Investigation of the Emergence of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Clonal Complex 398 in Thailand's Swine Industry using Whole-Genome Sequencing

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CONTENTS

ABBREVIATIONS

Abbreviation	Definition
AMGs	Aminoglycosides
ARG	Antimicrobial resistance gene
BLs	Beta-lactams
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CI	Composite island
CK10	Cytokeratin 10
ClfB	Clumping factor B
CoNS	Coagulase-negative staphylococci
CoPS	Coagulase-positive staphylococci
FOS	Fosfomycin
FQs	Fluoroquinolones
gDNA	Genomic deoxyribonucleic acid
GPAs	Glycopeptide antibiotics
GTR	General time-reversible
HA-MRSA	Healthcare-associated methicillin-resistant *Staphylococcus aureus*
HPD	Highest posterior density
IEC	Immune evasion gene cluster
Isd	Iron-regulated surface determinant protein
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
K2P	Kimura's two-parameter substitution model

LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LINs	Lincosamides
LmrS	Lincomycin resistance protein of *Staphylococcus aureus*
MATE	Multidrug and toxic compound extrusion
MCC	Maximum clade credibility
MCMC	Markov chain Monte Carlo
MDR	Multidrug-resistant
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
MLS_B	Macrolide-lincosamide-streptogramin B compounds
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
NP	Nakhon Pathom
NR	Nakhon Ratchasima
NT	Non-typeable
NZL	New Zealand
PB	Prachin Buri
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHEs	Phenicols
$PhLOPS_A$	Phenicols-oxazolidinones-lincosamides-pleuromutilins-streptogramin A compounds
PLS_A	Lincosamides-pleuromutilins-streptogramin A compounds

PVL	Panton–Valentine leukocidin
QACs	Quaternary ammonium compounds
RAPD	Random amplified polymorphic DNA
RB	Ratchaburi
RIF	Rifampicin
SB	Suphanburi
SCC*mec*	Staphylococcal cassette chromosome *mec*
SE	Staphylococcal enterotoxin
SEL	Staphylococcal enterotoxins-like toxin
SMR	Small multidrug resistance
SNP(s)	Single nucleotide polymorphism(s)
SSTIs	Skin and soft tissue infections
ST(s)	Sequence type(s)
TCs	Tetracyclines
TMP	Trimethoprim
TSST-1	Toxic shock syndrome toxin-1
UK	United Kingdom
USA	United States of America
WGS	Whole-genome sequencing

Symbol	**Definition**
-	Not detected
+	Detected

LIST OF PUBLICATIONS

Chapter I:

1. Narongpun P, Chanchaithong P, Yamagishi J *et al*. Whole-Genome Investigation of Zoonotic Transmission of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Clonal Complex 398 Isolated from Pigs and Humans in Thailand. *Antibiotics (Basel)*. 2023;12(12):1745.

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Chapter II:

1. Emergence of Livestock-Associated MRSA Clonal Complex 398 in Thailand's Swine Industry (currently under review for publication in the Journal of Antimicrobial Chemotherapy).

PREFACE

Staphylococcus species are Gram-positive cocci of the family Staphylococcaceae, commensally colonizing the mucous membranes, skin, and upper respiratory tract of diverse mammalian species [1,2]. In routine laboratory practice, these microorganisms are typically classified as coagulase-positive or coagulase-negative. While coagulase-negative staphylococci (CoNS) encompass various species, coagulase-positive staphylococci (CoPS) are predominantly represented by *Staphylococcus aureus* [2]. In humans, *S. aureus* can serve as an opportunistic pathogen, causing diseases that range from localized skin lesions to invasive infections, such as abscesses, skin and soft tissue infections (SSTIs), and bloodstream infections [3]. *S. aureus* can also adapt to various host environments in animal species, including companion animals and livestock. Isolates from dogs and cats often originate from human hosts and can be transmitted between pets and their owners [1]. In dairy farming, *S. aureus*, along with *Escherichia coli*, is a primary causative pathogen of bovine mastitis, resulting in economic and production losses in the dairy supply chain. *S. aureus* in swine and poultry is usually associated with skin infections and meat contamination, posing a potential risk for foodborne illness in humans [1]. These factors underscore the clinical significance of *S. aureus* in both human and veterinary medicine, emphasizing its role in zoonotic transmission and food safety [1,4].

Antibiotics refers to a class of antimicrobial agent that can kill bacteria or inhibit bacterial growth [5]. Salvarsan was the first antimicrobial compound used to treat bacterial infections. This arsenic-based compound was synthesized in 1910 by Paul Ehrlich and Sahachiro Hata for the treatment of syphilis caused by *Treponema pallidum*. The discovery of true antibiotics by Alexander Fleming in 1928 marked one of the greatest breakthroughs in medical history, revolutionizing therapeutic strategies involving antimicrobials [6]. Fleming observed that *S. aureus* failed to grow on a culture plate contaminated with *Penicillium notatum*, which secreted an antibacterial substance later identified as penicillin [7]. Penicillin is an inhibitor of cell wall biosynthesis by binding to penicillin binding proteins (PBPs), therefore blocking cross-linking of peptidoglycan—an essential structural component of the bacterial cell wall. This mechanism disrupts the integrity of bacterial cell wall, resulting in cell death. In addition to *S. aureus* infections, penicillin was also demonstrated to successfully treat streptococcal infections, including sepsis and meningitis [6,8,9]. Following this, the introduction of various antimicrobial classes, such as aminoglycosides, tetracyclines, and sulfonamides, significantly advanced bacterial infection treatment. However, the widespread use of these antimicrobial agents has driven the emergence of antimicrobial resistance, which has now become one of the most critical global health challenges [5].

S. aureus strains resistant to penicillin initially emerged in the early 1940s, soon after the introduction of antibiotics into medical treatments [5]. The *blaZ* gene, which encodes beta-lactamase

(penicillinase), enables *S. aureus* to inactivate penicillin by hydrolyzing the beta-lactam ring [10]. Initially, this penicillin-resistant strain was primarily disseminated in healthcare settings, but later spread to community environments [8]. To address the challenge of penicillin-resistant *S. aureus*, methicillin, a semi-synthetic antibiotic, was developed in 1959. Due to its chemical structure, methicillin is not susceptible to degradation by beta-lactamase. However, methicillin-resistant *Staphylococcus aureus* (MRSA) emerged as a nosocomial strain within hospitals in the UK only a year later [5]. It could exhibit resistance to a broad range of beta-lactams, subsequently limiting treatment options and increasing a risk of therapeutic failure [3]. So far, MRSA has become endemic in healthcare facilities worldwide and has been further spread in the community, affecting healthy individuals with no recent history of hospital exposure [3]. Since then, this spread has established MRSA as a significant threat for global public health. The Centers for Disease Control and Prevention (CDC) estimated that approximately 2% of the USA population carries MRSA. Its resistance to multiple antimicrobials contributes to increased morbidity and mortality, with around 80,000 invasive infections reported in the USA in 2011 and over 6,000 deaths in European countries in 2020 [11,12].

MRSA mediate resistance against methicillin and other β-lactam drugs through penicillin-binding protein 2a (PBP2a), which is encoded by the *mecA* gene. This gene resides on the staphylococcal chromosome cassette *mec* (SCC*mec*), a mobile genetic element located on *Staphylococcus* spp. chromosome. Due to its lower affinity for β-lactam antibiotics compared to native PBP2, this alternative protein enables MRSA to maintain cell wall synthesis and survive in hosts despite antimicrobial exposure [11]. In addition to *mecA* gene, this mobile genetic element can also contain additional resistance genes and other genetic determinants, such as *tet* genes encoding tetracycline resistance, *czrC* gene conferring cadmium and zinc resistance, and cassette chromosome recombinase (*ccr*) genes [13,14]. The *ccr* genes are believed to play a crucial role in the insertion and excision of SCC*mec*, facilitating horizontal gene transfer and contributing to the spread of antimicrobial resistance genes among staphylococcal species [14,15].

Since the 1960s, the rising prevalence of MRSA in healthcare settings such as hospitals, medical centers, and nursing homes led to the description of healthcare-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA). This nosocomial pathogen is commonly multidrug resistant due to antibiotic selection pressure in healthcare environments, affecting not only patients with prolonged hospitalization, but also those with recent healthcare contact [3]. Furthermore, HA-MRSA infections are frequently associated with the long-term use of indwelling medical devices, percutaneous catheters, orthopedic implants, and other invasive devices, which serve as major sites for MRSA colonization [16]. Notably, elderly adults and populations with compromised immune systems or chronic diseases have been shown to be the most susceptible to HA-MRSA infections [3,16,17]. Clinical presentations of HA-MRSA vary widely. While bloodstream infections and pneumonia are commonly observed in hospitalized patients, SSTIs and surgical wounds infection may also occur [3].

MRSA infections in humans are not confined to healthcare settings; they also occur in the community, where they are classified as community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) [17]. Since the 1980s, CA-MRSA has emerged in general populations without prior healthcare contact, contributing to outbreaks among children in schools, athletes on sports teams, and military personnel [18,19]. Unlike HA-MRSA, CA-MRSA primarily manifests as SSTIs, frequently involving abscesses or purulent lesions, which account for approximately 90% of reported cases. CA-MRSA is reported to have lower antibiotic resistance compared to HA-MRSA. However, CA-MRSA can also cause life-threatening infections, including necrotizing osteomyelitis, pneumonia, and septic shock [3,17]. These severe manifestations are believed to be associated with the presence of Panton–Valentine leukocidin (PVL) genes, which have been detected in over 95% of CA-MRSA isolates [3,17,18]. As a result, the unique epidemiological and clinical features of CA-MRSA distinguish it from HA-MRSA [20].

In addition to human-related MRSA, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) refers specifically to MRSA strains commonly found in livestock, such as pigs, cattle, and chickens, and occasionally in other animal species, including rabbits, horses, dogs, and cats [1,21]. Among these species, pigs are recognized as a major reservoir for LA-MRSA, which can be transmitted to humans and spillover to other animals [16,22,23]. Consequently, individuals in close contact with colonized animals, including swine workers, veterinarians, and slaughterhouse personnel, are at an increased risk of LA-MRSA nasal colonization and subsequent infection [16]. Importantly, nasal carriage of LA-MRSA in humans is often asymptomatic, yet it can pose a considerable concern for further transmission, enabling the spread of the pathogen between carriers and contacts [24,25].

Currently, LA-MRSA is increasingly recognized as an emerging concern in the swine industry, mainly due to occupational transmission [26]. LA-MRSA is generally considered less harmful to humans, as it typically harbors fewer virulence genes compared to HA-MRSA and CA-MRSA strains; however, severe or even fatal infections—such as endocarditis, bacteremia, and pneumonia—can still occur. [3,26,27]. The most predominant LA-MRSA clones in pigs belong to clonal complexes (CC) 9 and CC398, though strains from CC1 and CC97 have also been identified [28]. Notably, LA-MRSA CC9 is particularly prevalent in Asia's pig populations, whereas CC398 is more commonly found in European countries. Nevertheless, the spread of LA-MRSA CC398 has been observed across the globe, with recent studies reporting prevalence rates of 87.5% and 96.3% in pig herds in Thailand and China, respectively [21,28,29].

In addition to antimicrobial resistance profiling, a variety of molecular techniques, including SCC*mec* typing, *spa* typing, pulsed-field gel electrophoresis (PFGE), and multi-locus sequence typing (MLST), have become essential tools in epidemiological studies and outbreak investigations [30]. For

example, PFGE combined with minimum inhibitory concentration (MIC) assays were utilized to investigate MRSA outbreaks in Dutch hospitals [31]. SCC*mec* typing and MLST analysis suggested that bovine LA-MRSA in South Korea may have emerged from a hospital-associated MRSA lineage [32]. Importantly, these molecular methods have also played a pivotal role in investigating zoonotic transmission of LA-MRSA. In 2005, animal-to-human transmission caused by LA-MRSA was first recorded in the Netherlands [25]. Molecular characterization analyses revealed that LA-MRSA isolates from two family members exhibited identical random amplified polymorphic DNA (RAPD) patterns to a porcine LA-MRSA isolate from their farm, and all belonged to the *spa* type t108. Additionally, this same study also identified two clusters of human-to-human transmission [25]. These findings presented initial evidence that LA-MRSA can be transmitted both from animals to humans as well as between humans.

Although the molecular approaches for *S. aureus* typing are extensively utilized in both laboratories and healthcare settings, their limitations should be carefully considered. To date, the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) has defined 14 distinct types of SCC*mec* elements, assigned based on the specific combination of *mec* gene and *ccr* gene complexes [33]. This classification is typically determined using multiplex polymerase chain reaction (PCR) assay, which is restricted to identifying only known SCC*mec* types. While PFGE demonstrates strong discriminatory power, the absence of standardized protocols compromises the comparability of results between different laboratories [30]. *spa* typing is a simple technique that targets the polymorphic X-region of the *spa* gene encoding protein A, but it offers lower discriminatory power compared to PFGE, as it is single-locus sequence analysis [30,34]. Therefore, additional typing analyses are recommended to address this limitation. In contrast, MLST assigns sequence types (STs) to *S. aureus* isolates based on the analysis of seven housekeeping genes, providing higher discriminatory power than *spa* typing, but it poses challenges for routine investigations due to time consumption and high laboratory workload [30,35].

Whole genome sequencing (WGS) is an interdisciplinary laboratory technique that thoroughly analyzes microorganisms' DNA sequences, allowing for a comprehensive examination of complete or nearly complete microbial genomes [36]. By integrating with high-throughput sequencing technologies —whether short-read or long-read—WGS has become a powerful tool with broad applications across microbiological studies [37,38]. In the context of antimicrobial resistance, WGS-based antimicrobial susceptibility testing offers a rapid alternative to phenotypic assay, with genotype–phenotype concordance rates of up to 95% [39,40]. This genomic approach also facilitates the *in-silico* prediction of virulence genes, plasmids, and other mobile genetic elements, potentially uncovering novel determinants and leading to earlier, targeted clinical interventions [37,41]. Importantly, WGS can computationally differentiate bacterial isolates based on even a single nucleotide variation [38]. Consequently, these advantages position WGS as a higher-resolution, more effective alternative to

traditional molecular typing methods. Accordingly, this thesis adopted WGS as the primary investigative tool in both Chapters I and II, enabling a deeper understanding of the genomic characteristics and transmission dynamics of LA-MRSA CC398 in Thailand's swine industry.

In Chapter I, WGS was applied to investigate the first zoonotic transmission of LA-MRSA CC398 between Thia pigs and swine workers on two farms. Maximum-likelihood phylogenetic tree analysis based on core single nucleotide polymorphisms (SNPs) indicated close genetic relationships between LA-MRSA CC398 isolated from pigs and swine workers on two farms, providing evidence of zoonotic transmission. WGS-based strain typing also supported the results from the phylogenetic tree reconstruction. Overall, this chapter demonstrated empirical evidence for the utility and efficacy of WGS for elucidating the zoonotic transmission of LA-MRSA CC398 in Thailand.

As mentioned earlier, LA-MRSA CC398 had not been identified in Thai pigs or swine workers before 2019, it is considered as an uncommon lineage in Thailand. Accordingly, Chapter II aimed to examine the potential origin of LA-MRSA CC398 in Thailand's swine production chain and the factors leading to its first report in 2019. The genomes of the 13 LA-MRSA CC398 isolates, as introduced in the Chapter I, were hybrid assembled to generate complete genomes. Furthermore, five genomes of LA-MRSA CC398 isolates from retail pork and pig carcasses in Thailand were also included. To explore the evolutionary relationship between Thai and international LA-MRSA CC398 isolate, a total of 1,197 LA-MRSA CC398 genomes from 24 countries were phylogenetically compared to the Thai isolates. The maximum likelihood approach suggested the possible country of origin for LA-MRSA CC398 in Thailand, and the Bayesian phylogenetic tree estimated divergence times among the isolates. Additionally, antimicrobial and metal resistance profiling was integrated to provide a comprehensive understanding of the transmission dynamics. This study indicated that the CC398-SCC*mec* Vc (5C2&5)-t034 genotype in Thai pigs may have originated from swine herds in Denmark, possibly via commercial pigs. On the one hands, the CC398-composite SCC*mec*-t034 genotype evolved separately and showed no association with the CC398-SCC*mec* Vc (5C2&5)-t034 genotype and Danish swine.

CHAPTER I

Whole-genome investigation of zoonotic transmission of livestock-associated methicillin-resistant *Staphylococcus aureus* clonal complex 398 isolated from pigs and humans in Thailand

Introduction

LA-MRSA has been found in domestic pigs in Thailand since 2011, with the first identification reported by Anukool *et al.* [42]. The LA-MRSA isolates detected were assigned to CC9 based on classical MLST. SCC*mec* typing and *spa* typing were additionally performed to assess the clonal relatedness among isolates from pigs on farms. Subsequently, studies in different provinces of Thailand further confirmed the presence of LA-MRSA in Thai swine populations [43,44]. Significantly, Patchanee *et al.* reported a prevalence of LA-MRSA CC9 among swine workers, accounting for 2.53%. MLST-based phylogenetic tree analysis revealed that these human-derived isolates were clustered closely with pig-associated strains from the same geographic region, suggesting the zoonotic potential of the CC9 lineage in Thailand [43].

In 2019, zoonotic transmission of LA-MRSA CC398 in swine workers was documented [21]. This previous study employed multiple molecular typing approaches—including PFGE, classical MLST, SCC*mec* typing, and *spa* typing—to characterize the bacterial strains and trace the transmission from pigs to humans. The results suggested zoonotic transmission on two swine farms, as isolates from both porcine and human sources at the same locations shared identical molecular profiles. Interestingly, one porcine CC398 isolate involved in the transmission exhibited a non-typeable (NT) SCC*mec* element, while the other two isolates harbored multiple *ccr* gene complexes, which are not commonly identified in the SCC*mec* cassette. These findings highlight certain limitations of conventional molecular typing techniques, particularly PCR-based methods, which rely on detection of known genetic targets [30].

To date, molecular typing methods have been utilized to suggest zoonotic transmission of LA-MRSA CC9 and CC398 in Thailand. Nonetheless, no WGS-based investigation has been conducted to examine the close genetic relationship between Thai LA-MRSA CC398 isolates derived from pigs and those from workers. As a high-resolution approach, WGS provides powerful discrimination to detect genomic dissimilarity at the nucleotide level [37,38]. Accordingly, this study aimed to apply WGS to comprehensively elucidate the zoonotic transmission dynamics of LA-MRSA CC398 between Thai pigs and farm workers, as previously reported in 2019.

Importantly, since CC398 is an atypical strain of LA-MRSA within Thailand's swine industry, the present study also aimed to characterize the genomic features of LA-MRSA CC398, including antimicrobial resistance-associated genes, virulence determinants, plasmid replicons, as well as other mobile genetic elements using public international databases. Lastly, the study aimed to clarify the presence of non-typeable SCC*mec* elements and the SCC*mec* elements containing multiple *ccr* gene complexes, which have not been fully resolved by conventional molecular methods.

Materials and Methods

LA-MRSA isolates

In the present study, 16 representative isolates of LA-MRSA, previously characterized using molecular typing methods in 2019, were selected for WGS analyses [21]. These isolates were obtained from nasal swabs collected between May 2015 and April 2017 from 467 clinically healthy pigs on 27 farms located in five provinces of Thailand—Nakhon Pathom, Nakhon Ratchasima, Prachin Buri, Ratchaburi, and Suphanburi (Figure 1). In parallel, nasal swabs were also collected from 38 asymptomatic swine workers who voluntarily participated from 16 of these farms.

Nasal swab samples were initially subjected to selective enrichment in Müller–Hinton broth (Difco) with 4 mg/mL cefoxitin and 6.5% NaCl, and incubated at 32 °C for 48 hours. Following this, the enriched broth was transferred into phenol red mannitol broth containing 3.5 mg/mL cefoxitin and incubated under the same conditions. Next, the cultures were streaked onto tryptic soy agar with 5% sheep blood for colony selection. One to three colonies presenting hemolytic activity were selected for biochemical tests, including Gram staining, catalase test, and coagulase test, including biochemical tests, including Gram staining, catalase, and coagulase assays. The identification of *S. aureus* was further confirmed by the detection of *nuc* gene. Methicillin resistance was then tested using both the cefoxitin disk diffusion method and PCR detection of the *mecA* gene.

Eventually, a total of 63 LA-MRSA isolates were cultured from swine farmers ($n = 3$) and pigs ($n = 60$) across 11 swine farms. Molecular characteristics were identified through MLST, SCC*mec* typing, *spa* typing, and PFGE. Based on molecular profiles, farm origin, and host source, the isolates were categorized into 16 groups. One representative isolate from each group—comprising 13 porcine and three human isolates—was randomly selected for further steps.

DNA extraction

The heat-killed bacterial solution of the 16 representative isolates was transported to the Hokkaido University International Institute for Zoonosis Control, Japan, for DNA extraction. Genomic DNA (gDNA) was extracted by the bead-beating technique, as described in a previous study [45]. The concentration and quality of DNA were assessed using a Qubit 3.0 Fluorometer and a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Library preparation, WGS, and genome assembly

Paired-end libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, California, USA). The concentration of each extracted gDNA sample was diluted to 0.2 ng/μL before sequencing on an Illumina MiSeq (2 × 300 bp reads). Raw reads were quantified using FastQC v0.11.9 [46]. The initial 20 bases from the 5′ end were trimmed, and reads

shorter than 100 bp were excluded. Low-quality reads, defined as those with a Phred quality score <20, were then filtered out using Trim Reads Tool from CLC Genomics Workbench v22.0.1 (Qiagen, Hilden, Germany) (https://digitalinsights.qiagen.com/). The remaining trimmed reads were assembled into contigs using SPAdes v3.15.4 on the Galaxy platform [47,48]. Assembly statistics were evaluated with QUAST v5.2.0. An N50 value of at least 50,000 was required for downstream analyses [49,50].

Genomic characterization, in-silico identification of antimicrobial resistance, stress and virulence genes, and mobile genetic elements

In-silico MLST and *spa* typing were performed using MLST v2.0 and *spa*Typer v1.0, respectively [51,52]. SCC*mec* types were determined using SCC*mec*Finder v1.2 with a minimum gene coverage of 80% and minimum identity cut-off of 90% [53].

Acquired antimicrobial resistance genes, mutations in genes associated with antimicrobial resistance, and stress genes were *in-silico* identified using AMRFinderPlus v3.10.42 [54]. Virulence genes were primarily detected using VFDB on the BV-BRC platform v3.28.5 (https://www.bv-brc.org/) [55,56]; however, genes not included in the VFDB were additionally predicted by the AMRFinderPlus. For both bioinformatic tools, the minimum length and percentage identity were adjusted to 80% and 90%, respectively, to determine the absence/presence of a particular gene.

Mobile genetic elements and their associated antimicrobial resistance genes were identified using MobileElementFinder v1.0, with a coverage threshold of 95% and an identity cut-off of 90% [57].

Core genome alignment and phylogenetic tree reconstruction

The trimmed paired-end reads were aligned against a reference genome (LA-MRSA ST398 strain S0385; GenBank accession no. AM990992) using Snippy pipeline v4.6.0. Snippy-core v4.6.0 was used for SNP calling from core-genome alignment [58]. Gubbins was run to eliminate the polymorphic sites of recombination in the alignment [59]. Further, the number of pairwise SNP distances was computed using Snp-Dists v0.8.2 [60]. The phylogenetic tree of LA-MRSA CC398 based on SNPs in the core genome was reconstructed on MEGA v11 [61]. The maximum-likelihood method, together with Kimura's two-parameter substitution model (K2P), was utilized to infer the evolutionary tree [62]. The bootstrap support values of 1000 replicates were calculated to assess the robustness of each node on the resulting tree. The final phylogenetic tree was visualized and integrated with a set of metadata using iTOL v6.8 [63].

Results

Data preprocessing and genome assembly

The genomes of 16 LA-MRSA isolates were sequenced using a paired-end read approach, generating raw reads ranging from 35 to 301 bp in length (Table 1), with total read counts between 734,372 and 2,806,085. Following quality trimming, which included the removal of 20 nucleotides from the 5′ end, the read lengths were narrowed to 100–281 bp, and the number of reads was reduced to between 273,555 and 879,902. This reduction accounted for approximately 50–70% of the total reads.

Subsequent genome assembly was performed using SPAdes, with assembly metrics summarized in Table 2. The assembled genomes ranged from 2.79 Mbp to 2.87 Mbp, with contig counts per genome varying from 38 to 91. The largest contig with a length of 564,703 bp was observed in the strain D16.1, while the lowest N50 value, 53,208 bp, was identified in the strain M3.1.

WGS-based characteristics

The most prevalent genotype (sequence type-SCC*mec* type-*spa* type) was ST398-SCC*mec* V-t034 (9/16), followed by ST398-SCC*mec* composite island (CI)-t034 (4/16), ST9-SCC*mec* IX-t337 (2/16), and ST4576-SCC*mec* IX-t337 (1/16) (Table 3). The type V SCC*mec* element of the ST398-t034 strain contained a class C2 *mec* gene complex with a type 5 of *ccr* gene complex (*ccrC1*). The SCC*mec* type IX, present in all members of CC9 (ST9-t337 and ST4576-t337 isolates), comprised the class C2 *mec* gene complex and a type 1 *ccr* gene complex (*ccrA1B1*). Interestingly, the composite SCC*mec* elements identified in the four ST398-t034 isolates were organized by a combination of multiple *ccr* gene complexes, *ccrA1B1* and *ccrC1*, and the *mec* class C2 complex.

SNP-based analyses and transmission of LA-MRSA CC398

Figure 2 presents the phylogenetic tree based on SNPs in the core genome of the 13 LA-MRSA CC398 isolates. The isolates were divided into two distinct phylogenetic clades, namely, Clade I and Clade II. Almost all internal nodes had bootstrap support of 90% or greater. Clade I was occupied by the four LA-MRSA isolates carrying the composite SCC*mec* elements from Farm 1 and Farm 11. Two porcine isolates and one human isolate from Farm 1 apparently were clustered in the same sub-clade, elucidating a high degree of genome relatedness among them; however, one human isolate from Farm 11 was distantly related to those from Farm 1. Clade II was completely clustered by LA-MRSA isolates carrying the SCC*mec* type V. Three LA-MRSA isolates from Farm 3 and Farm 4, located in Suphanburi, were placed into the same sub-clade showing a geographical specificity. Like Farm 1, one porcine isolate from Farm 3 was clustered tightly with a human isolate obtained from the same location. The other six porcine isolates in Clade II, obtained from five different farms in Prachin Buri and Nakhon Ratchasima, resided together in a discrete sub-clade.

To verify all transmission events detected in this study, a pairwise SNP distance matrix made from the core genome alignment was investigated (Table 4). In Clade I, the pairwise SNP distances between the two porcine strains L3.1 and Z19.1 from Farm 1 were 13-core genome SNPs. These porcine strains were even closer to the human strain L43.2 from the same farm with 11 and 10 SNPs, respectively. On top of that, in Clade II, the porcine strain M3.1 from Farm 3 was only three SNPs distant from the human strain M31.1 from the same farm. This indicates that both isolates were genomically related and originated from the same root. For Farm 4, the porcine strain AA3.1 was separated from both isolates from Farm 3 (the strains M3.1 and M31.1) by two and three SNPs, respectively. The remaining porcine strains in this clade, collected from Prachin Buri and Nakhon Ratchasima, shared a common ancestor with a different number of SNP distances ranging from 14 to 38 SNPs.

Antimicrobial resistance-associated genes and stress genes

A total of 32 antimicrobial resistance-associated genes, mediating resistance to 14 antimicrobial groups, were detected: aminoglycosides (*n* = 6), beta-lactams (*n* = 3), fluoroquinolones (*n* = 2), fosfomycin (*n* = 3), glycopeptide antibiotics (*n* = 1), lincosamides (*n* = 1), lincosamides-pleuromutilins-streptogramin A compounds (PLS_A) (*n* = 3), macrolide-lincosamide-streptogramin B compounds (MLS_B) (*n* = 3), phenicols (*n* = 2), phenicols-oxazolidinones-lincosamides-pleuromutilins-streptogramin A compounds ($PhLOPS_A$) (*n* = 1), rifampicin (*n* = 1), tetracyclines (*n* = 4), trimethoprim (*n* = 1), and multidrug efflux MATE (multidrug and toxic compound extrusion) transporter (*n* = 1) (Table 5). It is noteworthy that PLS_A, MLS_B, and $PhLOPS_A$ phenotypes are related to cross-resistance, which refers to an ability of resistance to several classes of antimicrobials by a single mechanism [64,65].

It is revealed that the 16 LA-MRSA isolates could be defined as multidrug-resistant (MDR) MRSA as they carried resistance genes, which can confer resistance against at least three classes of antimicrobials. In addition, all isolates tested positive for *mecA*, S84L mutation in *gyrA*, *tet*(38), *tet*(M), *dfrG*, and *mepA*. However, vancomycin resistance gene *vanA* and mutation of *rpoB* gene were not presented in any isolates.

The *in-silico* detection of antimicrobial resistance gene patterns among LA-MRSA isolates within each genotype were highly similar. Within the CC398 subpopulation, the ST398-SCC*mec* CI-t034 genotype did not exhibit a significant difference with the ST398-SCC*mec* V-t034 genotype, except for *aac(6')-Ie/aph(2")-Ia,* mutations in *parC* and *erm* genes; however, it should be mentioned that, when comparing the patterns of gene carriage and mutations between the CC9-t337 and CC398-t034 subpopulations, the distribution of some resistance genes or mutations that was evidently associated with a particular subpopulation was observed. As shown in Table 5, the streptomycin resistance gene *str* was localized only in the CC9-t337 clone, but the other three aminoglycosides-related

genes, *ant(6)-Ia*, *ant(9)-Ia*, and *spw*, were specifically found in the CC398-t034 clone. While the CC9-t337 strains mediated resistance to fosfomycin by encoding protein FosB, all isolates with the CC398-t034 genotype conferred fosfomycin resistance through mutations of *glpT* A100V/F31I and *murA* D278E/E291D [66,67]. For the PLS_A resistance phenotype, the CC398-t034 clone expressed resistance through encoding *lsa*(E) gene; however, the CC9-t337 clone harbored *vga*(A) genes conferring resistance against PLS_A antibiotics. It is important to highlight that some resistance phenotypes were limited to either the CC9-t337 or CC398-t034 subpopulation. The *lnu*(B) gene, involving resistance to lincosamides, was present in all isolates with the CC398-t034 genotype. On the one hand, the strain Q10.1 from the CC9-t337 clone possessed *catA* gene, showing resistance to phenicols. Also, the strain BA3.1 carried phenicols resistance gene *fexA* as well as *cfr* gene exhibiting resistance to $PhLOPS_A$.

Also, the absence/presence of antimicrobial resistance genes distributed among the LA-MRSA isolates involved in zoonotic transmission was examined. Undoubtedly, the LA-MRSA isolates from swine workers showed an almost 100% identity of antimicrobial resistance gene carriage with those from pigs isolated from the same farm origin.

Besides screening for the antimicrobial resistance genes, stress genes, including biocide and metal resistance genes, were also determined. A total of five stress genes, including *arsB*, *arsC*, *lmrS*, *mco*, and *qacG*, were detected (Table 6). The most prevalent genes were *lmrS* and *mco*, carried by all LA-MRSA isolates. The arsenic resistance-related genes, *arsB* and *arsC*, were significantly associated with all LA-MRSA belonging to the ST398-SCC*mec* CI-t034 and CC9-SCC*mec* IX-t337 genotypes; however, *qacG* resulted positive for the strains Q10.1, Y1.3, and S2.1 from both CC9-t337 and CC398-t034 subpopulations.

Virulence gene repertoire

To assess whether pig-associated LA-MRSA would become a serious issue in human medicine, a total of 76 virulence genes were analyzed and further classified into five categories, according to their functions: adherence (n = 13), exoenzymes (n = 10), host immune evasion (n = 16), iron uptake and metabolism (n = 8), and toxins and type IV secretion systems (n = 29) (Table 7) [68]. Most of the virulence genes were distributed homogeneously in all subpopulations, except for some genes that were restrictedly occupied by either the CC9-t337 or CC398-t034 subpopulation. For example, the collagen adhesion gene (*cna*) and coagulase gene (*coa*) were exclusively found in the CC398-t034 strain. Conversely, *map*, *sdrD*, *aur*, *cap8F*, *essC*, *esxB*, *esxC*, staphylococcal enterotoxin (SE) genes *sei*, *sem*, *sen*, *seo*, *seu* and *sey*, and staphylococcal enterotoxins-like toxin (SEL) genes *sel27*, *sel28*, and *selX* were specifically found in the CC9-t337 strains; however, *sel26* was the only SEL gene widespread in all isolates.

Notably, nine adherence genes (*clfA*, *clfB*, *ebp*, *icaA*, *icaB*, *icaC*, *icaD*, *icaR*, and *sdrE*) and seven genes encoding the iron-regulated surface determinant protein (Isd) A-G (*isdA*, *isdB*, *isdC*, *isdD*, *isdE*, *isdF*, *and isdG*) resulted positive for all LA-MRSA isolates. The following virulence genes were, however, totally absent in all isolates: *sak*, *scn*, *chp*, *lukF-PV*, *lukS-PV*, *sea*, *sep*, and *tsst-1* genes.

Mobile genetic elements

MobileElementFinder discovered 10 plasmid replicon sequences with diverse combinations of carriage. The most widespread plasmid replicon belongs to types rep7a and repUS43 (16/16), followed by rep19b (9/16), and rep10 and rep21 (8/16). Furthermore, a total of four transposon elements, including Tn*551* (4/16), Tn*554* (4/16), Tn*558* (1/16), and Tn*6009* (9/16), were identified in CC398-t034 isolates, as demonstrated in Table 8.

The plasmid replicon repUS43 carried by different strains was obviously linked to various genetic determinants. It was shown to be associated with *mecA* and *tet*(M) genes in the CC9-t337 subpopulation, whereas the ST398-SCC*mec* CI-t034 and ST398-SCC*mec* V-t034 strains carried this plasmid coupled with the *tet*(M) gene and transposon Tn*6009*, respectively. Similarly, the plasmid replicon rep7a carrying *tet*(K) was predominately present in all CC398-t034 isolates, except for the strain M3.1. On the other hand, the rep7a carrying *str* was detected in all CC9-t337 isolates, one of which (the strain Q10.1) also harbored the chloramphenicol resistance gene *cat*(pC221) on this plasmid. Among the CC398-t034 isolates, both Tn*551* and Tn*554* were carried by all isolates with the composite SCC*mec* element. The *erm*(A) gene, together with *ant(9)-la*, was located on the transposon Tn*554*, but the *erm*(B) gene resided on the transposon Tn*551*. In contrast, the rep7a plasmid associated with the *erm*(C) gene was mainly restricted to the eight CC398-t034 isolates with the SCC*mec* type V.

Importantly, it is worth nothing that three interesting carriages of antimicrobial resistance genes with mobile genetic elements were found in two isolates. The resistance genes *aadD*, *erm*(B), and *tet*(L) were co-localized on the plasmid sequence of the strain BA3.1. On top of that, this strain also tested positive for the transposon Tn*558* carrying *fexA*. Another multi-resistance gene cluster resided on the rep22 belonging to the strain J101.2. It contained two aminoglycosides resistance genes, *aadD* and *ant(6)-la*, the *blaZ* gene, the lincosamides resistance gene *lnu*(B), and the *Isa*(E) encoding PLS_A phenotype.

Apart from the plasmid replicons and transposons, other mobile genetic elements were also determined. Within the CC398-t034 subpopulation, the insertion sequence IS*256* was present in only one isolate, the strain J101.2. The IS*Sau8* were predictably harbored by the following three strains: L43.2, D16.1, and X1.1; however, no association between these insertion sequences and antimicrobial resistance genes was observed.

Discussion

This study is the first documentation of a whole-genome investigation of zoonotic transmission caused by LA-MRSA CC398 in Thailand. The WGS approaches were used for the genotypic characterization of LA-MRSA. The resulting WGS profiles were nearly in accordance with those of molecular characterization in a previous study [21]. At that time, one porcine strain Z19.1 was identified as carrying a NT SCC*mec* element using multiplex PCR assays. Additionally, the PCR-based SCC*mec* typing could not specify an allotype of *ccrC* gene complex; however, these uncertainties were clarified by the WGS analyses in the present study. Together, these findings indicated a higher discriminatory power of WGS beyond other general typing approaches.

The occurrence of the SCC*mec* Cl in this study might imply an empirical impact on genetic recombination. As seen in Table 3, the composite SCC*mec* elements of the four CC398-t034 isolates contained both *ccrA1B1* and *ccrC1* gene complexes. The *ccrA1B1* and *ccrC1* gene complexes were also found in the CC9-SCC*mec* IX-t337 and CC398-SCC*mec* V-t034 subpopulation, respectively. Although LA-MRSA CC398 is uncommon in Asian countries, the CC398 isolates, particularly those carrying SCC*mec* V elements, were sporadically reported in pigs from Japan, South Korea, and Thailand, where live pigs are reportedly imported from overseas [45,69,70]. Based on these findings, it can be hypothesized that LA-MRSA CC398-t034 with SCC*mec* V may have been introduced into Thailand and subsequently acquired the additional gene complex (*ccrA1B1*) from the domestic strain (CC9-SCC*mec* IX-t337) via genetic recombination, leading to the emergence of a unique composite of the SCC*mec* element [71]. However, this chapter did not focus on the factors driving the emergence of LA-MRSA CC398 in Thai pigs; these will be explored in the following chapter.

Previous studies from China reported the carriage of multiple *ccr* gene complexes in clinical and porcine isolates of LA-MRSA ST9, as well as the presence of CoNS carrying multiple SCC*mec* elements [72,73]. The latter study demonstrated that the isolates with multiple SCC*mec* exhibited greater capability to maintain *mecA* gene transcription, which is important for cell wall synthesis. As a result, these isolates did not lose Gram positivity under antibiotic exposure when compared to those with a single SCC*mec* element [73]. However, phenotypic analyses to investigate the effect of multiple SCC*mec* element carriage were not performed in this study.

The zoonotic transmission of LA-MRSA CC398 in two farms was elucidated and verified using core-genome SNP-based analyses. The phylogenetic tree reconstruction illustrated a high genome similarity between porcine and human isolates. Genomic characteristics supported the phylogenetic tree as well as imply that the origin of human LA-MRSA CC398 would be pigs. Ultimately, the pairwise SNP analysis did not only confirm pig-to-human transmission; it also indicated the evolutionary changes in the core genome of LA-MRSA CC398 after the zoonotic spillover and colonization on human nares. One study in Denmark also used WGS to investigate the zoonotic transmission of

LA-MRSA between livestock and farmers. The phylogenetic tree based on core genome SNPs revealed that animal isolates differed from Danish worker isolates by only three to five SNPs [23]. The small number of pairwise SNPs is very similar to those in the present study.

Additionally, the phylogenetic analysis clearly revealed two distinct clades of LA-MRSA CC398 with their own specific SCC*mec* types. LA-MRSA isolates from the same province or their neighboring province were phylogenetically grouped into the same clade or sub-clade. It was exemplified by the six porcine isolates from Prachin Buri and Nakhon Ratchasima. Nakhon Ratchasima shares its provincial borders with Prachin Buri. Unsurprisingly, all porcine isolates from these two provinces in Clade II resided together in the same sub-clade; therefore, it can be stated that these clues speculated as to local transmission events of pig-associated LA-MRSA within the same province or between provinces.

Like the phenotypic susceptibility testing described earlier, the WGS analyses exhibited that all LA-MRSA isolates in the present study were identified as MDR as well as harboring several cross-resistance genes [21]. The overall distribution of antimicrobial resistance genes suggested the genetic homogeneity of LA-MRSA in each subpopulation. In other words, the isolates sharing the same genotypic characteristics showed a highly similar pattern of antimicrobial resistance gene carriage, although they were collected from different locations or hosts. The *tet*(M) gene, which is believed to be a common genetic characteristic among LA-MRSA CC398 isolated from pigs, was detected in all isolates from both Thai pigs and humans, supporting the evidence of zoonotic transmission [79]. In addition, LA-MRSA CC9 and CC398 possessed different resistance mechanisms against a particular antimicrobial group such as a resistance mechanism against fosfomycin or PLS_A. It is also important to emphasize that several cross-resistance genes identified do not only confer resistance against antimicrobial agents widely used in livestock, but also antibiotics of last resort in human medicine such as quinupristin/dalfopristin and linezolid; however, analysis of antimicrobial resistance gene carriage using WGS in LA-MRSA CC398 were previously explored by a study collecting porcine specimens from a province in central Thailand, and it is consistent with the results of this study [45,74].

Although the *lmrS* gene encoding a multidrug efflux pump is categorized as a stress gene according to the AMRFinderPlus database, it is proved that this gene is able to implicate in resistance to linezolid, aminoglycosides, macrolides, and phenicols, which is relatively similar to the $PhLOPS_A$ phenotype expressed by the *cfr* gene; hence, it can be assumed that all LA-MRSA isolates in this study were highly likely to potentiate resistance against oxazolidinones via the lincomycin resistance protein of *Staphylococcus aureus* (LmrS) [75]. Three LA-MRSA strains could develop quaternary ammonium compounds (QACs) resistance due to harboring the *qacG* gene. QAC-based disinfectants are commonly used in livestock farms to chemically kill microorganisms on non-living

surfaces; therefore, this gene would probably protect LA-MRSA from commercially available disinfectants promoting bacterial persistence in farm environment.

In this study, WGS approaches also disclosed an abundance and diversity of virulence determinants imposed by LA-MRSA isolates. As expected, the toxic shock syndrome toxin-1 (TSST-1)-encoding gene (*tsst-1*), PVL-encoding genes (*lukF-PV* and *lukS-PV*), as well as the immune evasion gene cluster (IEC), including *sak*, *scn*, *chp*, *sea*, and *sep*, were absent in all LA-MRSA isolates because they were rarely detected in livestock-derived isolates [76–79]. In addition, they are well known as crucial virulence determinants promoting the pathogenesis or severity of MRSA infections in humans. Only a few SE and SEL genes were found in the LA-MRSA isolates.

However, multiple genes, including *clfA-B*, *cna*, *icaA-D*, *icaR*, *isdA-G*, and *sdrC-E*, were carried by the LA-MRSA isolates. Specifically, the *clfB* genes encoding clumping factors B (ClfB) is determined to play an important role in nasal colonization in humans and experimental animals [80–82]. It can bind to the upper layer of epithelial cells in anterior nares by a ligand-receptor interaction with loricrin and is able to interact with cytokeratin 10 (CK10) expressed on skin epithelium. Furthermore, it can facilitate skin and SSTIs during the early stage of pathogenesis [83,84]. Seven LA-MRSA isolates had the *cna* gene, which is responsible for bacterial adherence to collagen in host tissue. With this function, the collagen-binding protein can influence staphylococcal infection in collagen-rich tissues such as heart, joints, and bones, and the cornea [85–88]. The *ica* operon, consisting of *icaA-D* genes and its negative regulator gene *icaR*, is involved in biofilm production. The slime formation does not only protect bacterial communities against host immunity, but also mediates the release of dispersed cells to new sites of infection and avoids penetration of antimicrobial agents through the staphylococcal biofilm [89,90]. Besides being involved in heme-iron acquisition systems, the IsdA is also able to bind to several host receptors such as loricrin and CK10, supporting the adherence of *S. aureus* to desquamated nasal epithelial cells in humans and colonization in animal experiments [91]. Only a few LA-MRSA isolates harbored *sdrC-D* genes even though *sdrE* gene was identified in all strains. The *sdrC-D* genes are related to adhesion to human epithelial cells; however, the *sdrE* gene is recognized as an inhibitor of both classical and alternative complement pathways [91,92].

Altogether, it can be summarized that all LA-MRSA isolates in this study posed the virulence genes that can potentiate intercellular adhesion and colonization in human nostrils, facilitate biofilm formation, and promote a wide spectrum of staphylococcal infections. These findings supported the conclusion that LA-MRSA can serve as an invasive pathogen threatening human health, especially in patients with immunocompromised or underlying medical conditions [24].

In-silico prediction of antimicrobial resistance gene-associated mobile genetic elements revealed two unique multi-resistant plasmids on draft genomes of LA-MRSA strains BA3.1 and J101.2; nevertheless, previous studies have reported the emergence of MRSA possessing multi-resistance gene

clusters either on chromosome or plasmid. To illustrate this, the co-existence of numerous antimicrobial resistance genes on the LA-MRSA genome J101.2, including *aadD*, *blaZ*, *lnu*(B), and *Isa*(E) genes, was detected on a chromosome of MRSA ST9 isolated from frozen food in China [93]. Additionally, a multi-resistance gene cluster on a plasmid was identified in MRSA ST9 from a Chinese pig [94]. Apart from the aminoglycosides, beta-lactams, lincosamides, and PLS_A resistance genes, the tetracycline resistance gene *tet*(L) and MLS_B resistance *erm*(B) were co-localized on this plasmid, which were also found on the LA-MRSA strain BA3.1. The co-carriage of diverse antimicrobial resistance genes on the plasmids demonstrated in this study raises an awareness of significant risk related to antimicrobial resistance gene transfer [94].

I, however, encountered some challenges in using short-read sequencing technologies. First, whole sequences of the SCC*mec* CI elements in four isolates of LA-MRSA CC398 could not be clarified. Thus, I could not whether the additional *ccr* gene complex was a result of two discrete integrated SCC*mec* elements [95]. Another limitation is that I was not able to visualize the genomic organization of multi-resistance gene clusters located on putative plasmid contigs. This would highlight that several antimicrobial resistance genes and other resistance genes can be horizontally transferred in a single event. To tackle these problems, the so-called hybrid genome assembly produced from long-read and short-read sequencing technologies will be implemented in the next chapter to reconstruct a complete bacterial chromosome or plasmid.

Conclusion

This study highlights the application of WGS technologies for the epidemiological investigation of zoonotic transmission of LA-MRSA CC398 in Thailand. WGS analyses with a high-resolution genomic approach reveal the genetic recombination during the evolutionary process, influenced by the introduction of exotic LA-MRSA CC398. Widespread distribution of multiple antimicrobial resistance-related genes was observed among the LA-MRSA isolates. The presence of cross-resistance genes underscores the judicious usage of antimicrobials in livestock production. In addition, the co-existence of several antimicrobial resistance genes on plasmids and the virulence gene repertoire reflects the robustness of biosecurity-associated strategies to confine horizontal gene transfer among bacterial communities outside agricultural areas as well as to reduce the risk of transmission at pre-harvest. These findings also accentuate the primary role of the One Health approach, collaboratively addressing antimicrobial resistance issues.

Table 1. Summary metrics of whole-genome sequencing data.

Strain name	Read	Total sequences		Total bases (Mbp)		Sequence length (bp)	
		Before trimming	After trimming	Before trimming	After trimming	Before trimming	After trimming
AA3.1	R1	2,326,167	794,112	276.4	142.4	35-301	100-281
	R2	2,326,167	794,112	276.4	138.3	35-301	100-281
BA3.1	R1	1,707,309	611,521	207.0	108.6	35-301	100-281
	R2	1,707,309	611,521	206.9	105.8	35-301	100-281
D16.1	R1	1,837,506	879,902	283.7	176.2	35-301	100-281
	R2	1,837,506	879,902	284.1	167.2	35-301	100-281
G2.1	R1	734,372	321,401	102.3	59.7	35-301	100-281
	R2	734,372	321,401	102.4	57.5	35-301	100-281
H49.1	R1	994,613	405,150	131.7	75.7	35-301	100-281
	R2	994,613	405,150	131.9	72.9	35-301	100-281
J101.2	R1	1,412,380	504,637	172.4	91.3	35-301	100-281
	R2	1,412,380	504,637	172.6	88.4	35-301	100-281
L3.1	R1	765,086	366,112	111.1	68.2	35-301	100-281
	R2	765,086	366,112	111.1	66.3	35-301	100-281
L43.2	R1	852,102	398,152	122.3	74.3	35-301	100-281
	R2	852,102	398,152	122.4	71.7	35-301	100-281
M3.1	R1	837,211	273,555	96.8	47.7	35-301	100-281
	R2	837,211	273,555	96.9	46.3	35-301	100-281
M31.1	R1	2,241,161	659,021	251.3	115.0	35-301	100-281
	R2	2,241,161	659,021	254.0	108.9	35-301	100-281
Q10.1	R1	885,174	293,268	102.3	50.7	35-301	100-281
	R2	885,174	293,268	102.5	49.1	35-301	100-281
S2.1	R1	1,501,109	438,850	163.0	75.3	35-301	100-281
	R2	1,501,109	438,850	163.5	72.9	35-301	100-281
X1.1	R1	2,806,085	849,296	310.9	148.6	35-301	100-281
	R2	2,806,085	849,296	311.5	143.8	35-301	100-281
Y1.2	R1	1,403,657	411,228	154.2	71.2	35-301	100-281
	R2	1,403,657	411,228	154.4	68.8	35-301	100-281
Y1.3	R1	1,053,368	405,542	134.3	73.7	35-301	100-281
	R2	1,053,368	405,542	134.6	71.1	35-301	100-281
Z19.1	R1	1,240,293	444,596	150.8	78.5	35-301	100-281
	R2	1,240,293	444,596	151.0	75.6	35-301	100-281

Table 2. Summary of genome assembly statistics.

Strain name	Parameter					
	Total length (bp)	Number of contigs	Largest contig (bp)	N50 (bp)	L50	GC content (%)
AA3.1	2,863,072	41	473,023	179,316	6	32.86
BA3.1	2,878,790	75	367,099	132,095	7	32.80
D16.1	2,858,494	39	564,703	205,806	4	32.85
G2.1	2,852,659	50	256,624	149,853	8	32.86
H49.1	2,847,713	38	539,003	204,117	4	32.87
J101.2	2,854,557	75	367,428	152,613	6	32.77
L3.1	2,834,929	52	510,257	149,084	6	32.78
L43.2	2,834,195	49	535,798	116,561	6	32.78
M3.1	2,859,102	91	134,954	53,208	18	32.87
M31.1	2,863,666	47	472,256	179,316	6	32.86
Q10.1	2,790,395	88	268,947	62,591	13	32.79
S2.1	2,857,426	45	463,198	201,005	5	32.87
X1.1	2,849,646	45	524,839	178,486	5	32.87
Y1.2	2,849,228	42	464,546	287,050	4	32.86
Y1.3	2,791,683	58	280,245	89,853	9	32.79
Z19.1	2,833,076	54	468,712	96,277	7	32.77

Table 3. WGS-based characteristics of the 16 livestock-associated methicillin-resistant *Staphylococcus aureus* isolates.

Strain name	Farm	Location	ST	CC	SCC*mec* type	*spa* type	*mec* complex class	*ccr* gene complex			
								Type	*ccrA1*	*ccrB1*	*ccrC1*
Q10.1	6	PB	9	9	IX	t337	C2	1	+	+	-
Y1.3	8	PB	4576	9	IX	t337	C2	1	+	+	-
BA3.1	2	RB	9	9	IX	t337	C2	1	+	+	-
J101.2	11	PB	398	398	CI	t034	C2	NT	+	+	+
L3.1	1	NP	398	398	CI	t034	C2	NT	+	+	+
L43.2	1	NP	398	398	CI	t034	C2	NT	+	+	+
Z19.1	1	NP	398	398	CI	t034	C2	NT	+	+	+
AA3.1	4	SB	398	398	V	t034	C2	5	-	-	+
M3.1	3	SB	398	398	V	t034	C2	5	-	-	+
M31.1	3	SB	398	398	V	t034	C2	5	-	-	+
Y1.2	8	PB	398	398	V	t034	C2	5	-	-	+
S2.1	7	PB	398	398	V	t034	C2	5	-	-	+
H49.1	10	NR	398	398	V	t034	C2	5	-	-	+
G2.1	9	NR	398	398	V	t034	C2	5	-	-	+
D16.1	5	PB	398	398	V	t034	C2	5	-	-	+
X1.1	7	PB	398	398	V	t034	C2	5	-	-	+

CI, composite island; NP, Nakhon Pathom; NR, Nakhon Ratchasima; NT, non-typeable; PB, Prachin Buri; RB, Ratchaburi; SB, Suphanburi; +, detected; -, not detected.

Table 4. Pairwise SNP distance matrix of the 13 livestock-associated methicillin-resistant *Staphylococcus aureus* CC398 isolates based on core genome alignment.

Pairwise SNP distance matrix of the 13 LA-MRSA CC398 isolates			L3.1	L43.2	Z19.1	M3.1	M31.1	AA3.1	D16.1	S2.1	X1.1	Y1.2	G2.1	H49.1	J101.2
			Farm 1	Farm 1	Farm 1	Farm 3	Farm 3	Farm 4	Farm 5	Farm 7	Farm 7	Farm 8	Farm 9	Farm 10	Farm 11
			Pig	Human	Pig	Pig	Human	Pig	Pig	Pig	Pig	Pig	Pig	Pig	Human
L3.1	Farm 1	Pig	0	11	13	503	506	505	506	500	508	476	501	491	239
L43.2	Farm 1	Human	11	0	10	503	506	505	506	500	508	476	501	491	238
Z19.1	Farm 1	Pig	13	10	0	504	507	506	507	501	509	477	502	492	240
M3.1	Farm 3	Pig	503	503	504	0	3	2	51	45	53	37	48	40	557
M31.1	Farm 3	Human	506	506	507	3	0	3	38	46	54	38	49	41	560
AA3.1	Farm 4	Pig	505	505	506	2	3	0	51	45	53	37	48	40	559
D16.1	Farm 5	Pig	506	506	507	51	52	51	0	38	14	29	23	21	560
S2.1	Farm 7	Pig	500	500	501	45	46	45	38	0	40	24	35	27	554
X1.1	Farm 7	Pig	508	508	509	53	54	53	14	40	0	30	25	23	562
Y1.2	Farm 8	Pig	476	476	477	37	37	37	29	24	30	0	26	20	515
G2.1	Farm 9	Pig	501	501	502	48	49	48	23	35	25	26	0	18	555
H49.1	Farm 10	Pig	491	491	492	40	41	40	21	27	23	20	18	0	545
J101.2	Farm 11	Human	239	238	240	557	560	559	560	554	562	515	555	545	0

Table 5. Distribution of 32 antimicrobial resistance-associated genes among the 16 livestock-associated methicillin-resistant *Staphylococcus aureus* CC9 and CC398.

Strain Name		Q10.1	Y1.3	BA3.1	J101.2	L3.1	L43.2	Z19.1	AA3.1	M3.1	M31.1	Y1.2	S2.1	H49.1	G2.1	D16.1	X1.1
Origin		Pig	Pig	Pig	Human	Pig	Human	Pig	Pig	Pig	Human	Pig	Pig	Pig	Pig	Pig	Pig
Farm		6	8	2	11	1	1	1	4	3	3	8	7	10	9	5	7
Location		PB	PB	RB	PB	NP	NP	NP	SB	SB	SB	PB	PB	NR	NR	PB	PB
ST		9	4576	9	398	398	398	398	398	398	398	398	398	398	398	398	398
CC		9	9	9	398	398	398	398	398	398	398	398	398	398	398	398	398
SCC*mec* type		IX	IX	IX	CI	CI	CI	CI	V	V	V	V	V	V	V	V	V
spa type		t337	t337	t337	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034
AMGs	*aac(6')-Ie/aph(2")-Ia*																
	aadD1																
	ant(6)-Ia																
	ant(9)-Ia																
	spw																
	str																
BLs	*blaPC1*																
	blaZ																
	mecA																
FQs	Mutation of *gyrA* S84L																
	Mutation of *parC* E84G																
	Mutation of *parC* S80F																
	Mutation of *parC* S80Y																
FOS	*fosB*																
	Mutation of *glpT* A100V																
	Mutation of *glpT* F3I																
	Mutation of *murA* D278E																
	Mutation of *murA* E291D																
GPAs	*vanA*																
LINs	*lnu*(B)																
PLS_A	*lsa*(E)																
	vga(A)																
	vga(A)-LC																
MLS_B	*erm*(A)																
	erm(B)																
	erm(C)																
PHEs	*catA*																
	fexA																
$PhLOPS_A$	*cfr*																
RIF	Mutation of *rpoB*																
TCs	*tet*(38)																
	tet(K)																
	tet(L)																
	tet(M)																
TMP	*dfrG*																
MATE transporter	*mepA*																

Grey shading indicates the presence of genes or mutations in each isolate.

AMGs, aminoglycosides; BLs, beta-lactams; FQs, fluoroquinolones; FOS, fosfomycin; GPAs, glycopeptide antibiotics; LINs, lincosamides; PLS_A, lincosamides-pleuromutilins-streptogramin A compounds; MLS_B, macrolide-lincosamide-streptogramin B compounds; PHEs, phenicols; $PhLOPS_A$, phenicols-oxazolidinones-lincosamides-pleuromutilins-streptogramin A compounds; RIF, rifampicin; TCs, tetracyclines; TMP, trimethoprim; MATE, multidrug and toxic compound extrusion.

Table 6. Distribution of five stress genes identified by the AMRFinderPlus database among the 16 livestock-associated methicillin-resistant *Staphylococcus aureus* CC9 and CC398.

Strain name	Origin	Farm	Location	Sequence type	Clonal complex	SCC*mec* type	*spa* type	Arsenite efflux transporter membrane subunit *arsB*	Thioredoxin-dependent arsenate reductase *arsC*	Multidrug efflux MFS transporter *lmrS*	Multi-copper oxidase *mco*	Quaternary ammonium compound efflux SMR transporter *qacG*
Q10.1	Pig	6	Prachin Buri	9	9	IX	t337	+	+	+	+	+
Y1.3	Pig	8	Prachin Buri	4576	9	IX	t337	+	+	+	+	+
BA3.1	Pig	2	Ratchaburi	9	9	IX	t337	+	+	+	+	-
J101.2	Human	11	Prachin Buri	398	398	CI	t034	+	+	+	+	-
L3.1	Pig	1	Nakhon Pathom	398	398	CI	t034	+	+	+	+	-
L43.2	Human	1	Nakhon Pathom	398	398	CI	t034	+	+	+	+	-
Z19.1	Pig	1	Nakhon Pathom	398	398	CI	t034	+	+	+	+	-
AA3.1	Pig	4	Suphanburi	398	398	V	t034	-	-	+	+	-
M3.1	Pig	3	Suphanburi	398	398	V	t034	-	-	+	+	-
M31.1	Human	3	Suphanburi	398	398	V	t034	-	-	+	+	-
Y1.2	Pig	8	Prachin Buri	398	398	V	t034	-	-	+	+	-
S2.1	Pig	7	Prachin Buri	398	398	V	t034	-	-	+	+	+
H49.1	Pig	10	Nakhon Ratchasima	398	398	V	t034	-	-	+	+	-
G2.1	Pig	9	Nakhon Ratchasima	398	398	V	t034	-	-	+	+	-
D16.1	Pig	5	Prachin Buri	398	398	V	t034	-	-	+	+	-
X1.1	Pig	7	Prachin Buri	398	398	V	t034	-	-	+	+	-

MFS, the major facilitator superfamily; SMR, small multidrug resistance.

Table 7. Distribution of 76 virulence determinants among the 16 livestock-associated methicillin-resistant *Staphylococcus aureus* CC9 and CC398.

Strain name		Q10.1	Y1.3	BA3.1	J101.2	L3.1	L43.2	Z19.1	AA3.1	M3.1	M31.1	Y1.2	S2.1	H49.1	G2.1	D16.1	X1.1
Origin		Pig	Pig	Pig	Human	Pig	Human	Pig	Pig	Pig	Human	Pig	Pig	Pig	Pig	Pig	Pig
Farm		6	8	2	11	1	1	1	4	3	3	8	7	10	9	5	7
Location		PB	PB	RB	PB	NP	NP	NP	SB	SB	SB	PB	PB	NR	NR	PB	PB
ST		9	4576	9	398	398	398	398	398	398	398	398	398	398	398	398	398
CC		9	9	9	398	398	398	398	398	398	398	398	398	398	398	398	398
SCC*mec* type		IX	IX	IX	CI	CI	CI	CI	V	V	V	V	V	V	V	V	V
spa type		t337	t337	t337	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034
Virulence genes related to adherence	*clfA*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	clfB	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cna					■	■		■		■			■		■	■
	ebp	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	icaA	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	icaB	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	icaC	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	icaD	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	icaR	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	map	■	■	■													
	sdrC		■	■		■							■				
	sdrD	■	■	■													
	sdrE	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Virulence genes related to exoenzymes	*adsA*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	aur	■	■	■													
	coa				■	■	■	■	■	■	■	■	■	■	■	■	■
	geh	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	hysA	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	lip	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	*sak**																
	sspA	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	sspB	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	sspC	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Virulence genes related to host immune evasion	*cap8A*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8B	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8C	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8D	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8E	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8F	■	■	■													
	cap8G	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8L	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8M	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8N	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8O	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	cap8P	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	chp																
	*scn**																
	sbi	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	spa	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■
Virulence genes related to iron uptake & metabolism	*isdA*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	isdB	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	isdC	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	isdD	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	isdE	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	isdF	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	isdG	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
	srtB	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■

Table 7. Continued

Strain name		Q10.1	Y1.3	BA3.1	J101.2	L3.1	L43.2	Z19.1	AA3.1	M3.1	M31.1	Y1.2	S2.1	H49.1	G2.1	D16.1	X1.1
Origin		Pig	Pig	Pig	Human	Pig	Human	Pig	Pig	Pig	Human	Pig	Pig	Pig	Pig	Pig	Pig
Farm		6	8	2	11	1	1	1	4	3	3	8	7	10	9	5	7
Location		PB	PB	RB	PB	NP	NP	NP	SB	SB	SB	PB	PB	NR	NR	PB	PB
ST		9	4576	9	398	398	398	398	398	398	398	398	398	398	398	398	398
CC		9	9	9	398	398	398	398	398	398	398	398	398	398	398	398	398
SCC*mec* type		IX	IX	IX	CI	CI	CI	CI	V	V	V	V	V	V	V	V	V
spa type		t337	t337	t337	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034	t034
Virulence genes related to toxins and type IV secretion	*esaA*																
	esaB																
	essA																
	essB																
	essC																
	esxA																
	esxB																
	esxC																
	hla																
	hlb																
	hld																
	hlgA																
	hlgB																
	hlgC																
	lukF-PV																
	lukS-PV																
	*sea**																
	*sep**																
	*sei**																
	*sel26**																
	*sel27**																
	*sel28**																
	*selx**																
	*sem**																
	*sen**																
	*seo**																
	*seu**																
	*sey**																
	tsst-1																

Grey shading indicates the presence of genes or mutations in each isolate.

* Virulence genes additionally predicted by the AMRFinderPlus.

Table 8. Distribution of mobile genetic elements with their associated antimicrobial resistance genes carried by the 16 livestock-associated methicillin-resistant *Staphylococcus aureus* CC9 and CC398.

Strain name	Origin	Farm	Location	ST	CC	SCC*mec* type	*spa* type	ARG-associated plasmid replicon and transposon	Insertion sequence
Q10.1	Pig	6	PB	9	9	IX	t337	rep5d-*vga*(A)-LC rep7a-*str*-*cat*(pC221) rep13-*qacG* rep21 repUS43-*mecA*-*tet*(M)	-
Y1.3	Pig	8	PB	4576	9	IX	t337	rep5d-*vga*(A)-LC rep7a-*str* rep13-*qacG* rep21 repUS43-*mecA*-*tet*(M)	-
BA3.1	Pig	2	RB	9	9	IX	t337	rep7a-*str* rep10b-*vga*(A)-LC repUS18-*aadD*-*erm*(B)-*tet*(L) repUS43-*mecA*-*tet*(M) Tn*558*	-
J101.2	Human	11	PB	398	398	CI	t034	rep7a-*tet*(K) rep22-*aadD*-*ant(6)*-*la*-*blaZ**-*lnu*(B)-*lsa*(E) repUS43-*tet*(M) Tn*551*-*erm*(B) Tn*554*-*ant(9)*-*la*-*erm*(A)	*IS*256
L3.1	Pig	1	NP	398	398	CI	t034	rep7a-*tet*(K) rep22-*aadD* repUS43-*tet*(M) Tn551-*erm*(B) Tn*554*-*ant(9)*-*la*-*erm*(A)	-
L43.2	Human	1	NP	398	398	CI	t034	rep7a-*tet*(K) rep22-*aadD* repUS43-*tet*(M) Tn*551*-*erm*(B) Tn*554*-*ant(9)*-*la*-*erm*(A)	*IS*Sau8
Z19.1	Pig	1	NP	398	398	CI	t034	rep7a-*tet*(K) rep22-*aadD* repUS43-*tet*(M) Tn*551*-*erm*(B) Tn*554*-*ant(9)*-*la*-*erm*(A)	-
AA3.1	Pig	4	SB	398	398	V	t034	rep7a-*tet*(K) rep10-*erm*(C) rep19b-*blaZ* rep21 repUS43-*tet*(M)-Tn*6009*	-
M3.1	Pig	3	SB	398	398	V	t034	rep7a rep10-*erm*(C) rep19b rep21 repUS43-*tet*(M)-Tn*6009*	-

Table 8. Continued

Strain name	Origin	Farm	Location	ST	CC	SCC*mec* type	*spa* type	ARG-associated plasmid replicon and transposon	Insertion sequence
M31.1	Human	3	SB	398	398	V	t034	rep7a-*tet*(K) rep10-*erm*(C) rep19b-*blaZ* rep21 repUS43-*tet*(M)-Tn*6009*	-
Y1.2	Pig	8	PB	398	398	V	t034	rep7a-*tet*(K) rep10 rep19b repUS43-*tet*(M)-Tn*6009*	-
S2.1	Pig	7	PB	398	398	V	t034	rep7a-*tet*(K) rep10-*erm*(C) rep13-*qacG* rep19b-*blaZ* rep21 repUS43-*tet*(M)-Tn*6009*	-
H49.1	Pig	10	NR	398	398	V	t034	rep7a-*tet*(K) rep10-*erm*(C) rep19b-*blaZ* repUS43-*tet*(M)-Tn*6009*	-
G2.1	Pig	9	NR	398	398	V	t034	rep7a-*tet*(K) rep10-*erm*(C) rep19b-*blaZ* rep21 repUS43-*tet*(M)-Tn*6009*	-
D16.1	Pig	5	PB	398	398	V	t034	rep7a-*tet*(K) rep10-*erm*(C) rep13 rep19b-*blaZ* rep21 repUS43-*tet*(M)-Tn*6009*	*lS*Sau8
X1.1	Pig	7	PB	398	398	V	t034	rep7a-*tet*(K) rep10-*erm*(C) rep19b-*blaZ* repUS43-*tet*(M)-Tn*6009*	*lS*Sau8

ARG, antimicrobial resistance gene.

* This gene was not detected in the human strain J101.2 using the AMRFinderPlus.

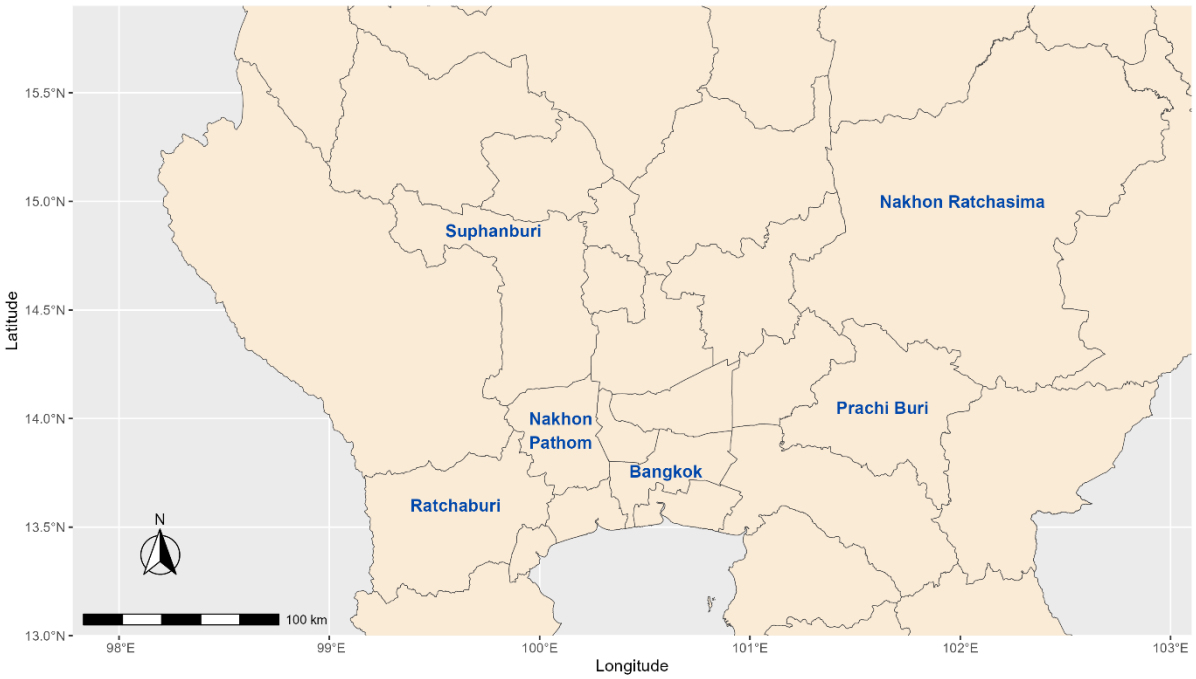

Figure 1. Sampling locations of the 16 livestock-associated methicillin-resistant *Staphylococcus aureus* isolates in central Thailand.

It presents the location of six cities, five of which are sources of the 16 representative isolates of livestock-associated methicillin-resistant *Staphylococcus aureus* CC9 and CC398. The capital city of Bangkok is used as a landmark. The map was created by several packages, including ggplot2, raster, sf and tidyverse, on RStudio v2023.06.1+524 (https://rstudio.com/) [96–99]. Geometric data of province boundaries were downloaded from the Database of Global Administrative Areas v4.1 (https://gadm.org/).

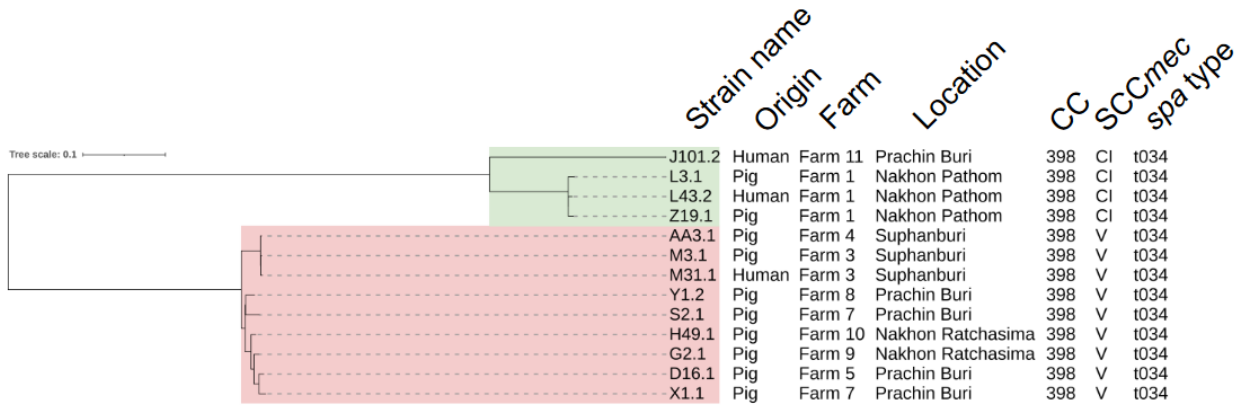

Figure 2. Phylogenetic analysis of the 13 livestock-associated methicillin-resistant *Staphylococcus aureus* CC398 isolates based on core genome SNPs.

Clades I and II are highlighted in green and red, respectively.

CHAPTER II

Emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* clonal complex 398 in Thailand's swine industry

Introduction

As discussed in the Chapter I, it has been shown that LA-MRSA CC398 in Thailand has already emerged in the Thailand's swine industry. Among the 13 LA-MRSA CC398 isolates identified across the five provinces, six harbored the type V of SCC*mec* element and *spa* type t034. These genetic characteristics are commonly associated with porcine-derived LA-MRSA strains reported in European and North American countries, including Denmark—where LA-MRSA CC398 is well-established [79]. The remaining four isolates of CC398 were also found to carry the *spa* type t034; however, these CC398 isolates possessed the composite SCC*mec* elements containing multiple *ccr* gene complexes (*ccr*A1B1 and *ccr*C1), which did not correspond to any SCC*mec* types currently recognized by the IWG-SCC [33]. This unusual combination of *ccr* gene complexes has not been ubiquitously identified among LA-MRSA CC398 lineage in Thailand [45].

In 2021, the second emergence of LA-MRSA CC398 in Thailand was documented, involving isolates collected from a slaughtered pig and retail pork products [45]. More importantly, all LA-MRSA CC398 isolates identified also carried the SCC*mec* V element. This underscores a potential contamination route throughout the pork production chain—from farm to fork and indicates a genotypic association between the Thai LA-MRSA CC398 lineage and the SCC*mec* V element.

The emergence of LA-MRSA CC398 in swine productions have also been sporadically reported other Asian countries [69,70]. To clarify, Furuno *et al.* investigated the prevalence of LA-MRSA in breeding pigs imported to Japan during quarantine inspections. These pigs had been sourced from five different countries in Europe and North America. Twelve representative isolates were characterized using MLST and SCC*mec* typing. All isolates belonged to CC398, with nine harboring the SCC*mec* type V element [69]. These findings support the hypothesis that the international movement of livestock could contribute to the introduction and dissemination of LA-MRSA CC398 in Asian swine production systems. Similarly, clonal expansion of LA-MRSA with the CC398-SCC*mec* V-t034 genotype was observed in pigs raised in South Korea [70]. Even though the original source of these domestic pigs could not be conclusively determined, data from the Ministry of Food, Agriculture, Forestry and Fisheries indicate that pigs have been imported from Denmark, Canada, and the USA to South Korea for breeding.

Together, previous studies from Japan and South Korea have suggested the potential source and underlying factors contributing to the emergence of LA-MRSA CC398 in the countries. However, such an investigation has never been officially reported in Thailand. To address this gap in evidence, the present study aimed to trace the potential original source of LA-MRSA CC398 lineage—specifically the CC398-SCC*mec* V-t034 and CC398-SCC*mec* CI-t034 genotypes—in Thailand, and to investigate the factors associated with its emergence in the Thai swine industry.

Materials and Methods

Genome collection and preprocessing

A total of 13 representative LA-MRSA CC398 isolates from the Chapter I were selected for long-read sequencing and hybrid genome assembly. These isolates, obtained through nasal swab sampling, included ten from pigs and three from swine workers [21]. High-molecular-weight genomic DNA was subsequently extracted from pure cultures using the Nucleospin Microbial DNA Kit (Macherey-Nagel, Düren, Germany). The DNA libraries were constructed using the Native Barcoding Kit (SQK-NBD114.96) for Oxford Nanopore PromethION 2 Solo (Oxford Nanopore Technologies, Oxford, UK), following the manufacturer's guidelines. Super-accurate basecalling and adapter trimming were conducted using Dorado v0.6.0 (https://github.com/nanoporetech/dorado). The trimmed reads were then assembled using Flye v2.9.3 to reconstruct draft genomes [101]. The assembly statistics were calculated with QUAST v5.2.0 for quality assessment [102]. To correct sequencing errors in the nanopore-based assemblies, short-read sequencing data, generated in the Chapter I, were integrated using Polypolish v0.5.0 [103,104]. Specifically, the Illumina MiSeq system was employed for paired-end sequencing (2 x 300 bp). The raw reads were first processed through adapter trimming and quality filtering using BBDuk2 in FoodQCpipeline v0.11.5 (https://bitbucket.org/genomicepidemiology/foodqcpipeline/src/master/). The trimmed reads were then quality-checked using FastQC v0.11.5 [46]. The complete genomes have been deposited in the National Center for Biotechnology Information (NCBI) GenBank with BioProject accession number PRJNA1158956 (Table 9).

Additionally, this study included five genomes of porcine LA-MRSA CC398 isolates in Thailand, which were sequenced in a previous study using the Illumina MiSeq platform [74]. Then, these genomes were preprocessed following the previously described workflow. Genome assembly was eventually carried out using SPAdes v3.11.0 [47]. The assembled genomes were required to have ≤150 contigs and high completeness [105].

Finally, a total of 1,653 deposited *S. aureus* CC398 genomes, including a reference genome (named S0385; GenBank accession no. AM990992), were downloaded from Pathogenwatch (https://pathogen.watch/). Genomes that did not match the predefined criteria were excluded. Of these, 1,197 high-quality genomes were qualified for downstream analyses. This international dataset encompassed genomes collected from various sources, including humans and pigs, in 24 countries between 1998 and 2021. However, it is important to note that two of these 1,197 draft genomes were derived from human isolates in Thailand (Table 10).

Phylogenetic tree reconstruction

In this study, SNP-based phylogenetic trees were inferred genetically using two different methods: maximum likelihood and Bayesian inference. A total of 1,215 *S. aureus* CC398 genomes were aligned against the reference genome S0385 to call high-quality SNPs through the CSI Phylogeny pipeline v1.4 with default settings [106]. The maximum-likelihood tree generated was displayed using iTOL v6.9.1 [63]. For the Bayesian approach, only a subset of 148 *S. aureus* CC398 isolates that clustered closely with 20 isolates of Thai *S. aureus* CC398 was focused, as indicated by the maximum-likelihood tree. The Bayesian phylogenetic tree was reconstructed using the BEAST package. To clarify, a NEXUS alignment of 168 *S. aureus* CC398 genomes was produced from the output of the CSI Phylogeny pipeline. The best-fitting substitution model for the alignment was selected from a set of candidate models predicted in MEGA v11.0.3 [61]. The NEXUS file was converted to an XML format, under the strict molecular clock model and the general time-reversible (GTR) model using BEAUti v1.10.4 [107–109]. The Markov chain Monte Carlo (MCMC) analysis was run for 100 million generations with samples taken every 10,000 MCMC generations using BEAST v1.10.4 [110,111]. The convergence of MCMC samples was then inspected using Tracer v1.10.4 [112]. Lastly, TreeAnnotator v1.10.4 discarded the first 10% burn-in and generated a final maximum clade credibility (MCC) tree [111]. The inferred tree was drawn using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

In-silico SCCmec typing and spa typing

SCC*mec*Finder v1.2, with default settings, was used for *in-silico* SCC*mec* identification from the assembled genomes [53]. Additionally, *spa* typing was performed using *spa*Typer v0.3.3 (https://github.com/HCGB-IGTP/spaTyper) to assign *spa* types in accordance with the Ridom SpaServer scheme.

In-silico detection of acquired antimicrobial resistance genes and cadmium-zinc resistance gene

Acquired antimicrobial resistance genes were identified using ResFinder v4.6.0 under default settings [113]. Furthermore, the reference sequence of the cadmium-zinc resistance gene, *czrC* (accession number KF593809.1), was retrieved from the NCBI Nucleotide database and later integrated into the ResFinder database. The presence of the *czrC* gene was determined using ResFinder, with a minimum gene coverage of 80% and a minimum identity threshold of 90%.

Results

Data preprocessing and genome assembly

Table 11 presents the summary statistics of long-read sequencing data after basecalling and adapter trimming using Dorado. The depth of coverage ranged from 101× to 419×, calculated based on

the genome size of the reference strain S0385 (2,872,582 bp). Additionally, genome assembly statistics are summarized in Table 12. The total assembly lengths of all genomes were compared to that of the reference genome to assess completeness. Most genomes were assembled into a single contig, indicating high assembly continuity. In contrast, strains J101.2, L3.1, and L43.2 contained multiple contigs (ranging from 3 to 5), suggesting possible assembly fragmentation or the presence of plasmid sequences.

Collection of S. aureus CC398 genomes in Thailand

Table 10 provides details of the 20 *S. aureus* CC398 genomes from Thailand included in this study. Of these, 18 were livestock-associated isolates obtained from both porcine and human sources, including pork, pigs and swine workers. The remaining two were from human clinical isolates.

Maximum-likelihood phylogenetic analysis

Figure 3 and Figure 4 depict the maximum-likelihood phylogenetic relationships of 1,215 *S. aureus* CC398 isolates from 24 countries, including Thailand, based on their core SNPs. The 20 *S. aureus* CC398 isolates from Thailand were positioned into three distinct locations within the phylogeny, designated as Sub-clades I, II, and III. Sub-clade I comprised seven isolates from Thailand and the USA. The strains SAMN17305089, SAMN17305092, J101.2, L3.1, L43.2, and Z19.1 were of Thai origin and shared a common ancestor. The closest relative to their ancestral *S. aureus* was a methicillin-susceptible *Staphylococcus aureus* (MSSA), named SAMN09906595, which was obtained from a human blood culture in the USA. Importantly, all six Thai strains carried SCC*mec* CI, consisting of *ccrA1B1* and *ccrC1*, and *spa* type t034 (Table 13). It is believed that the *ccr* gene complex plays a crucial role in the integration and precise excision of SCC*mec* elements [114]. However, the MSSA strain SAMN09906595 did not harbor an SCC element, though its *spa* type also belonged to t034 as same as the others.

Eleven European-sourced isolates and three porcine isolates from Thailand (TUM-B-P12/2/1, TUM-C-P26/1/1, and TUM-C-P27/1/1), shared a common ancestral node within Sub-clade II. These European isolates were recovered from various hosts, including bovines and humans in Denmark and Germany, and pigs in Italy. Interestingly, all isolates within this sub-clade were found to harbor SCC*mec* type Vc (5C2&5) (or type V in Chapter I), but they exhibited distinct *spa* types. The strains SAMEA104188246, SAMN14133959, TUM-B-P12/2/1, TUM-C-P26/1/1, and TUM-C-P27/1/1 from Germany, Italy, and Thailand possessed *spa* type t034, which was the most predominant type in this group, while *spa* type t011 was identified in over half of the Danish strains (except for SAMN08367605, SAMN08367609, and SAMN08367610).

Lastly, Sub-clade III clearly indicated that seven human isolates from Denmark clustered with the majority of isolates from Thailand. Remarkably, all Thai isolates were genotypically characterized

as SCC*mec* Vc (5C2&5)-t034, identical to the Danish strain SAMN14421326. The other six Danish isolates also harbored SCC*mec* type Vc (5C2&5); however, five of these isolates displayed *spa* type 011. The *spa* type for the Danish strain SAMN14421358 could not be determined and was labeled as NT.

Bayesian phylogenetic analysis and core genome-based pairwise SNP distance matrix

To estimate the emergence period of *S. aureus* CC398 in the Thai swine industry, Bayesian phylogenetic analysis with the targeted subset of 168 *S. aureus* CC398 genomes was conducted to reconstruct an MCC tree. The leaf labels (strain name) for these genomes are shown in green in Figure 3. Similar to the maximum-likelihood phylogenetic tree, the 20 *S. aureus* CC398 isolates from Thailand were placed in three different positions, highlighted in pale grey, in the Bayesian phylogenetic tree (Figure 5). As illustrated, six *S. aureus* CC398 isolates from pigs and humans (SAMN17305089, SAMN17305092, J101.2, L3.1, L43.2, and Z19.1) in Thailand exclusively resided together in a discrete sub-clade, which was distantly related to the other taxa in the tree. Significantly, it was estimated that their common ancestor descended from the oldest common ancestor of all included taxa in 1975 (95% HPD: 1969.87-1980.02). The timing of branching events within this group was scaled distinctly from 1992 (95% HPD: 1988.93-1996.35) to 2015 (95% HPD: 2014.66-2015.66). In addition, their closest taxon was the MSSA strain SAMN09906595 from the USA, as previously identified in the maximum-likelihood tree.

Phylogenetically, three porcine strains from Thailand (TUM-B-P12/2/1, TUM-C-P26/1/1, and TUM-C-P27/1/1) were closely related to the bovine strain SAMN08367610 from Denmark. It should be emphasized that these isolates from both countries diverged from a shared ancestor dating back to 2008 (95% HPD: 2007.06-2010.32). Subsequently, divergence events took place twice: once in 2015 (95% HPD: 2014.41-2016.12) and again in mid-2016 (95% HPD: 2016.12-2016.91). To assess genetic distances between Thai and Danish *S. aureus* CC398, the core genome-based pairwise SNP matrix from the CSI Phylogeny was further examined. It is observed that all porcine isolates from Thailand were evolutionarily distant from the bovine isolate from Denmark, with differences ranging from 51 to 53 SNPs.

Similarly, the remaining 11 *S. aureus* isolates from both human and porcine origins in Thailand clustered tightly with the human strain SAMN14421329 from Denmark. Furthermore, when examining the phylogenetic relationships retrospectively, this Danish strain SAMN14421329 clustered closely with six additional Danish isolates. The Bayesian phylogenetic inference estimated a shared evolutionary relationship between *S. aureus* from the two countries, tracing back to a common ancestor in 2008 (95% HPD: 2007.75-2009.00). Following this, multiple phylogenetic bifurcations within the Thai *S. aureus* lineage were elucidated from 2009 (95% HPD: 2008.77-2011.18) to mid-2016

(95% HPD: 2016.21-2016.91). The pairwise SNP distances between *S. aureus* from both countries varied between 22 and 45 SNPs, remarkably smaller than those previously mentioned.

Characterization of acquired antimicrobial resistance and cadmium-zinc resistance gene profiles

Further, I explored resistance genes of the 20 *S. aureus* CC398 isolates from Thailand and their sister taxa, including the strains SAMN08367610 and SAMN14421329 from Denmark. These isolates were categorized into three different groups —A, B, and C—based on their relationships in the Bayesian phylogeny (Table 14).

In total, 16 acquired antimicrobial resistance genes were predicted to be widely distributed among the 22 *S. aureus* CC398 isolates. These genes included those that phenotypically mediate resistance against six antimicrobial groups: aminoglycosides (n = 5), beta-lactams (n = 2), MLS_B (n = 5), phenicols (n = 1), tetracyclines (n = 2), and trimethoprim (n = 1).

When comparing antimicrobial resistance gene profiles among Thai isolates within Group A, it was found that the clinical strain SAMN17305092 exhibited a resistance pattern similar to those of the porcine and swine worker isolates, with the exception of the *aadD*, *ant(6)-Ia*, *lnu*(B), and *lsa*(E) genes. On the other hand, the clinical strain SAMN17305089 was found to carry nearly all the antimicrobial resistance genes identified in the porcine and swine worker isolates (except for *tet*(K)).

Remarkably, the Danish strain SAMN08367610 from Group B harbored a diverse assortment of antimicrobial resistance genes, including *ant(9)-Ia*, *str*, *blaZ*, *mecA*, *lnu*(B), *lsa*(E), *tet*(K), *tet*(M), and *dfrG*. These genes were consistently detected in all isolates from Thailand. However, *erm*(C) and *cat(pC221)* were uniquely observed in the Thai strain TUM-C-P27/1/1.

Finally, seven of the 11 Thai *S. aureus* isolates in Group C displayed an exact match in their antimicrobial resistance gene carriage to the Danish strain SAMN14421329, further highlighting a close genetic relationship between these isolates from the two countries Notably, the remaining four Thai isolates also exhibited a similar antimicrobial resistance gene pattern to the Danish strain; however, they harbored additional genes. To clarify, *ant(9)-Ia* and *erm*(A) were found in the Thai strains AA3.1, M3.1, and M31.1, whereas *erm*(C) was present in the Thai strain TUM-B-P22/2/2, but all were absent in the Danish strain SAMN14421329.

Aside from the antibiotic resistance genes, the *czrC* gene was also screened. Interestingly, none of the *S. aureus* CC398 within Group A tested positive for this heavy metal resistance gene. In contrast, it was detected in all isolates belonging to Groups B and C. It is believed that the *czrC* gene can serve as a genetic marker for pig-related strains [22].

Discussion

This study provides the epidemiological evidence on the genetic relatedness between LA-MRSA CC398 in Thailand and *S. aureus* CC398 from foreign countries. The SNP-based phylogenetic analyses using both maximum likelihood and Bayesian inference, clearly revealed that LA-MRSA CC398 in Sub-clades II and III from Thailand shared a high level of genome similarity with isolates from three European countries: Denmark, Italy, and Germany.

All Thai isolates from Sub-clades II and III belonged to the SCC*mec* Vc (5C2&5)-t034 genotype. In contrast, SCC*mec* Vc (5C2&5)-t011 was the most prevalent genotype among the European isolates, followed by the SCC*mec* Vc (5C2&5)-t034 genotype. These genotypes were widely disseminated in LA-MRSA, originating from pigs and cattle in Europe [79,115,116]. Notably, one of these Thai isolates was collected from a swine worker, and a previous study confirmed that it was transmitted from pigs through occupational exposure [104]. Taken together, these findings indicated that the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype in Thailand may have originated from either pigs or cattle in Europe. This hypothesis is further supported by the WGS-based characterization, which demonstrated a close genetic relationship between the Thai and European isolates.

It can be hypothesized that the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype in Thailand is unlikely to have originated from humans, despite most porcine isolates from Thailand were clustered closely with multiple human isolates from Europe. This is supported by the observation that these human isolates were primarily isolated from individuals with a history of livestock contact, for example, the human strain SAMN14421329 from Denmark [117]. This human strain also tested positive for the *tet*(M), *tet*(K) and *czrC* genes, which are typically found in LA-MRSA associated with pigs [22,79]

Although LA-MRSA CC398 has historically been less prevalent in Asia, several recent reports have suggested that the emergence of LA-MRSA CC398 among Asian pig populations might be driven by international pig trade [69,70,100,118]. Accordingly, I hypothesized that the introduction of LA-MRSA CC398 into Thai swine herds may be similarly associated with pig importation from overseas. To investigate this hypothesis, the Bayesian phylogenetic tree and data on Thailand's pig import volumes from global suppliers were simultaneously discussed in this study.

According to the Bayesian analysis, phylogenetic divergence events between the most recent common ancestors of the CC398-SCC*mec* Vc (5C2&5) genotype in Thailand and the two isolates from Denmark (SAMN08367610 and SAMN14421329) were estimated to have occurred in 2008 (95% HPD: 2007.06-2010.32 and 2007.75-2009.00, respectively). Subsequently, multiple lineage separations within the Thai lineage were detected serval times during 2009 (95% HPD: 2008.77-2011.18) to mid-2016 (95% HPD: 2016.21-2016.91), indicating ongoing changes

in transmission dynamics within Thailand's pig production system during that period. These findings align with the Trade Statistic Report System of the Ministry of Commerce of Thailand, which officially reports that Denmark has exported live swine to Thailand annually since 2003 (https://tradereport.moc.go.th/th) (Figure 6).

Although three porcine isolates (TUM-B-P12/2/1, TUM-C-P26/1/1, and TUM-C-P27/1/1) and the bovine strain SAMN08367610 from Denmark appeared to have descended from a common bacterial ancestor, it can be speculated that the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype in Thailand is more likely to have originated from Danish pigs rather than Danish cattle. This inference is supported by the fact that this bovine isolate was reported as evidence of spillover from pigs to cattle production in Denmark [22]. The isolate also lacked the *vwb* gene, which encodes the von Willebrand factor specific to ruminant hosts but carried the porcine-associated *czrC* gene [22]. Furthermore, the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype in Thailand is unlikely to have originated from Italian pigs, as there have been no records of pig imports from Italy to Thailand (Figure 6).

Ultimately, it is possible that the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype was introduced into Thailand's pig industry through global livestock trade. This echoes findings from a 2018 study in Italy, which detected LA-MRSA CC398 in pig farms that had purchased pigs from Denmark. These porcine isolates were phylogenetically related to the dominant lineages of Danish LA-MRSA CC398 [119]. However, imports of pork products and meat products derived from other livestock species can also contribute to the global dissemination of LA-MRSA. For instance, LA-MRSA CC398 was detected in pork and chicken meat available for sale in England. These meat products were sourced from continental Europe [120,121]. Similarly, Thailand has imported pork products (fresh, refrigerated, or frozen) from mainland Europe, including Denmark. Nevertheless, this may not be the case for this study, as such imports from Denmark were only documented in 2012 and 2013 during the 2010s, based on data from the Ministry of Commerce of Thailand (data not shown).

Besides animal movements and global trade, human activities may also play a role in the introduction of LA-MRSA in particular circumstances. For example, various MRSA CCs, including CC398, were identified in illegally imported food products confiscated from travelers arriving in the EU [122]. A healthcare worker was found to have introduced MRSA CC398 into a hospital in Denmark after overseas travel [123]. Thus, these examples suggest that a ban on the importation of pigs and pork may not be the most optimal strategy to address public health concerns of LA-MRSA CC398 in Thailand. Instead, from a One Health perspective, I personally believed that robust on-farm biosecurity coupled with the prudent use of antimicrobials would be a more practical solution to prevent occupational transmission from livestock to workers.

The LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 isolates in Thailand exhibited a moderate to high degree of similarity in their antimicrobial resistance gene profiles compared to their closely

related Danish isolates. However, some Thai isolates were found to carry additional antimicrobial resistance genes, including *ant(9)-Ia*, *cat(pC221)*, *erm*(A), and *erm*(C), which were absent in the related Danish strains. These genes are usually associated with mobile genetic elements, such as plasmids or transposons, in staphylococcal species [124]. This observation suggested that the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 isolates in Thailand might have acquired these additional resistance genes horizontally from other *Staphylococcus* spp. via mobile genetic elements after the introduction.

The final significant observation is that both phylogenetic analyses yielded consistent results. To clarify, the LA-MRSA CC398-SCC*mec* CI-t034 genotype evolved independently from the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype, indicating a separate evolutionary line. The common ancestor of the composite genotype is believed to have existed prior to 2008, suggesting that this genotype is unlikely to be associated with porcine-associated CC398 lineage in Denmark.

Conclusion

This study highlights the potential risk of introducing the exotic LA-MRSA CC398 strain into Thailand's swine industry due to transboundary animal movement. WGS-based phylogenetic analyses provide substantial genetic evidence indicating that the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype found in Thailand may have originated from pigs in Denmark. This hypothesis is further supported by Thailand's international trade statistics, which document the exportation of pigs from Denmark to Thailand. Nevertheless, the absence of LA-MRSA CC398 isolates from imported pigs represents a limitation of this study. As such, monitoring pig imports from MRSA-positive countries should be prioritized in Thailand. Conversely, the LA-MRSA CC398-SCC*mec* CI-t034 genotype may have evolved separately from the LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype. This finding indicates that the composite genotype is unlikely to be associated with the international movement of animals.

Table 9. Complete genomes of the 13 livestock-associated methicillin-resistant *Staphylococcus aureus* CC398 isolates in this study deposited in the NCBI GenBank database under the BioProject accession number PRJNA1158956.

BioSample	Strain name	Year of collection	Source	Country
SAMN43554680	J101.2	2016	Human	Thailand
SAMN43554681	L3.1	2016	Swine	Thailand
SAMN43554682	L43.2	2016	Human	Thailand
SAMN43554683	Z19.1	2017	Swine	Thailand
SAMN43554684	AA3.1	2017	Swine	Thailand
SAMN43554685	M3.1	2016	Swine	Thailand
SAMN43554686	M31.1	2016	Human	Thailand
SAMN43554687	Y1.2	2017	Swine	Thailand
SAMN43554688	S2.1	2017	Swine	Thailand
SAMN43554689	H49.1	2016	Swine	Thailand
SAMN43554690	G2.1	2016	Swine	Thailand
SAMN43554691	D16.1	2016	Swine	Thailand
SAMN43554692	X1.1	2017	Swine	Thailand

Table 10. Details of the 20 *Staphylococcus aureus* CC398 genomes in Thailand analyzed in this study.

Strain name	Source	Detailed source	Year of collection	MRSA/MSSA	Reference
TUM-B-P12/2/1	Swine	Retail pork	2017	MRSA	Tanomsridachchai
TUM-B-P22/2/2	Swine	Retail pork	2017	MRSA	Tanomsridachchai
TUM-C-P26/1/1	Swine	Retail pork	2017	MRSA	Tanomsridachchai
TUM-C-P27/1/1	Swine	Retail pork	2017	MRSA	Tanomsridachchai
TUS-A2-N16/1	Swine	Slaughtered pig	2018	MRSA	Tanomsridachchai
J101.2	Human	Swine worker	2016	MRSA	This study
L3.1	Swine	Pig	2016	MRSA	This study
L43.2	Human	Swine worker	2016	MRSA	This study
Z19.1	Swine	Pig	2017	MRSA	This study
AA3.1	Swine	Pig	2017	MRSA	This study
M3.1	Swine	Pig	2016	MRSA	This study
M31.1	Human	Swine worker	2016	MRSA	This study
Y1.2	Swine	Pig	2017	MRSA	This study
S2.1	Swine	Pig	2017	MRSA	This study
H49.1	Swine	Pig	2016	MRSA	This study
G2.1	Swine	Pig	2016	MRSA	This study
D16.1	Swine	Pig	2016	MRSA	This study
X1.1	Swine	Pig	2017	MRSA	This study
SAMN17305089	Human	Patient	2017	MRSA	Pathogenwatch
SAMN17305092	Human	Patient	2017	MRSA	Pathogenwatch

MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.

Table 11. Summary metrics of whole-genome sequencing data using long-read sequencing.

	Parameter			
Strain name	Total bases (Mbp)	Mean read length (bp)	Total reads	Depth of coverage (x)
AA3.1	1178.4	9,618	122,518	419
D16.1	414.7	10,315	40,203	147
G2.1	502.2	6,681	75,169	178
H49.1	460.3	6,211	74,101	164
J101.2	376.0	8,333	45,129	134
L3.1	447.2	6,795	65,818	159
L43.2	780.0	9,708	80,348	277
M3.1	1059.9	9,874	107,338	377
M31.1	677.6	10,738	63,103	241
S2.1	842.4	10,296	81,816	299
X1.1	362.5	8,080	44,866	129
Y1.2	364.2	7,788	46,760	129
Z19.1	285.6	10,551	27,067	101

Table 12. Summary of long-read genome assembly statistics.

Strain name	Parameter					
	Total length (bp)	Number of contigs	Largest contig (bp)	N50 (bp)	L50	GC content (%)
AA3.1	2,893,072	1	2,893,072	2,893,072	1	33.00
D16.1	2,882,692	1	2,882,692	2,882,692	1	33.01
G2.1	2,882,716	1	2,882,716	2,882,716	1	33.01
H49.1	2,882,691	1	2,882,691	2,882,691	1	33.01
J101.2	3,085,606	5	2,035,461	2,035,461	1	32.98
L3.1	2,883,445	4	2,836,728	2,836,728	1	32.91
L43.2	2,886,738	3	2,839,237	2,839,237	1	32.91
M3.1	2,893,071	1	2,893,071	2,893,071	1	33.00
M31.1	2,893,070	1	2,893,070	2,893,070	1	33.00
S2.1	2,884,278	1	2,884,278	2,884,278	1	33.01
X1.1	2,882,367	1	2,882,367	2,882,367	1	33.01
Y1.2	2,882,389	1	2,882,389	2,882,389	1	33.01
Z19.1	2,935,222	4	2,849,074	2,849,074	1	32.93

Table 13. WGS-based characteristics of *Staphylococcus aureus* CC398 isolates clustered within Sub-clade I, II and III.

Sub-clade	Strain name	SCC*mec* type	*spa* type	Country	Source	*mec* complex class	*ccr* gene complex			
							Type	*ccrA1*	*ccrB1*	*ccrC1*
I	SAMN09906595	-	t034	USA	Human	-	-	-	-	-
	SAMN17305089	CI	t034	Thailand	Human	C2	NT	+	+	+
	SAMN17305092	CI	t034	Thailand	Human	C2	NT	+	+	+
	J101.2	CI	t034	Thailand	Human	C2	NT	+	+	+
	L3.1	CI	t034	Thailand	Swine	C2	NT	+	+	+
	L43.2	CI	t034	Thailand	Human	C2	NT	+	+	+
	Z19.1	CI	t034	Thailand	Swine	C2	NT	+	+	+
II	SAMEA6648566	Vc (5C2&5)	t011	Denmark	Human	C2	5	-	-	+
	SAMEA104188246	Vc (5C2&5)	t034	Germany	Human	C2	5	-	-	+
	SAMN08367605	Vc (5C2&5)	t4677	Denmark	Bovine	C2	5	-	-	+
	SAMN08367608	Vc (5C2&5)	t011	Denmark	Bovine	C2	5	-	-	+
	SAMN08367609	Vc (5C2&5)	t693	Denmark	Bovine	C2	5	-	-	+
	SAMN08367610	Vc (5C2&5)	t4677	Denmark	Bovine	C2	5	-	-	+
	SAMN08367611	Vc (5C2&5)	t011	Denmark	Bovine	C2	5	-	-	+
	SAMN08367612	Vc (5C2&5)	t011	Denmark	Bovine	C2	5	-	-	+
	SAMN08367613	Vc (5C2&5)	t011	Denmark	Bovine	C2	5	-	-	+
	SAMN11954862	Vc (5C2&5)	NT	Italy	Swine	C2	5	-	-	+
	SAMN14133959	Vc (5C2&5)	t034	Italy	Swine	C2	5	-	-	+
	TUM-B-P12/2/1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	TUM-C-P26/1/1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	TUM-C-P27/1/1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
III	SAMN14421326	Vc (5C2&5)	t034	Denmark	Human	C2	5	-	-	+
	SAMN14421329	Vc (5C2&5)	t011	Denmark	Human	C2	5	-	-	+
	SAMN14421343	Vc (5C2&5)	t011	Denmark	Human	C2	5	-	-	+
	SAMN14421358	Vc (5C2&5)	NT	Denmark	Human	C2	5	-	-	+
	SAMN14421361	Vc (5C2&5)	t011	Denmark	Human	C2	5	-	-	+
	SAMN14421389	Vc (5C2&5)	t011	Denmark	Human	C2	5	-	-	+
	SAMN14421390	Vc (5C2&5)	t011	Denmark	Human	C2	5	-	-	+
	AA3.1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	D16.1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	G2.1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	H49.1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	M3.1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	M31.1	Vc (5C2&5)	t034	Thailand	Human	C2	5	-	-	+
	S2.1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	TUM-B-P22/2/2	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	TUS-A2-N16/1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	X1.1	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+
	Y1.2	Vc (5C2&5)	t034	Thailand	Swine	C2	5	-	-	+

CI, composite island; NT, non-typeable; +, detected; -, not detected.

Table 14. Acquired antimicrobial resistance genes and cadmium-zinc resistance gene present in the 20 *Staphylococcus aureus* CC398 isolates from Thailand and their closest relatives, as illustrated in the Bayesian phylogenetic tree.

						AMGs					BLs							MLS_B		PHEs	TCs		TMP	Cadmium and Zinc
Group	Strain name	SCC*mec* type	*spa* type	Country	Source	*aac(6')-aph(2")*	*aadD*	*ant(6)-Ia*	*ant(9)-Ia*	*str*	*blaZ*	*mecA*	*erm*(A)	*erm*(B)	*erm*(C)	*lnu*(B)	*lsa*(E)	*cat(pC221)*	*tet*(K)	*tet*(M)	*dfrG*	*czrC*
A	SAMN17305089	CI	t034	Thailand	Human	+	+	+	+	-	+	+	+	+	-	+	+	-	-	+	+	-
	SAMN17305092	CI	t034	Thailand	Human	+	-	-	+	-	+	+	+	+	-	-	-	-	+	+	+	-
	J101.2	CI	t034	Thailand	Human	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	+	-
	L3.1	CI	t034	Thailand	Swine	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	+	-
	L43.2	CI	t034	Thailand	Human	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	+	-
	Z19.1	CI	t034	Thailand	Swine	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+	+	-
B	SAMN08367610	Vc (5C2&5)	t4677	Denmark	Bovine	-	-	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+
	TUM-B-P12/2/1	Vc (5C2&5)	t034	Thailand	Swine	-	-	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+
	TUM-C-P26/1/1	Vc (5C2&5)	t034	Thailand	Swine	-	-	-	+	+	+	+	-	-	-	+	+	-	+	+	+	+
	TUM-C-P27/1/1	Vc (5C2&5)	t034	Thailand	Swine	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+
C	SAMN14421329	Vc (5C2&5)	t011	Denmark	Human	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+
	AA3.1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	+	-	+	+	+	-	-	+	+	-	+	+	+	+
	D16.1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+
	G2.1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+
	H49.1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+
	M3.1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	+	-	+	+	+	-	-	+	+	-	+	+	+	+
	M31.1	Vc (5C2&5)	t034	Thailand	Human	-	+	+	+	-	+	+	+	-	-	+	+	-	+	+	+	+
	S2.1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+
	TUM-B-P22/2/2	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	+	+	+	-	+	+	+	+
	TUS-A2-N16/1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+
	X1.1	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+
	Y1.2	Vc (5C2&5)	t034	Thailand	Swine	-	+	+	-	-	+	+	-	-	-	+	+	-	+	+	+	+

AMGs, aminoglycosides; BLs, beta-lactams; CI, composite island; MLS_B, macrolide-lincosamide-streptogramin B compounds; PHEs, phenicols; TCs, tetracyclines; TMP, trimethoprim; +, detected; -, not detected.

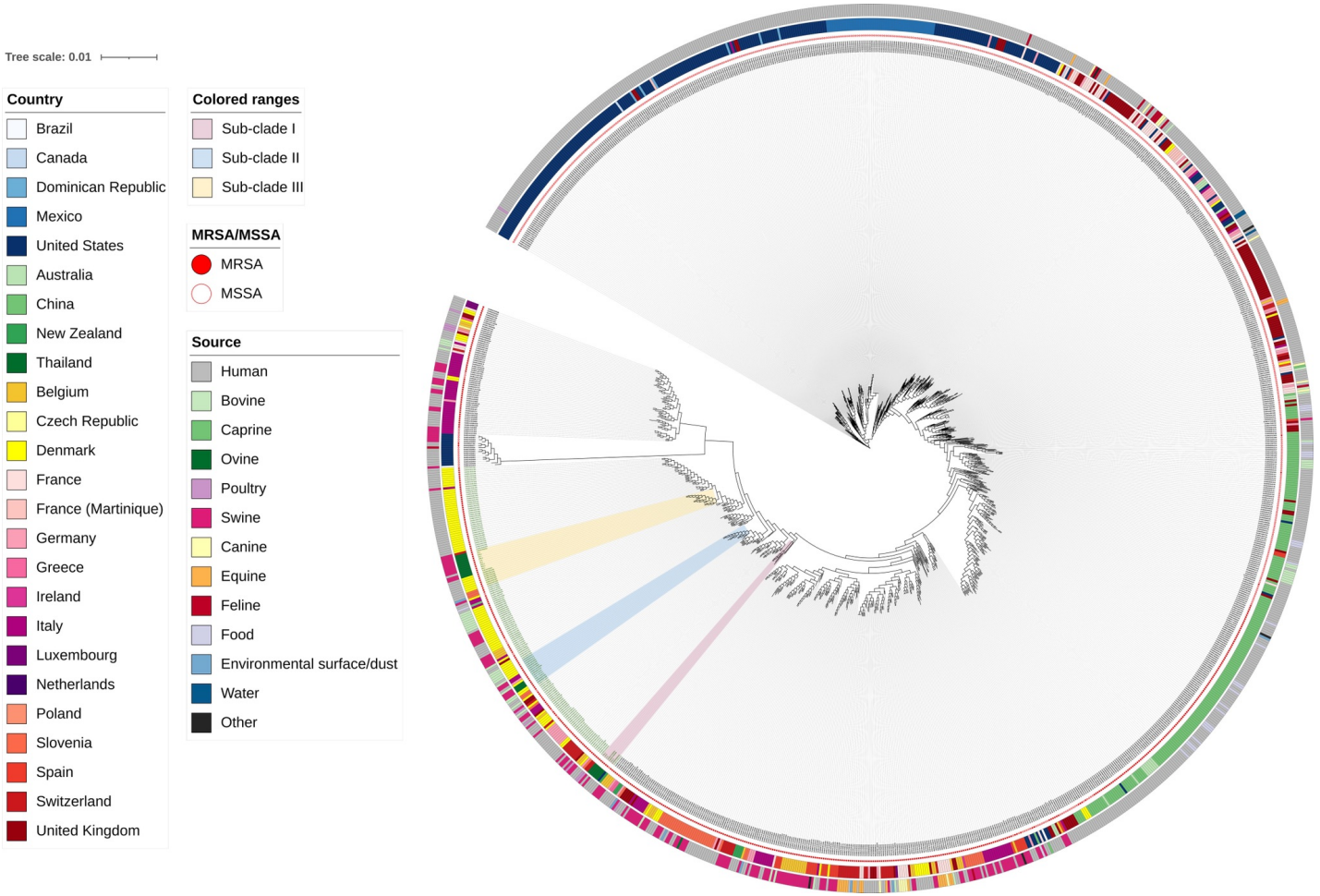

Figure 3. Maximum-likelihood phylogeny of the 1,215 *Staphylococcus aureus* CC398 isolates reconstructed from 39,642 core SNPs.

Methicillin resistance is indicated by filled and empty red circles. Metadata for the country (inner ring) and source of origin (outer ring) are represented by distinct colors. Sub-clades I, II, and III are highlighted in pastel pink, light blue, and pale yellow, respectively. The leaf labels (strain name) in green denote the subset of 168 *Staphylococcus aureus* CC398 isolates selected for Bayesian phylogenetic tree analysis.

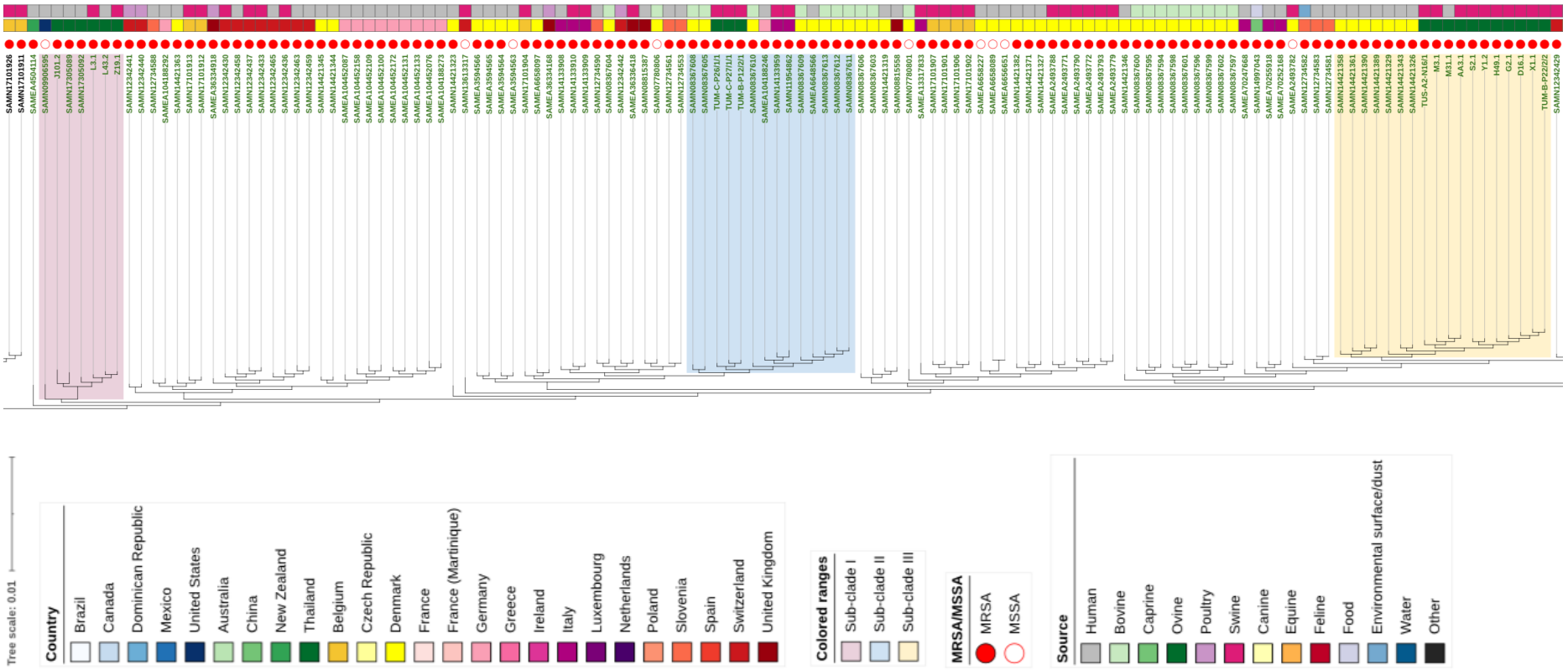

Figure 4. Maximum-likelihood phylogeny of the 1,215 *Staphylococcus aureus* CC398 isolates reconstructed from 39,642 core SNPs (rectangular layout; partially cropped).

Methicillin resistance is indicated by filled and empty red circles. Metadata for the country (left column) and source of origin (right column) are represented by distinct colors. Sub-clades I, II, and III are highlighted in pastel pink, light blue, and pale yellow, respectively. The leaf labels (strain name) in green denote the subset of 168 *Staphylococcus aureus* CC398 isolates selected for Bayesian phylogenetic tree analysis.

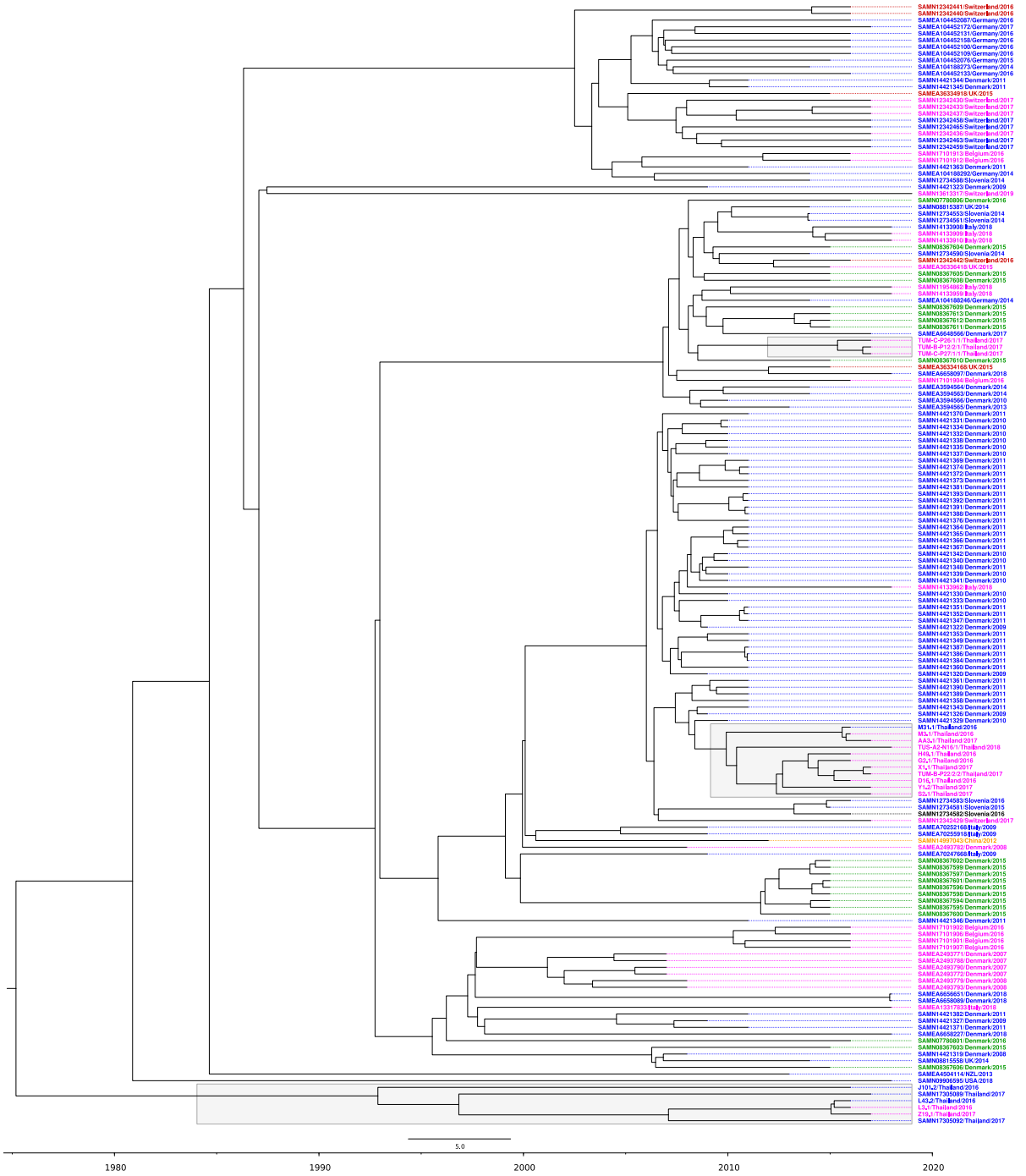

Figure 5. Bayesian phylogenetic tree of the subset of 168 *Staphylococcus aureus* CC398 isolates, generated using the BEAST package.

The 20 *Staphylococcus aureus* CC398 isolates from Thailand are positioned in three different positions, highlighted in pale grey. Divergence times are estimated with the 95% highest posterior density (HPD) intervals. Isolates are labeled with their strain name, country, and year of collection. Labels are color-coded by source of origin: navy blue for humans, pink for swine, green for bovine, dark red for poultry, and black for environmental surfaces/dust. Country abbreviations are as follows: NZL, New Zealand; UK, United Kingdom; USA, United States of America.

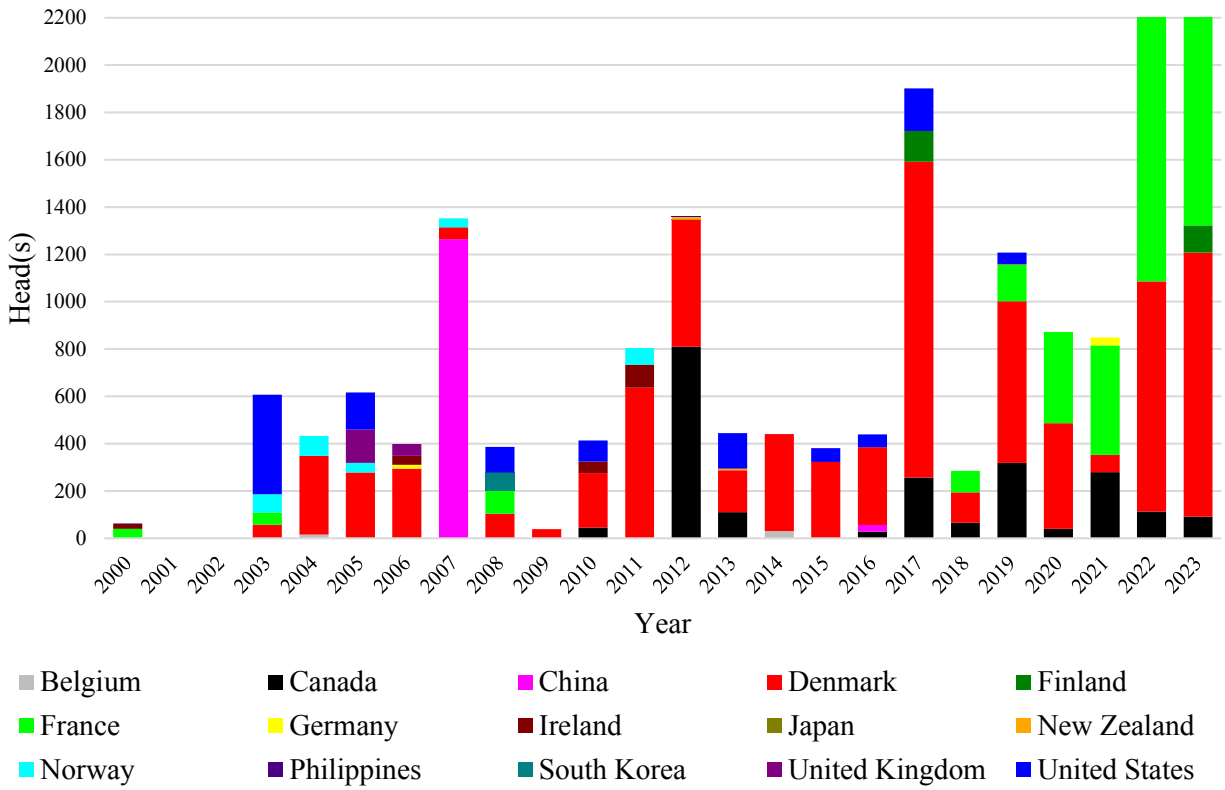

Figure 6. Annual number of live pig imports to Thailand from international suppliers (2000–2023).

Each country is represented by a different color. The data was retrieved from the Trade Statistics Report System of the Ministry of Commerce, Thailand, on August 28, 2024 (https://tradereport.moc.go.th/th).

GENERAL CONCLUSION

Chapter I systematically integrates comparative genomics, antimicrobial resistance profiling, virulome characterization to investigate the transmission dynamics of LA-MRSA CC9 and CC398 among Thai pigs and swine workers. Furthermore, the study empirically validates the application of WGS as an effective approach to verify the zoonotic transmission of LA-MRSA CC398 on two swine farms. Core genome SNP-based maximum-likelihood phylogenetic analysis reveals a close genetic relationship between LA-MRSA CC398 isolates from pigs and swine workers, with pairwise SNP distances ranging from 3 to 11. The phylogenetic tree further demonstrates local dissemination of porcine-derived LA-MRSA CC398 within the same province and between neighboring provinces. The LA-MRSA CC398-SCC*mec* CI-t034 genotype appears to have acquired the additional *ccrA1B1* gene complex via genetic recombination. Importantly, All LA-MRSA isolates identified—from both CC398 and CC9 lineages—are characterized as MDR LA-MRSA, harboring numerous antimicrobial resistance genes, including those associated with cross-resistance on putative plasmid contigs. The antimicrobial resistance profile also highlights the potential for these LA-MRSA isolates to confer resistance to antimicrobials used in livestock as well as critically important antibiotics in human medicine. Although these isolates lack major virulence determinants typically associated with severe human MRSA diseases, they can encode multiple genes implicated in nasal colonization and persistence in human hosts. Collectively, these findings raise public health concerns related to occupational exposure and therapeutic limitations in colonized individuals and susceptible populations.

Chapter II provides epidemiological evidence on the genetic relationships between LA-MRSA CC398 in Thailand and *S. aureus* CC398 from other countries. The SNP-based phylogenetic analyses of the 18 Thai LA-MRSA CC398 isolates, using both maximum likelihood and Bayesian inference methods, reveals three distinct evolutionary lines. The LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype, identified in Sub-clades II and III, shows close genetic relatedness to human and livestock-associated isolates from Denmark, Italy, and Germany. The Bayesian phylogenetic tree clearly indicates a shared ancestral origin between this genotype and two Danish isolates: the human strain SAMN14421329 and bovine strain SAMN08367610. Based on the presence of the *czrC* gene—commonly associated with pig- associated MRSA—in both Danish isolates, it is hypothesized that the Thai LA-MRSA CC398-SCC*mec* Vc (5C2&5)-t034 genotype may have originated from porcine-associated *S. aureus* CC398 strains circulating in Denmark. In addition, the international clonal expansion of *S. aureus* CC398 into Thailand is likely associated with cross-border movements of livestock. In contrast, the ancestral source of the CC398-SCC*mec* CI-t034 genotype within Sub-clade I remains unsolved, possibly due to the limited availability of genome data for this variant.

This emphasizes the essential need for ongoing genomic surveillance and monitoring of the CC398-SCC*mec* CI-t034 genotype in Thailand.

In conclusion, this dissertation presents comprehensive whole-genome-based analyses of LA-MRSA CC398, identified for the first time in Thailand's swine industry. The study also suggests the potential origins and contributing factors underlying the emergence and dissemination of LA-MRSA CC398 in Thailand. More importantly, it underscores that the global livestock movement can facilitate the introduction of an exotic bacterial strain with zoonotic potential and extensive antimicrobial resistance. The importation of animals from endemic regions may therefore represent a considerable public health risk.

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