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Original Article

Occurrence and characterization of livestock-associated methicillin-resistant *Staphylococcus aureus* in pig industries of northern Thailand

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This study was conducted to determine the prevalence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in pigs, farm workers, and the environment in northern Thailand, and to assess LA-MRSA isolate phenotypic characteristics. One hundred and four pig farms were randomly selected from the 21,152 in Chiang Mai and Lamphun provinces in 2012. Nasal and skin swab samples were collected from pigs and farm workers. Environmental swabs (pig stable floor, faucet, and feeder) were also collected. MRSA was identified by conventional bacterial culture technique, with results confirmed by multiplex PCR and multi locus sequence typing (MLST). Herd prevalence of MRSA was 9.61% (10 of 104 farms). Among pigs, workers, and farm environments, prevalence was 0.68% (two of 292 samples), 2.53% (seven of 276 samples), and 1.28% (four of 312 samples), respectively. Thirteen MRSA isolates (seven from workers, four from environmental samples, and two from pigs) were identified as Staphylococcal chromosomal cassette *mec* IV sequences type 9. Antimicrobial sensitivity tests found 100% of the MRSA isolates resistant to clindamycin, oxytetracycline, and tetracycline, while 100% were susceptible to cloxacillin and vancomycin. All possessed a multidrug- resistant phenotype. This is the first evidence of an LA-MRSA interrelationship among pigs, workers, and the farm environment in Thailand.

Keywords: environment, MRSA, pigs, Thailand, workers

Introduction

Staphylococcus (*S.*) *aureus* is an opportunistic bacterium and a part of the microflora in humans as well as various animals [27]. It frequently colonizes the anterior nares [30] and may cause infection when the host immune system becomes compromised. This microorganism has developed resistance to methicillin (*i.e.*, methicillin-resistant *S. aureus*; MRSA) by acquiring the *mec*A gene that is part of a large mobile genetic element [26] as first reported in 2003 [19]. MRSA has become a pathogen of increasing importance in hospitals, the community, and livestock operations [10]. To date, livestock-associated MRSA (LA-MRSA) has been found worldwide, particularly among people who are involved with livestock farming [9,22,29]. These bacteria can be transmitted to humans in close contact with MRSA-colonized animals [23]. Livestock, especially pigs, can serve as reservoirs for LA-MRSA [15]. The prevalence of LA-MRSA among pigs, agriculture workers, and the environment varies according to geographic area. The majority of LA-MRSA strains belong to sequence type (ST) 398 in Europe and America while ST9 is found in Asia [6,7,18,20]. In Thailand, MRSA has been isolated from healthy pigs [1,14] and pork [28]. However, LA-MRSA prevalence in livestock, especially among pigs

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in Thailand, is unknown. The goals of this study were therefore to determine the prevalence of MRSA in pig farms, farm workers, and the farm environment in northern Thailand as well as investigate the genotypic and phenotypic characteristics of MRSA to evaluate potential relationships between humans, animals, and the farm environment.

Materials and Methods

Study design and study populations

This cross-sectional study was conducted on pigs, workers, and the environment of pig farms in Chiang Mai and Lamphun provinces of northern Thailand in 2012. Farm operations varied from large industrial facilities to small organizations. Target populations of the pig farms located in both provinces were from 21,152 farms based on a 2012 pig farm registry from the Department of Livestock Development, Ministry of Agriculture (Thailand). The appropriate sample size was calculated for the pig farms with an expected prevalence of 20% [16], accepted error of 10%, and 95% confidence interval using Win Episcope 2.0 [25]. One hundred and five pig farms were determined to be needed, and proportional sampling was conducted with a 7 : 1 ratio of Chiang Mai farms (n = 18,508) to Lamphun farms (2,644). Finally, 62 and 53 pig farms from Chiang Mai and Lamphun provinces, respectively, were randomly selected for the investigation.

Sample and data collection

Demographic data for the farms and information about farm management practices including farm types, number of pigs, herd size, period of operation, and personnel protective equipment used by the workers were collected. Swab samples from pigs, workers, and the environment were also taken using the following protocols:

Pigs: At each farm, groups of weaning pigs, fattening pigs, and sows were sampled if present. For each group, nasal and skin swabs were collected from five randomly selected pigs by a veterinarian. The nasal and skin samples were collected from both sides of the external nares and auxiliary regions using sterile cotton swabs (Oxoid, UK). Swab samples from each group of five pigs for each site (nasal and skin swabs) were pooled.

Workers: Farm workers were invited to participate in the study if they had been employed by the farm for at least 1 year. A maximum of two workers were recruited for each farm. All eligible participants were asked to provide written informed consent. Samples from both sides of the participants' external nares and auxiliary regions were collected using sterile cotton swabs.

Environment: Environmental samples were collected from pig stables. Five sites including the stable floor, faucet, and feeder were assayed using cotton swabs.

All swab samples were stored separately in and stored in Stuart transport medium (Oxoid), kept cool in an ice box and transported to the central laboratory at Chiang Mai University, Faculty of Veterinary Medicine within 24 h for further analysis.

MRSA isolation and identification

All swab samples were incubated for 48 h at 37°C in pre-enrichment media containing tryptic soy broth (Oxoid) with 10 mL of 10% NaCl. Next, the samples were streaked onto mannitol salt agar (Merck, USA) with 6 mg/L of oxacillin (Oxoid) and incubated at 37°C for 24 h. Three single colonies suspected to be *S. aureus* derived from each sample were selected and identified by Gram staining with Gram-positive cocci and catalase activity.

The colonies were re-streaked onto tryptic soy agar plates (Oxoid) and incubated at 37°C for 24 h. A coagulase test was carried out, and the presumptive positive samples were further screened for methicillin resistance by disc diffusion with 1 μg oxacillin (diameter of the inhibition zone for MRSA must be less than 10 mm.) [4]. Identification of MRSA isolates was further confirmed by multiplex PCR specific for the *mec*A gene. All MRSA isolates were kept in brain-heart infusion broth (Oxoid) with 15% glycerol at −70°C and sent for molecular testing at central laboratory at Chiang Mai University, Faculty of Veterinary Medicine.

Molecular analyses

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing: The type of the SCC*mec* gene complex in the isolates was determined using a previously described multiplex PCR method. Bacterial cultures were sedimented and transferred in 200 μL of Chelex 100 buffer (Bio-Rad Laboratories, USA). DNA was extracted by boiling bacterial suspensions [5]. Multiplex PCR was applied to test for mecA detection and to classify the SCC*mec* type and subtype followed by Zhang *et al*. [32]. DNA master mixed (50 μL total volume) contained 0.5 μL of DNA, 50 mM $MgCl_2$, 5 μL of 10× buffer, 0.2 mM dNTPs, 1.0 U *Taq* DNA polymerase (Thermo Fisher Scientific, USA) and 0.2 μM primers. Amplification (PTC 200 Thermal Cycler; Bio-Rad Laboratories) was carried out as follows: 94°C for 5 min; followed by 10 cycles of 94°C for 45 sec, 65°C for 45 sec and 72°C for 90 sec; followed by 25 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 90 sec; followed by a final heating at 72°C for 10 min. Amplicons were visualized under UV light in 2% agarose gel-electrophoresis, which ethidium bromide stained (Geldoc 200; Bio-Rad Laboratories).

Multi locus sequence typing (MLST): MLST analysis was performed according to a method previously described by Enright *et al*. [8]. Amplification analyses of seven housekeeping genes (*arc*, *aro*, *glp*, *gmk*, *pta*, *tpi*, and *yqi*)

were performed following the *S. aureus* MLST website. The PCR products were purified and sequenced according to the manufacturer's protocol (Bio Basic Canada, Canada). Allelic profiles and sequence types of the MRSA isolates were identified using the *S. aureus* MLST database. Finally, phylogenetic tree of isolates tested was generated and compared with 68 MRSA isolates from Thailand provided by the *S. aureus* MLST website using Bionumerics 3.5 with character data module (Applied Maths NV, Belgium).

Antimicrobial susceptibility test (AST)

An AST was performed using the Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [4] with a 24-h incubation period at 35°C. Discs with the following compounds were used: amoxicillin-clavulanic acid, cefoxitin, ceftriaxone, cephazolin, chloramphenicol, clindamycin, penicillin, cloxacillin, doxycycline, gentamycin, oxytetracycline, sulfa-trimethoprim, tetracycline, and vancomycin (Oxoid).

Data analyses

Data management and analyses were performed using Win Episcope 2.0 [25]. The farm was the unit of analysis. Descriptive statistics including the proportion, mean, median, prevalence ratio, and 95% confidence interval were calculated. A farm was considered to be MRSA-positive if MRSA isolates were found in at least one swab sample (from pigs, workers, or the environment) from that farm.

Results

In total, 880 samples from 104 farms were processed in this study. One farm was excluded because the business had closed. Out of the 208 workers from the study farms, 138 were recruited for the study because they had more than 1 year of experience on the farm.

Information pertaining to the isolation and identification of *S. aureus*, methicillin-sensitive *S. aureus* (MSSA), and methicillin-resistant *S. aureus* (MRSA) is shown in Table 1. Thirteen MRSA isolates were collected. The MRSA prevalence was 0.68% among pigs (two out of 292 samples), 2.53% among workers (seven out of 276 samples), and 1.28% in the environment (four out of 312 samples). Herd prevalence of LA-MRSA was 9.61% (10 out of 104 farms). There was one farm in Chiang Mai where LA-MRSA was isolated from both a pig and the environment (Table 2).

Molecular typing was performed for the 13 MRSA isolates. All isolates belonged to ST9 (alleic profile: 3-4-1-1-1-1-10) and carried the SCC*mec* IV gene complex (Fig. 1). A phylogenetic tree containing the 13 ST9 MRSA isolates from the pigs, workers, and environment recovered in this study along with 68 submitted isolates from previous

Table 1. Prevalence and 95% confidence interval (CI) of *Staphylococcus* (*S.*) *aureus*, methicillin-sensitive *S. aureus*, and methicillin-resistant *S. aureus* (MRSA) among pigs, workers,and environment of farms in Chiang Mai and Lamphun (Thailand)

	N = 880	%*S. aureus* (n)	95% CI	%MSSA (n)	95% CI	%MRSA (n)	95% CI
Nursery pigs							
Nasal	24	ND	ND	ND	ND	ND	ND
Skin	23	ND	ND	ND	ND	ND	ND
Fattening pigs							
Nasal	65	1.5 (1)	0.0−4.5	ND	ND	1.5 (1)	0.0−4.5
Skin	69	4.3 (3)	0.0−9.2	2.9 (2)	0.0−6.9	1.4 (1)	0.0−4.3
Sows							
Nasal	57	ND	ND	ND	ND	ND	ND
Skin	54	ND	ND	ND	ND	ND	ND
Sub-total	292	1.4 (4)	0.0−2.7	0.7 (2)	0.0−1.6	0.7 (2)	0.0−1.6
Environment							
Stable floor	104	ND	ND	ND	ND	ND	0.0
Nipple	104	2.9 (3)	0.0−6.1	1.9 (2)	0.0−4.5	1.0 (1)	0.0−2.8
Feeder	104	2.9 (3)	0.0−6.1	ND	ND	2.8 (3)	0.0−6.1
Sub-total	312	1.9 (6)	0.3−3.4	0.6 (2)	0.0−1.5	1.3 (4)	0.0−2.5
Workers							
Nasal	138	7.9 (11)	3.5−12.5	5.1 (7)	1.4−8.7	2.9 (4)	0.0−5.7
Skin	138	2.9 (4)	0.0−5.7	0.7 (1)	0.0−2.1	2.8 (3)	0.0−4.6
Sub-total	276	5.4 (15)	2.8−8.1	2.9 (8)	0.9−4.9	2.5 (7)	0.7−4.4

N: total number of samples, n: number of samples in each column, ND: not detected.

Table 2. Distribution of MRSA and antibiotic resistance patterns of isolates from pig farms in Chiang Mai and Lamphun (Thailand)

Sample ID	Farm ID	Location	Sample type			Antibiotic resistance patterns
			Pig	Worker	Environment	
W1A4	4	CM		■		CN-DA-DO-OT-P-SXT-TE
W2A4	4	CM		■		CN-DA-OT-P-SXT-TE
W1N6	6	CM		■		DA-FOX-OT-P-SXT-TE
J8	8	CM			■	DA-FOX-OT-P-SXT-TE
R8	8	CM			■	DA-OT-P-SXT-TE
R10	10	CM			■	CRO-DA-FOX-OT-P-SXT-TE
W1A12	12	CM		■		CN-DA-DO-FOX-OT-P-SXT-TE
W1N13	13	CM		■		DA-FOX-OT-P-SXT-TE
FN29	29	CM	■			AMC-C-CN-DA-FOX-KZ-OT-P-TE
R29	29	CM			■	AMC-C-CN-CRO-DA-DO-FOX-KZ-OT-P-SXT-TE
W1N43	43	LP		■		AMC-CN-CRO-DA-DO-FOX-KZ-OT-P-SXT-TE
FA60	60	LP	■			AMC-C-CN-DA-DO-FOX-OT-P-SXT-TE
W1N66	66	LP		■		AMC-C-CN-DA-FOX-OT-P-SXT-TE

CM: Chiang Mai, P: penicillin, C: chloramphenicol. CN; gentamycin, DA; clindamycin, DO; doxycycline, OT; oxytetracycline, SXT; sulfa-trimethoprim, TE; tetracycline, FOX; cefoxitin, CRO; ceftriaxone, AMC; amoxicillin, KZ; cephazolin, LP: Lamphun.

studies in Thailand was generated by the computer software. These findings are presented in Fig. 2.

The 13 MRSA isolates were tested for antimicrobial susceptibility. Sources of the isolates were from pigs (two isolates), workers (seven isolates), and the farm environment (four isolates). Susceptibility testing revealed that 100% of the isolates were resistant to clindamycin, oxytetracycline, tetracycline, and penicillin. No resistance to cloxacillin or vancomycin was observed (Fig. 3). There were 11 different patterns of antimicrobial resistance among the pig, worker, and farm environment isolates (Table 2). All were resistant to at least five antimicrobial agents (Fig. 4). One was resistant to 12 antimicrobial drugs (environment isolate AMC-C-CN-CRO-DA-DO-FOX-KZ-OT-P-SXT-TE).

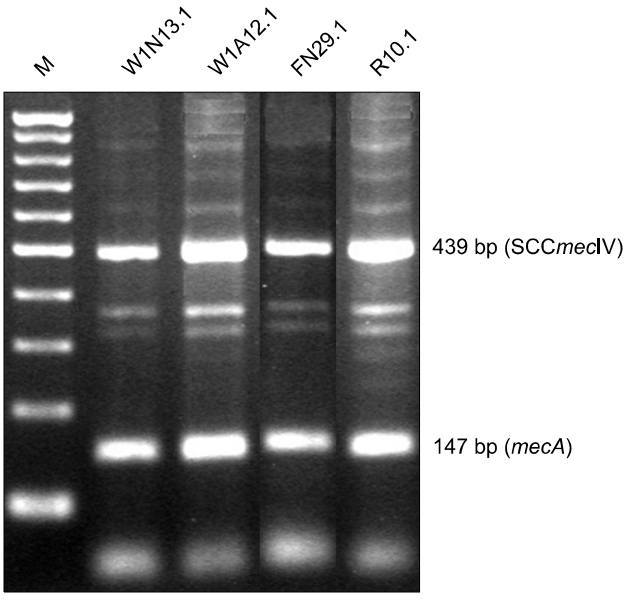

Fig. 1. SCC*mec*-specific multiplex PCR results for worker, pig, and environment samples. M, marker; W1N13.1 and W1A12.1, worker; FN29.1, pig; R10.1, environment.

Discussion

Findings from this study represent basic information regarding the burden of MRSA associated with pig industries. The herd prevalence of LA-MRSA among pig farms in northern Thailand was 9.61%. This figure is quite similar to one reported in a previous study (10.0%) conducted in the same region [15] but lower than rates found in other countries between 2008 and 2013 including 22.7% in Korea [16], 26% in Canada [13], 36% in the USA [22], 39% in the Netherlands [7], and 49% in Germany [24]. However, prevalence can vary depending on many factors including geographical region, sampling methods, laboratory testing methods [3], and age of the pigs tested [13]. The large size of commercial farms in the USA and European countries may facilitate more opportunities for pathogen transfer and higher prevalence of MRSA compared to the smaller farms in Thailand evaluated in the present study.

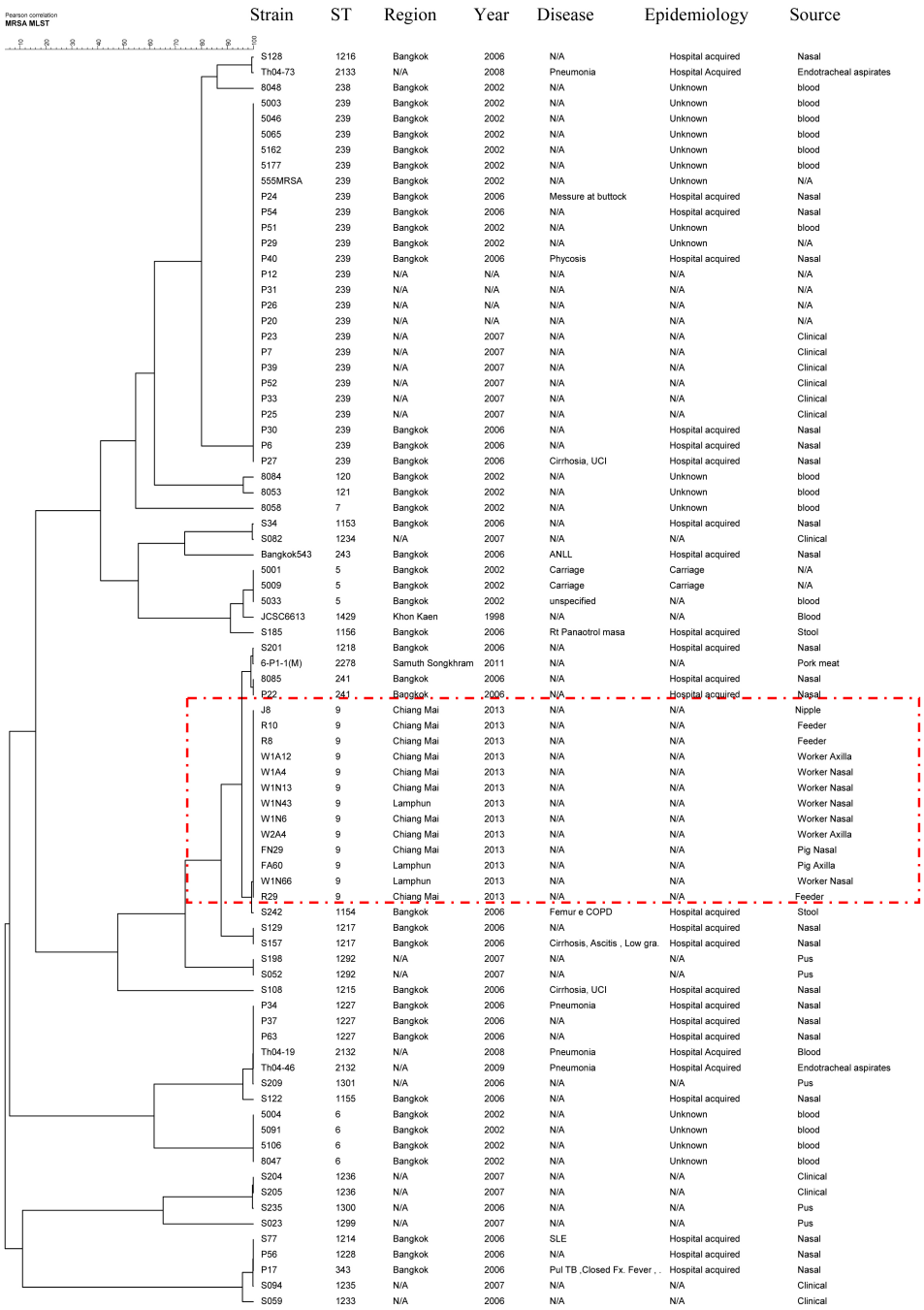

Fig. 2. Phylogenetic tree showing the relationships of MRSA ST9 from pigs, farm workers, and the environment of pig farms in Chiang Mai and Lamphun (Thailand) with 68 MRSA isolates from Thailand over the *S. aureus* multi locus sequence typing website. ST: sequence type.

MRSA colonization among pig workers in northern Thailand was low (2.53%) compared to rates found among pig workers in Europe [17] and the USA [13]. MRSA prevalence for at-risk populations including slaughterhouse workers and veterinarians in Europe was found to range between 3% and 12.5% [12,26,31]. Our results revealed that pig workers in northern Thailand are at a lower risk of MRSA colonization than employees from other countries, perhaps because of the prevalence of MRSA among pigs is lower.

The prevalence of MRSA isolated from the environment in this study was 1.28%, which was lower than the rate (17.3%) previously reported in the USA [10]. Staphylococci in the farming environment could serve as a source for

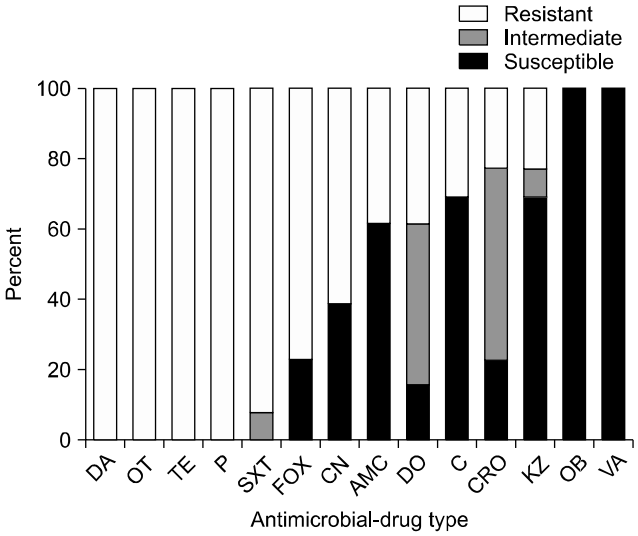

Fig. 3. Antimicrobial susceptibility test results for the MRSA isolates from pig farms in Chiang Mai and Lamphun (Thailand). OB; cloaxacillin, VA; vancomycin.

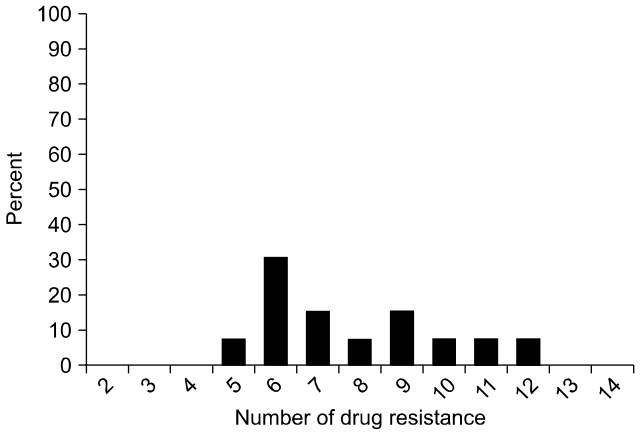

Fig. 4. Proportion of multi-drug resistant MRSA isolates from pig farms in Chiang Mai and Lamphun (Thailand).

MRSA generation as this microorganism is easily detected in both pigs and the environment [11]. We found that MRSA-positive samples were simultaneously collected from both pigs and the environment on only one farm. MRSA-positive samples from both pigs and workers were not simultaneously collected at any of the farms.

Our study revealed that all LA-MRSA isolates were ST9 with the SCC*mec* IV gene complex. ST9 represents the most common sequence type in Asia [5,14,18], and is a dominant clone among pig and pig-related workers [2]. Recent reports indicate that colonization by MRSA ST9 with different SCC*mec* types occurs in pigs and pork from the northern and central regions of Thailand [1,14,28]. As shown in Fig. 2, the sequence type was similar among isolates from workers and the environment, which suggests that MRSA ST9 was circulating among workers and through the environment.

MRSA is a human bacterial pathogen that has emerged as a major threat in hospitals (as a nosocomial infection) and the cause of community-acquired infection among high-risk groups such as slaughterhouse workers [10]. The use of antibiotics in livestock production has promoted the development of multi-drug resistance. In the current study, various resistance phenotypes of the MRSA isolates from farm workers and the environment were observed with combined resistance to clindamycin, cefoxitin, tetracycline, penicillin, and sulfa-trimethoprim. Similar patterns of MRSA resistance to clindamycin, cefoxitin, tetracycline, and ciprofloxacin were found in other studies of MRSA ST9 from China [6]. These antibiotics are commonly used in both human medicine and food animal health management. Overuse or misuse of medically important antibiotics in animals is emerging as a public health concern due to increased incidence of community-associated antibiotic-resistant infection [21].

To the best of our knowledge, our study is the first to demonstrate the prevalence of LA-MRSA among pigs, farm workers, and the environment in Thailand. Continuous efforts to monitor MRSA in on the farms and among these populations are required to detect epidemiological changes and implement effective control measures to protect livestock and human health. Further investigations in different areas in Thailand, such as the central or eastern part of the country where the largest pig populations reside, should also be performed.

One limitation of the present study was that the sampling method did not include the use a stratified sampling technique to study a specific production system. Additionally, the study was somewhat underpowered because the observed MRSA prevalence was lower than the expected MRSA prevalence used for sample size calculations. Moreover, only one farm out of 104 had MRSA-positive samples from pigs. Alternative techniques for isolating and detecting MRSA should be considered. Other works have indicated that the prevalence of MRSA among pigs in Thailand might be quite high given that a small number of samples have been tested [1,14,27].

In conclusion, our study is the first to determine MRSA prevalence among pigs, farm workers, and the environment in Thailand. The prevalence of MRSA was low among pig farms in northern Thailand compared to rates reported in other countries. Isolates from the workers and environment were identified as sequences type 9 with Staphylococcal chromosomal cassette *mec* IV (ST9 SCC*mec* IV). In addition, multi-drug resistant MRSA isolates were observed. Continued efforts are required to monitor MRSA among at-risk populations including livestock and slaughterhouse workers to detect changes in epidemiology and implement

effective control measures.

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Conflict of Interest

There is no conflict of interest.

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