirs of diverse SCC*mec* elements and drug-resistant genes. Monitoring of prevalence and antibiotic resistance profile among MRCNS in livestock is necessary for preventing and controlling the spread of these strains to the community and hospitals.

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