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Emergence of livestock-associated MRSA clonal complex 398 in Thailand's swine industry

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Running title: Emergence of LA-MRSA CC398 in Thai swine industry

Abstract

Background: Livestock-associated MRSA (LA-MRSA) clonal complex (CC) 398 is primarily found among pigs across Europe. However, CC398 has also been sporadically identified in several Asian countries, including Thailand. This study aimed to examine the drivers contributing to the emergence of LA-MRSA CC398 in Thailand's swine industry.

Methods: Whole-genome sequencing data of 18 LA-MRSA CC398 isolates from pigs, retail pork, and swine workers in Thailand were analyzed. Additionally, a total of 1,197 qualified genomes from various sources in 24 countries were retrieved from Pathogenwatch. Subsequently, two SNP-based phylogenetic trees were reconstructed using maximum likelihood ($n = 1,215$) and Bayesian inference ($n = 168$).

Results: Our findings revealed that *S. aureus* isolates with the CC398-SCCmec Vc (5C2&5)-t034 genotype were closely related to European isolates, including those from Denmark. In contrast, *S. aureus* isolates belonging to the CC398-composite SCCmec-t034 formed a phylogenetically distinct cluster.

Conclusion: This study reports that the CC398-SCCmec Vc (5C2&5)-t034 genotype identified in Thai pigs may have originated from Danish swine populations, possibly introduced into Thailand through international livestock movement. The CC398-composite SCCmec-t034 genotype was also detected in domestic pigs; however, it appears to have evolved independently from the CC398-SCCmec Vc (5C2&5)-t034 genotype. Bayesian phylogenetic analysis estimates that the common ancestor of the composite genotype emerged earlier than that of the CC398-SCCmec Vc (5C2&5)-t034 genotype, indicating a separate evolutionary origin.

Introduction

MRSA is recognized as one of the most serious threats to human health due to extensive dissemination in healthcare facilities worldwide.¹ Livestock-associated MRSA (LA-MRSA) is a specific type of MRSA colonizing food-producing animals, primarily pigs.² In Thailand, LA-MRSA clonal complex (CC) 9 has historically been the dominant lineage identified in swine holdings.^{3–5} However, LA-MRSA CC398—widely spread among European pigs—was first detected in Thai swine in 2019.^{2,6} Despite this finding, a comprehensive investigation into the emergence of LA-MRSA CC398 in Thailand has never been officially reported. Consequently, the present study aimed to trace the potential original source of LA-MRSA CC398 in Thailand and to investigate the factors associated with the emergence of LA-MRSA CC398 in the Thai swine industry.

Methods

Genome preprocessing

A total of 13 LA-MRSA CC398 isolates, previously recovered from pigs and swine workers in Thailand, were selected for WGS.⁶ 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). Super-accurate basecalling and adapter trimming were performed using Dorado v0.6.0 (<https://github.com/nanoporetech/dorado>). The trimmed reads were then assembled using Flye v2.9.3.⁷ To polish the nanopore-based assemblies, previously generated short-read data were integrated using Polypolish v0.5.0.^{5,8}

In addition, this study included five genomes of porcine LA-MRSA CC398 isolates in Thailand, which were sequenced in a previous study.⁹

For global comparison, 1,653 publicly available *S. aureus* CC398 genomes, including the reference genome S0385 (GenBank accession no. AM990992), were retrieved from Pathogenwatch (<https://pathogen.watch/>). Low-quality genomes containing ≥ 150 contigs were filtered out.¹⁰ 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.

Phylogenetic tree reconstruction

SNP-based phylogenetic trees were inferred genetically using two methods: maximum likelihood and Bayesian inference. A total of 1,215 *S. aureus* CC398 genomes were aligned against the reference genome S0385 to call SNPs through the CSI Phylogeny v1.4.¹¹ The resulting maximum-likelihood tree was visualized using iTOL v6.9.1.¹² For the Bayesian approach, we focused on a subset of 148 *S. aureus* CC398 isolates that clustered closely with 20 isolates of Thai *S. aureus* CC398, as indicated by the maximum-likelihood tree. The Bayesian phylogenetic tree was reconstructed under the strict molecular clock model and the general time-reversible model using BEAST package.^{13,14} 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.^{14,15} The convergence of MCMC samples was then inspected using Tracer v1.10.4.¹⁶ TreeAnnotator v1.10.4 discarded the first 10% burn-in and

generated a final maximum clade credibility (MCC) tree.¹⁴ Lastly, the inferred tree was drawn using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

SCCmec typing and spa typing

In silico SCCmec typing was conducted using SCCmecFinder v1.2, while *spa* typing was performed with spaTyper v0.3.3 (<https://github.com/HCGB-IGTP/spaTyper>).¹⁷

Detection of cadmium-zinc resistance gene

Cadmium-zinc resistance gene, *czrC* (accession number KF593809.1), was identified using ResFinder v4.6.0, with minimum gene coverage of 80% and minimum identity threshold of 90%.¹⁸

Results

***S. aureus* CC398 genomes from Thailand**

Table 1 summarizes the genomic characteristics of the 20 *S. aureus* CC398 genomes from Thailand included in this study. Of these, six isolates were identified as the CC398-SCCmec composite island (CI)-t034 genotype. The remaining 14 belonged to the CC398-SCCmec Vc (5C2&5)-t034 genotype. The composite SCCmec element was composed of both *ccrA1B1* and *ccrC1* gene complexes, whereas the SCCmec Vc (5C2&5) harbored only the *ccrC1* gene complex.

Maximum-likelihood phylogenetic analysis

Figure S1 depicts the maximum-likelihood phylogenetic relationships of 1,215 *S. aureus* CC398 isolates from 24 countries based on their core SNPs. The 20 *S. aureus* CC398 isolates from Thailand were positioned into three distinct locations within the tree. Six Thai isolates harboring the CC398-SCCmec CI-t034 genotype—SAMN17305089, SAMN17305092, J101.2, L3.1, L43.2, and Z19.1—shared a common ancestor with an MSSA isolate (SAMN09906595) from the USA.

Nine European isolates shared a common ancestral node with three Thai isolates (TUM-B-P12/2/1, TUM-C-P26/1/1, and TUM-C-P27/1/1) from the CC398-SCCmec Vc (5C2&5)-t034 genotype. These European isolates were recovered from various hosts, including bovine and humans in Denmark and Germany, and pigs in Italy. The remaining Thai CC398-SCCmec Vc (5C2&5)-t034 isolates formed a closely related cluster with two human isolates from Denmark.

Bayesian phylogenetic analysis

To estimate the emergence period of *S. aureus* CC398 in the Thai swine industry, we conducted Bayesian phylogenetic analysis on the targeted subset of 168 *S. aureus* CC398 genomes to reconstruct an MCC tree. The leaf labels (strain name) for these genomes are shown in green in **Figure S1**. 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 1**). As illustrated, the CC398-SCCmec CI-t034 genotype comprising six isolates from pigs and humans 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).

Phylogenetically, three porcine strains with the CC398-SCCmec Vc (5C2&5)-t034 genotype in Thailand were closely related to the bovine strain SAMN08367610 from Denmark. This Danish isolate was found to harbor SCCmec type Vc (5C2&5) and *spa* type t4677 (**Table S1**). It should be emphasized that the isolates from both countries diverged from a shared ancestor dating back to 2008 (95% HPD: 2007.06-2010.32). Subsequently, divergence events were estimated to have occurred in 2015 (95% HPD: 2014.41-2016.12) and mid-2016 (95% HPD: 2016.12-2016.91).

Similarly, the remaining 11 *S. aureus* isolates with the CC398-SCCmec Vc (5C2&5)-t034 genotype from both human and porcine origins in Thailand clustered tightly with the human strain SAMN14421329 from Denmark, which carried SCCmec Vc (5C2&5)-t011. 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).

Cadmium-zinc resistance gene

We also examined the presence of the *czrC* gene among the 20 *S. aureus* CC398 isolates from Thailand and their sister taxa, as shown in **Figure 1** and **Figure S1**, from Denmark, Germany, and Italy. Interestingly, none of the *S. aureus* CC398 with the composite SCCmec element tested positive for this heavy metal resistance gene (**Table S1**). In contrast, it was detected in all isolates harboring the SCCmec Vc (5C2&5). It is believed that the *czrC* gene can serve as a genetic marker for pig-related *S. aureus*.¹⁹

Discussion

This study presents epidemiological evidence on the genetic relatedness between *S. aureus* CC398 isolates from Thailand and those from foreign countries. The maximum likelihood phylogenetic tree showed that the CC398-SCCmec Vc (5C2&5)-t034 genotype identified in Thailand shared a high level of genome similarity with *S. aureus* CC398 isolates from three European countries, including Denmark. However, the Bayesian phylogenetic inference revealed a particularly close genetic relationship between the CC398-SCCmec Vc (5C2&5)-t034 genotype in Thailand and *S. aureus* CC398 isolates in Denmark.

We hypothesized that the CC398-SCCmec Vc (5C2&5)-t034 genotype in Thai pigs is more likely to have originated from Danish pigs, even though our porcine isolates were clustered closely with multiple Danish isolates from humans and bovine. This hypothesis is supported by the observation that the human isolates SAMN14421326 and SAMN14421329, were isolated from individuals with occupational exposure to pigs.²⁰ Additionally, the bovine

isolates SAMN08367610, SAMN08367611, SAMN08367612, and SAMN08367613 were reported as evidence of spillover from pigs to cattle production systems in Denmark.¹⁹

The Bayesian phylogenetic analysis estimated that the common ancestors of the CC398-SCC*mec* Vc (5C2&5) genotype in Thailand diverged from the two isolates from Denmark (SAMN08367610 and SAMN14421329) around 2008. Subsequently, multiple lineage separations within the Thai lineage were detected several times during 2009 to mid-2016, indicating ongoing changes in transmission dynamics within Thailand's swine industry. 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 S2).

Both SNP-based phylogenetic analyses consistently revealed that the CC398-SCC*mec* CI-t034 genotype in Thailand evolved independently from the CC398-SCC*mec* Vc (5C2&5) genotype, indicating a separate evolution. Furthermore, 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.

In conclusion, this study highlights the significant impact of international livestock movement in the transmission of LA-MRSA CC398 across borders, possibly facilitating the introduction of the CC398-SCC*mec* Vc (5C2&5)-t034 genotype from Denmark to Thailand. The independent evolution of the CC398-SCC*mec* CI-t034 genotype underscores the genetic complexity within the CC398 lineage in Thailand, emphasizing the necessity for continued surveillance.

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Transparency declaration

None to declare.

Author contributions

P.N., P.C., P.L., F.A, C.N. and Y.S. contributed to conceptualization; all authors contributed to the methodology; P.C., J.Y., P.L., F.A, C.N. and Y.S. contributed to validation; all authors

contributed to investigation; P.N., P.C., W.T., J.Y. and O.S. contributed to resources; P.N., J.Y., P.L. and F.A contributed to data curation and formal analysis; P.N., P.L., F.A., C.N. and Y.S. contributed to writing—original draft; all authors contributed to reviewing and editing the manuscript; C.N. and Y.S. contributed to supervision and project administration; Y.S. contributed to funding acquisition. All authors have read and agreed to the published version of the manuscript.

Data availability

The complete genome sequencing data of the 13 LA-MRSA CC398 isolates analyzed in this study are accessible in GenBank under BioProject accession number PRJNA1158956 (Table S2).

Supplementary data

Figures S1 and S2, and Tables S1 and S2 are available as Supplementary data at JAC Online.

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298 **Table 1.** WGS-based characteristics of the 20 *Staphylococcus aureus* CC398 genomes in Thailand analyzed in this study.

Strain name	Source	Detailed source	Year of collection	SCC <i>mec</i>	<i>spa</i> type	<i>mec</i> complex	<i>ccr</i> gene complex			Reference	
				type		class	Type	<i>ccrA1</i>	<i>ccrB1</i>		<i>ccrC1</i>
J101.2	Human	Swine worker*	2016	CI	t034	C2	NT	+	+	+	This study
L3.1	Swine	Pig*	2016	CI	t034	C2	NT	+	+	+	This study
L43.2	Human	Swine worker*	2016	CI	t034	C2	NT	+	+	+	This study
SAMN17305089	Human	Blood (sepsis)	2017	CI	t034	C2	NT	+	+	+	Pathogenwatch
SAMN17305092	Human	Blood (sepsis)	2017	CI	t034	C2	NT	+	+	+	Pathogenwatch
Z19.1	Swine	Pig*	2017	CI	t034	C2	NT	+	+	+	This study
AA3.1	Swine	Pig*	2017	Vc (5C2&5)	t034	C2	5	-	-	+	This study
D16.1	Swine	Pig*	2016	Vc (5C2&5)	t034	C2	5	-	-	+	This study
G2.1	Swine	Pig*	2016	Vc (5C2&5)	t034	C2	5	-	-	+	This study
H49.1	Swine	Pig*	2016	Vc (5C2&5)	t034	C2	5	-	-	+	This study
M3.1	Swine	Pig*	2016	Vc (5C2&5)	t034	C2	5	-	-	+	This study
M31.1	Human	Swine worker*	2016	Vc (5C2&5)	t034	C2	5	-	-	+	This study
S2.1	Swine	Pig*	2017	Vc (5C2&5)	t034	C2	5	-	-	+	This study
TUM-B-P12/2/1	Swine	Retail pork	2017	Vc (5C2&5)	t034	C2	5	-	-	+	Tanomsridachchai ⁹
TUM-B-P22/2/2	Swine	Retail pork	2017	Vc (5C2&5)	t034	C2	5	-	-	+	Tanomsridachchai ⁹
TUM-C-P26/1/1	Swine	Retail pork	2017	Vc (5C2&5)	t034	C2	5	-	-	+	Tanomsridachchai ⁹
TUM-C-P27/1/1	Swine	Retail pork	2017	Vc (5C2&5)	t034	C2	5	-	-	+	Tanomsridachchai ⁹
TUS-A2-N16/1	Swine	Slaughtered pig	2018	Vc (5C2&5)	t034	C2	5	-	-	+	Tanomsridachchai ⁹
X1.1	Swine	Pig*	2017	Vc (5C2&5)	t034	C2	5	-	-	+	This study
Y1.2	Swine	Pig*	2017	Vc (5C2&5)	t034	C2	5	-	-	+	This study

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

300 * Clinically healthy.

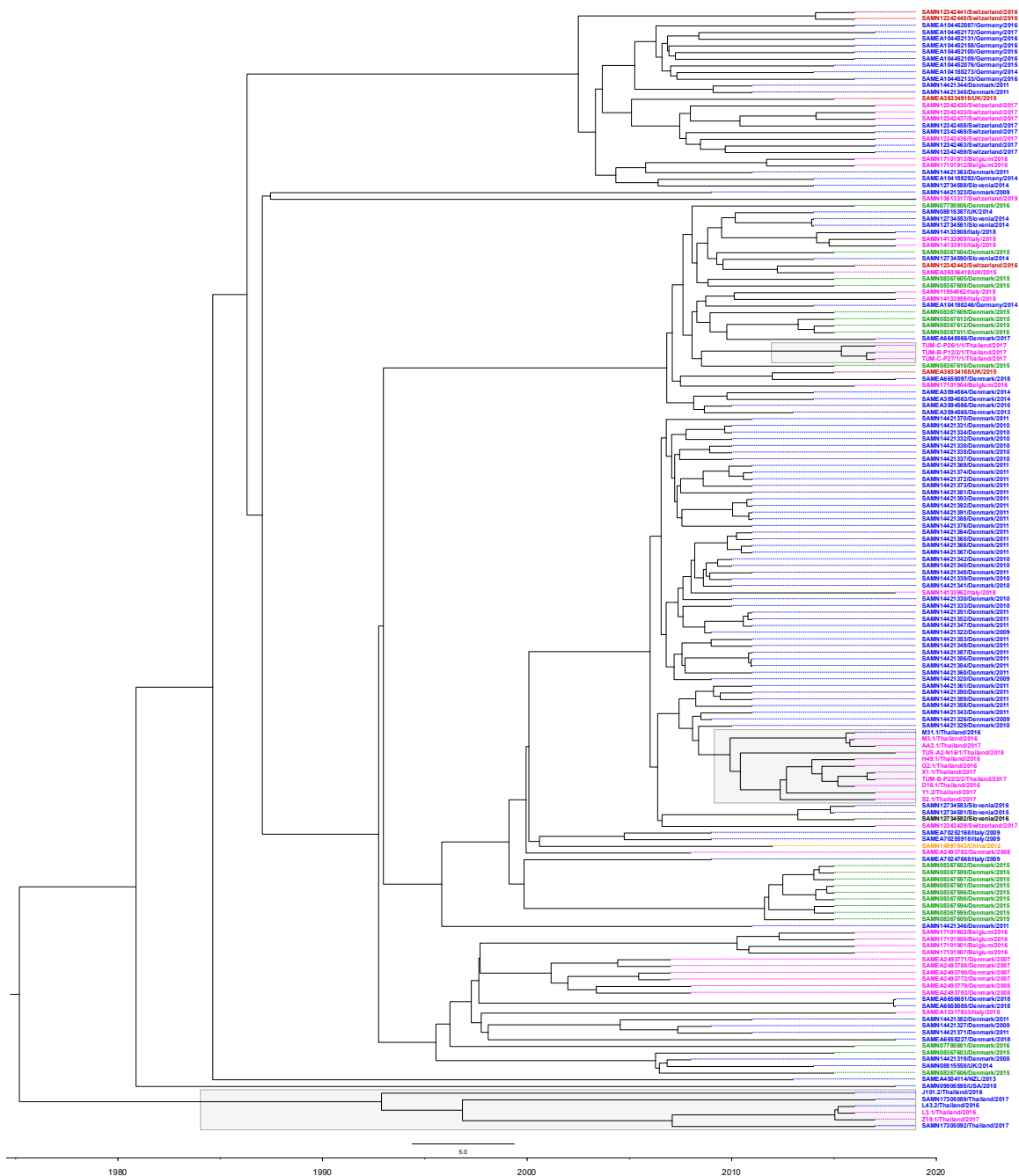


Figure 1. Bayesian phylogenetic tree of the subset of 168 *Staphylococcus aureus* CC398 isolates, generated using the BEAST package. The 20 *Staphylococcus aureus* CC398 isolates from Thailand are positioned in three different positions, highlighted in pale grey. Divergence times are estimated with the 95% highest posterior density (HPD) intervals. Isolates are labeled with their strain name, country, and year of collection. Labels are color-coded based on 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.