ASEAN
Journal of Scientific and Technological Reports
Online ISSN:2773-8752

Research article

Prevalence, Antimicrobial Resistance, and Genetic Relationship of Methicillin-Resistant *Staphylococcus aureus* from Meats, Hat-Yai, Thailand

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Citation:
Sukhumungoon, P.; Nuwilai, L.; Boontaworn, M; Rattanachuay, P. Prevalence, antimicrobial resistance, and genetic relationship of methicillin-resistant *Staphylococcus aureus* from neats, Hat-Yai, Thailand . *ASEAN J. Sci. Tech. Report.* **2025**, *28*(3), e256114. https://doi.org/10.55164/ajstr.v28i3.256114.

Article history:
Received: September 30, 2024
Revised: March 7, 2025
Accepted: March 26, 2025
Available online: April 26, 2025

Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a dangerous pathogen that can cause serious illnesses, leading to morbidity and mortality worldwide. Food items, especially meats, can contaminate it. In this study, a total of 100 meat samples were investigated for MRSA. The results demonstrated the MRSA prevalence in meats as 2.84% (4/141 isolates). Three MRSA strains were obtained from pork, while 1 from beef (p-value < 0.05). It was discovered that all 4 MRSA showed the same virulence gene profiles. The α-hemolysis and catalase production were positive for all 4 MRSA strains. Antimicrobial susceptibility assay showed that all but one MRSA strains were resistant to streptomycin, chloramphenicol, and tetracycline. MRSA strain LN-4 from beef was resistant to an extra antimicrobial agent, clindamycin. MRSA typing using BOX-PCR discovered that 3 MRSA strains (LN-1 to LN-3) from pork and 1 (LN-4) from beef were identical in DNA fingerprint, suggesting the close genetic relationship among strains from 2 different meat samples. This study demonstrates the presence of MRSA from meats in the south of Thailand. Even though they exist in low amounts, their virulence traits are thought to be harmful and cause diseases. Thus, MRSA contamination in meats should be immediately paid attention to for public health.

Keywords: *Staphylococcus aureus*; *mecA*; MRSA; meat

1. Introduction

Nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) have emerged as important problems worldwide [1-3]. Methicillin resistance is mediated through the *mec* operon, a part of the Staphylococcal cassette chromosome *mec* (*SCCmec*) [4]. MRSA carries *mecA* encoding penicillin-binding protein 2a (PBP2a), responsible for low binding affinity to β-lactam antibiotics [5]. This β-lactam antibiotic-resistant capability reinforces *S. aureus* to be more virulent, thereby exacerbating the severity of the disease and prolonging the therapeutic period [6]. The severity of the disease could be worsened in elderly and immunocompromised patients [7]. In addition to its role in nosocomial infection, MRSA is also associated with intestinal tract infections by producing staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST) [7]. There was a report of an acute community-acquired

gastroenteritis outbreak involving infection of staphylococcal enterotoxin C-producing MRSA and the consumption of coleslaw from a delicatessen [8]. Thus, foods are thought to be the important vehicle of MRSA. Meat is one of the important vehicles transporting several ferocious bacteria, including MRSA [9-11], and those MRSA strains carry numerous virulence genes and are resistant to antimicrobial agents. Therefore, surveillance of MRSA in food sources, especially meats, remains indispensable to prevent large morbidities and mortalities.

Thus, this study aims to report the prevalence, virulence profiles, antimicrobial susceptibility profiles, and genetic relationship of MRSA from meats marketed in southern Thailand. This information should be of importance to the public health of Thailand.

2. Materials and Methods

2.1 Sample collection

100 meat samples (40 beef, 30 pork, and 30 chicken) were collected from open markets throughout Hat-Yai City, Songkhla province, southern Thailand, from November 2022 to January 2023. Samples were processed within 2 hours, as previously described by Bunnoeng et al. [10]. In short, a 10 g of meat was mixed using a CIR-400 stomacher (Seward, West Sussex, UK) with 90 ml of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD, USA) for 1 minute. The liquid portion was separated and incubated at 37°C for 6 hours. One hundred µl aliquot of 10^{-3} dilution of the culture broth was spread on Mannitol Salt agar (MSA) (Becton Dickinson) and incubated at 37°C for 18 hours. Five to 10 typical colonies per sample were randomly selected for further molecular analyses.

2.2 MRSA identification by detecting *mecA* and *nuc*

Genomic DNA (gDNA) of the bacterial isolates was prepared as described by Pannuch et al. [12]. In brief, a single colony was cultured in 3 ml of TSB for 3 hours at 37°C with 150 rpm agitation. One ml aliquot of culture solution was boiled for 10 minutes, then placed on ice for 5 minutes, centrifuged at 11,000 *g* for 10 minutes, and the supernatant was diluted 10 folds with sterile deionized water. Amplification of *mecA* and *nuc* was performed as described by Bunnueang et al. [7] using specific primer pairs (Table 1). PCR was performed in a 25-µl reaction mixture comprising 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM each primer pair, 0.5 U Go*Taq* DNA polymerase (Promega, Madison, WI, USA), 1X Go*Taq* Flexi green buffer, and 2 µl of bacterial gDNA. Thermocycling was conducted in a T100TM Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: 95°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute (for both *mecA* and *nuc*), and 72°C for 1 minute, with a final step at 72°C for 5 minutes. Amplified products were analyzed using 1.0 % agarose gel electrophoresis, stained with ethidium bromide, and imaged using WSE-5200 Printpraph 2M gel imaging system (ATTO Corp., Tokyo, Japan). *S. aureus*, which carries both *mecA* and *S. aureus*-specific *nuc* genes, was classified as MRSA.

2.3 Virulence gene assay

Five virulence genes other than *mecA* (*coa*, *luk-PV*, *vWbp*, *spa*, and *sea*) were examined using uni-plex PCR as previously described [13]. The 25-µl reaction consisted of 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM each primer pair (listed in Table 1), 0.5 U Go*Taq* DNA polymerase (Promega), 1X Go*Taq* Flexi buffer, and 2 µl of gDNA solution. Thermocycling conditions were as follows: 95°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 50 °C for 1 minute (*coa*, *vWbp*, *sea*) or 55°C for 1 minute (*spa*), or 57°C for 1 minute (*luk-PV*), and 72°C for 1 minute (1.5 minutes for *spa*). The reaction was finalized at 72°C for 5 minutes. Amplicons were analyzed as described above.

2.4 Hemolysis and other virulent characteristics of MRSA

Hemolysis on blood agar and catalase production were also examined to seek their additional virulent characteristics. An overnight bacterial colony was spotted on blood agar for hemolysis assay and incubated at 37°C for 18 hours. Alpha (α), beta (β), or gamma (γ) hemolysis was observed macroscopically. Catalase production assay was examined using a 3% H_2O_2 solution. Additionally, a coagulase tube and slide test using rabbit serum were also employed to observe the coagulation by MRSA.

Table 1. Oligonucleotide primers used in the study

Target gene	Name	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
mecA	mecA1	GTAGAAATGACTGAACGTCCGATAA	310	[28]
	mecA2	CCAATTCCACATTGTTTCGGTCTAA		
luk-PV	luk-PV1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	[29]
	luk-PV2	GCATCAASTGTATTGGATAGCAAAAGC		
coa	COA1	CGAGACCAAGATTCAACAAG	730	[30]
	COA2	AAAGAAAACCACTCACATCAGT		
vWbp	vWbp-F	GCTGGATTAAATGGTGAAAGTCATG	320	[10]
	vWbp-R	GTTTATTAAAACGTTTTTGATGACC		
spa	SPA1	ATCTGGTGGCGTAACACCTG	1,500	[30]
	SPA2	CGCTGCACCTAACGCTAATG		
sea	SEA-F	GCAGGGAACAGCTTTAGGC	520	[31]
	SEA-R	GTTCTGTAGAAGTATGAAACACG		
nuc	nuc1	GTAGGTGGCAAGCGTTATCC	279	[32]
	nuc2	CGCACATCAGCGTCAG		
boxA	BOXA1R	CTACGGCAAGGCGACGCTGACG	Variable	[15]

2.5 Antimicrobial susceptibility assay

All MRSA strains were examined for their antimicrobial susceptibility using the disk diffusion method [14]. Ten common antimicrobial agents used in this assay were as follows: amikacin (30 μg), ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), erythromycin (30 μg), gentamicin (10 μg), kanamycin (30 μg), tetracycline (30 μg), and streptomycin (10 μg) (Oxoid Hampshire, UK). Vernier caliper measured the clear zone.

2.6 MRSA typing by BOX-PCR

BOX-PCR of MRSA strains was carried out as previously described by Versalovic et al. [15]. Shortly, a 25-μl reaction mixture comprising 0.2 μM BOXA1R primer (Table 1), 0.2 mM dNTPs, 1X Go*Taq* DNA polymerase buffer (Promega), 3.0 mM MgCl 2, 1.25 U Go*Taq* DNA polymerase (Promega) and 50 ng of DNA [prepared by a glass fiber matrix spin column method (Geneaid, Taipei, Taiwan)] was subjected to thermocycling as described above. Thermocycling condition was as follows: 95°C for 3 minutes; then 30 cycles of 94°C for 3 seconds, 92°C for 30 seconds, 50°C for 60 seconds, and 65°C for 8 minutes. Amplified products were separated using 1.5% agarose gel-electrophoresis at 100V for 1 hour and imaged as described above.

2.7 Statistical analysis

One-way ANOVA was employed to compare the prevalence of MRSA among meat types. Significance was set at p-value < 0.05.

3. Results and Discussion

3.1 Prevalence of MRSA from meats

In this study, we tried to isolate *S. aureus* as much as possible. A total of 141 typical colonies on MSA from 100 meat samples could be obtained. Among them, 4/141 isolates (2.84%) from 2 meat samples (prevalence of 2%) were identified as MRSA (Table 2). Three MRSA were obtained from pork while 1 from beef (p-value < 0.05). The absence of MRSA from chicken in this study was thought that bacterial contamination on meat did not reach the detectable limit. The quantity of bacterial contamination on meat samples varied from study to study. One investigation conducted in the same geographical area in 2013 revealed a high level of MRSA contamination in chicken at 57.14% with 40 MRSA strains isolated [10]. In addition, 37.1% of MRSA prevalence was found in frozen chicken meat in Bangladesh. On top of that, they all were multi-drug resistant strains [16].

The contamination of MRSA (strains LN-1 to LN-4) on meats in this study was found to be from the same open market. *S. aureus* contamination in raw meat and meat products is presumably the result of poor food hygienic handling or contamination directly from the animals themselves [17]. During slaughtering

processes, MRSA can pollute carcasses, leading to high contamination rates of MRSA on meats at the retail level [18]. Various rates of MRSA contamination in meats have been reported from numerous areas of the world. Many kinds of retail meat were investigated in the Netherlands [19]. They were found to carry MRSA as 10.6%, 10.7%, and 16% in beef, pork, and chicken, respectively [19]. In an additional report from Canada, Weese et al. [20] investigated MRSA in four provinces and found MRSA as 9.6%, 5.6%, and 1.2% in pork, beef, and chicken, respectively. In Thailand, it was thought that the prevalence of MRSA in meats depends, in part, on the time point of sampling and geographical location. This study revealed MRSA isolation rates in pork as 9.38% (3/32 samples), while the study in the same geographical area from Bunnoeng et al. [10] revealed 50% (8/16 samples). This suggests that there can be different MRSA contamination rates in different time intervals. Likewise, Tanomsridachchai et al. [21] investigated MRSA in pork samples in the central part of Thailand and found MRSA at a high level of 44.8% (52/116).

Table 2. Prevalence of methicillin-resistant *Staphylococcus aureus* from meats

Meat type	No. of positive sample / No. of sample (%)	No. of MRSA-positive isolates / Total isolates (%)
Chicken	0/30^{C} (0)	0/80 (0)
Pork	1/30^{B} (3.33)	3/32 (9.38)
Beef	1/40^{A} (2.5)	1/30 (3.33)
Total	2/100 (2)	4/141 (2.84)

Uppercase letters indicate significant differences among meat types (p-value < 0.05).

3.2 Virulence gene and other virulent traits

Virulence genes frequently found in *S. aureus* were also investigated in 4 MRSA strains (LN-1 to LN-4) in this study. It was discovered that all 4 MRSA showed the same virulence gene profiles (Table 3) and exhibited catalase production and α-hemolysis on blood agar. Both coagulase tube and slide tests were negative, corresponding to the lack of *coa* and vWbp genes. The absence of important virulence genes in MRSA in this study is unsurprising since a similar phenomenon can be found in our previous work in 2016 [10]. Of 185 MRSA isolates from meats, none exhibited the presence of *coa* (coding for coagulase responsible for plasma clotting), *luk-PV* (coding for Panton-valentine leukocidin, a pore-forming toxin responsible for necrotizing disease), and *sea* (coding for staphylococcal enterotoxin A responsible for food poisoning). However, a few strains revealed the presence of *vWbp* (coding for von Willebrand factor binding protein) and *spa* (coding for Staphylococcal protein A) [10]. It is inconclusively shown that MRSA strains LN-1 to LN-5 are naive since they may be equipped with many virulence factors to combat host defense systems. Catalase production in these MRSA is one of their virulence traits that should not be overlooked since it can protect them from H_2O_2-mediated leukocytic bactericidal mechanism, resulting in bacterial survival [22].

Table 3. Virulence characteristics of 4 MRSA strains isolated from meats

Sample number (meat type)	Strain designation	Virulence gene						Hemolysis	Catalase	Drug resistance
		mecA	*coa*	*luk-PV*	*spa*	*vWbp*	*sea*			
No. 40	LN-1	+	-	-	-	-	-	α	+	C, S, TE
(pork)	LN-2	+	-	-	-	-	-	α	+	C, S, TE
	LN-3	+	-	-	-	-	-	α	+	C, S, TE
No. 93	LN-4	+	-	-	-	-	-	α	+	C, S, TE,
(beef)										DA

*C, chloramphenical; DA, clindamycin; S, streptomycin; TE, tetracycline.

3.3 Antimicrobial susceptibility assay

Antimicrobial susceptibility assay revealed that all but one MRSA strains were resistant to streptomycin, chloramphenicol, and tetracycline. MRSA strain LN-4 from beef also showed extra resistance to clindamycin (Table 3). Various MRSA strains from clinical and raw meat samples around the world are documented to be resistant to many antimicrobial agents [23,18]. In the Western hemisphere, O'Brien et al.

[23] investigated MRSA from pork products at the retail level in Iowa, Minnesota, and New Jersey, USA. They discovered that MRSA isolates were resistant to clindamycin, erythromycin, and tetracycline at 34.6%, 38.5%, and 65.4%, respectively. Also, Jackson et al. [24] collected retail pork and beef products (100 each) from 14 retail food stores in Athens, Georgia, USA, in 2009 to search for MRSA. Twenty-five percent (1/4 strains) of MRSA from beef samples was shown to resist clindamycin and tetracycline, while 33.3% (1/3 strains) of MRSA from pork was resistant to clindamycin. In the Eastern hemisphere, the antimicrobial resistance rate was much higher. A nationwide investigation of MRSA from numerous retail food items was performed in China between 2011 and 2016 [25]. Among 108 MRSA strains obtained, 41/108 (38%), 86/108 (79.6%), and 71/108 (65.7%) MRSA strains were resistant to chloramphenicol, clindamycin, and tetracycline, respectively. Focusing on tetracycline, the study from China and our present study concord with the work from Pakistan that demonstrated 100% resistance [26]. High bacterial resistance to tetracycline is not surprising since this antimicrobial agent is inexpensive. Thus, it has been extensively used for prophylaxis and therapy of humans and animals. Moreover, it is used at sub-therapeutic levels as an animal growth promoter [27]. More importantly, tetracycline-resistant genes are effectively transferred among bacterial species. Therefore, the effectiveness of this antimicrobial agent in treating MRSA infections should be noted.

3.4 BOX-PCR profiling of MRSA

According to MRSA's genotype and phenotype similarities in this current study, DNA typing using BOX-PCR was performed to seek their genetic relationship. It was revealed that 3 MRSA strains (LN1 to LN-3) from pork and 1 (LN-4) from beef showed identical DNA fingerprints by BOX-PCR (Figure 1) even though MRSA strain LN-4 demonstrated an extra clindamycin resistance compared to the other three. This is probably explained by the dissociation between *boxA* and the clindamycin-resistant gene, resulting in the undisturbed DNA fingerprint. Interspersed repetitive-element PCR (rep-PCR) is broadly employed for bacterial typing since the repetitive elements dispersed throughout the bacterial genome are greatly conserved. BOX elements demonstrate a striking ability for DNA fingerprinting generation among repetitive elements. Among 3 BOX subunits, *boxA* (57 bp), *boxB* (43 bp), and *boxC* (50 bp), only *boxA* is highly conserved [15]. Therefore, *boxA* is used to generate reliable and reproducible DNA fingerprints. BOX-PCR is mainly inexpensive and easy to perform. It is thus an excellent approach for investigating bacterial genetic relationships.

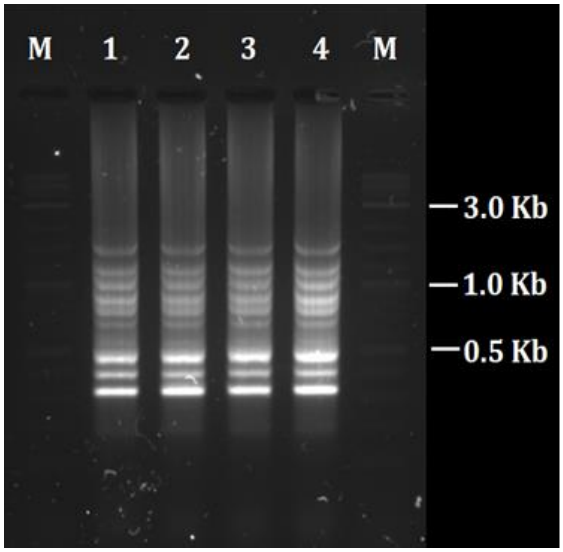

Figure 1. BOX-PCR of MRSA from meats. Lanes 1-4 are MRSA strain LN-1 to LN-4, respectively. Lane M is 2 2-log DNA ladder (New England Biolabs, USA).

4. Conclusions

Pathogenesis by *S. aureus* is a multifactorial process dependent on the presence and expression of several virulence factors. Moreover, some *S. aureus* strains show resistance to various antimicrobial agents. Thus, the contamination of MRSA in meats should be paid immediate attention. Surveillance of MRSA in

meats should be performed to gain data on prevalence and virulence, including the antimicrobial-resistant pattern, for the benefit of the population in Thailand.

5. Acknowledgements

We thank the Department of Microbiology, Division of Biological Science, Faculty of Science, Prince of Songkla University for providing the essential facilities.

Author Contributions: PS, conceptualization, original draft preparation, methodology, review, editing, validation, resource; LN, methodology, technical assistance; MB, technical assistance; PR, original draft preparation, methodology. All authors have read and agreed to the published version of the manuscript.

Funding: The study was partly funded by the Faculty of Science, Prince of Songkla University, fiscal year 2023.

Conflicts of Interest: The authors declare no conflict of interest.

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