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Incidence of Enterotoxin-Producing MRSA in Bovine Mastitis Cases, Bulk Milk Tanks and Processing Plants in Thailand

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Abstract: Methicillin Resistant *S. aureus* (MRSA) are virulent strains of *S. aureus* which have become resistant to most antibiotics. The emergence of MRSA is a serious public health concern worldwide. The present study sought to determine the distribution of enterotoxin-producing MRSA in Thailand using multiplex PCR. A total of 375 *S. aureus* isolates obtained from 598 mastitis cases, 376 bulk tank milk samples and 46 pasteurized milk samples were investigated for phenotypic methicillin resistance. Of these 375 isolates, 74 were found to be methicillin resistant. Variation in the SE encoding genes was detected. A total of 61 isolates harbored at least one classical *SE* gene, 30 isolates possessed only one type of enterotoxin gene and the remaining 31 were found to be positive for more than one toxin gene. The genes most frequently detected were seb and sed. Isolates obtained from mastitis cases had the highest incidence of enterotoxin genes followed by bulk milk isolates. On comparing the data relative to the different dairy locations, the isolates from Khon Kaen province harbored most detected enterotoxin genes. This was the only location where MRSA isolates from both mastitis milk and bulk milk were found harboring enterotoxin genes. Among the 5 *S. aureus* strains isolated from pasteurized milk only one isolate was MRSA. The strain which was isolated in Mahasarakham was positive for the *sed* gene. The current study has detected enterotoxigenic MRSA in mastitis milk, bulk milk and also pasteurized milk from Thailand. Further detailed analysis of functional genomics is now warranted to gain a better understanding of enterotoxin activity and virulence.

Key words: MRSA, enterotoxin, milk, isolates, harbored, virulence

INTRODUCTION

Staphylococcus aureus is one of the most pathogenic bacteria causing contagious bovine mastitis. Although, a variety of antibiotics can be used against this organism, *S. aureus* mastitis has been found to respond poorly to antibiotic treatment (Barkema *et al*., 2006). Methicillin Resistant *S. aureus* (MRSA) is one of a number of virulent *S. aureus* strains which have become resistant to most antibiotics (Van Loo *et al*., 2007). The spread of *S. aureus* especially, MRSA has been remarkable and its control has become a major challenge in Thailand.

Some strains of *S. aureus* can express a large number of virulence factors including Staphylococcal Enterotoxins (SEs). SEs are recognized as the main agents of Staphylococcal Food Poisoning (SFP) in humans (Dego *et al*., 2002; Peacock *et al*., 2002).

SEs are remarkably heat resistant and it is possible for them to retain biological activity after the thermal process of pasteurization (Rall *et al*., 2008; Normanno *et al*., 2007). The classical SEs have been characterized into five serological types (SEA-SEE) on the basis of their antigenicity (Bergdoll *et al*., 1974). These toxins are responsible for food poisoning outbreaks and are important in terms of food safety.

Many studies indicate that enterotoxin production can be observed in bovine milk and a great diversity of SE encoding genes have been found among *S. aureus* isolates (Akineden *et al*., 2001; Katsuda *et al*., 2005; Cremonesia *et al*., 2005; Moon *et al*., 2007; Jorgensen *et al*., 2005).

Little data is available on the epidemiology of MRSA strains from animal origin in Thailand. The aim of the present investigation was to study distribution of MRSA

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harboring the major *SE* genes using multiplex PCR. Such information can give an indication of the virulence of MRSA present in different areas on the basis of comparison between different sources of milk. To ensure safety of milk consumers, surveilance of the entire production chain for pathogens is required. Therefore, this survey compared different sources of milk including mastitis cases, bulk milk tanks and pasteurized milk-processing plants.

MATERIALS AND METHODS

Source of *S. aureus* isolates: The eight milk-producing provinces in Thailand, Khon Kaen, Mahasarakham, Udon Thani, Loei, Sakon Nakhon, Nakhon Ratchasima, Lop Buri and Sara Buri (Fig. 1) were surveyed to investigate the emergence of MRSA. For mastitis milk sample collection, the California Mastitis Test (CMT) was applied in 598 farms to diagnose bovine mastitis cows with somatic cell counts greater than 5×10^5 cells mL^{-1}. One sample from each farm was aseptically collected (~10 mL). Bulk tank milk samples from 376 bulk lots of milk were collected from 28 milk collection centers. For pasteurized milk samples, researchers collected 46 samples from 3 districts where pasteurized milk plants were located. An aliquot of each sample was simultaneously spread onto a plate containing Baird-Parker's medium (Oxoid Ltd., Thailand) and incubated under aerobic conditions at 37°C for 24 h. All grey-black shiny convex 1-1.5 mm diameter colonies obtained were presumed to be *S. aureus*. Identification of these presumptive *S. aureus* colonies was based on standard biological tests including gram staining, colony morphology, catalase test and coagulase test using human plasma. All *S. aureus* isolates were examined for methicillin resistance.

Detection of MRSA: MRSA isolates were detected by antibiotic disc diffusion using 1 μg oxacillin and 30 μg cefoxitin (Oxoid Ltd., Thailand). The usefulness of this method for accurately detecting MRSA has recently been described by many researchers (Anand *et al*., 2009; Zeeshan *et al*., 2007; Stepanovic *et al*., 2006; Velasco *et al*., 2005; Brown *et al*., 2005). Mueller-Hinton agar was used as recommended by the Clinical and Laboratory Standards Institute (CLSI). Bacterial suspensions equal to a 0.5 McFarland standard for 1×10^8 CFU mL^{-1} were prepared in 0.9% saline. In order to monitor the susceptibility test, methicillin sensitive *S. aureus* ATCC 25923 and methicillin resistant *S. aureus* DMST 20625 were used as negative and positive controls, respectively. Both reference strains were from the Department of Medical Sciences, Ministry of Public Health, Thailand. Zones of inhibition were measured after 24 h of incubation at 37°C and results were interpreted according to CLSI recommendations. Each isolate was tested in duplicated and mean zone of inhibition diameters were determined. Isolates with both oxacillin resistances (inhibition zone diameter≤10 mm) and cefoxitin resistances (inhibition zone diameter≤21 mm) were identified as MRSA and selected for detection of SE genes.

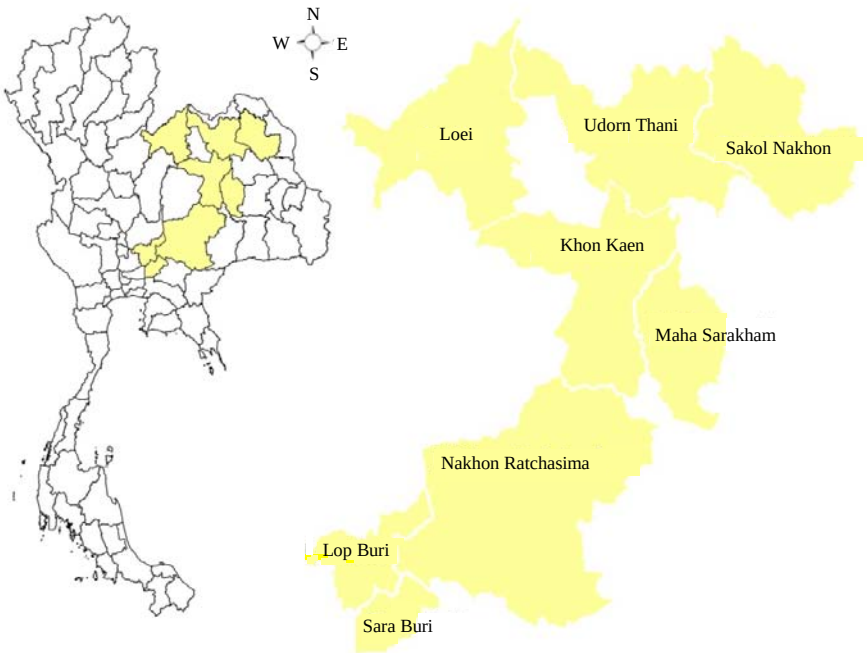

Fig. 1: Geographical location of study area

Table 1: Oligonucleotide primers for amplification of genes encoding staphylococcal enterotoxins

Genes	Sequence (5'-3')	Base pair	Annealing Temp (°C)	References
sea	ttggaaacggttaaaacgaa gaaccttcccatcaaaaaca	120	50	Johnson *et al.* (1991)
seb	tcgcatcaaactgacaaacg gcaggtactctataagtgcc	478	50	Johnson *et al.* (1991)
sec	gacataaaagctaggaattt aaatcggattaacattatcc	257	50	Johnson *et al.* (1991)
sed	ctagtttggtaatatctcct taatgctatatcttataggg	317	50	Johnson *et al.* (1991)

Genomic DNA extraction: MRSA isolates were grown overnight in Brain-Heart Infusion broth (BHI) (Oxoid Ltd., Thailand) at 37°C. DNA extraction was carried out using a Genomic DNA Extraction Kit (RBC bioscience, Taiwan). Purity and concentration of the DNA were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). About 100 ng of the genomic DNA was used for mPCR analysis.

mPCR testing for genes encoding staphylococcal enterotoxins: Detection of enterotoxin genes by multiplex PCR was performed. After DNA isolation, amplification of selected genes (enterotoxin genes *sea*, *seb*, *sec* and *sed*) was achieved using 4 primer sets in a 25 μL reaction mixture containing 1 μL DNA sample, 12.5 μL Bluemix DNA Polymerase Mastermix® (RBC bioscience, Taiwan) and molecular-grade water. All DNA primers (Table 1) were synthesized by Biodesign (Thailand). DNA amplification was performed using the following conditions: initial denaturation for 5 min at 94°C followed by 30 cycles of denaturation (94°C for 2 min), annealing and extension (72°C for 1 min). The oligonucleotide primers and annealing temperatures used were those recommended by Johnson and are shown in Table 1. A final extension step (72°C for 5 min) was performed after completion of the cycles. *S. aureus* strains harboring known enterotoxins were used as positive controls. These included *S. aureus* ATCC 13565 (sea), *S. aureus* ATCC 14458 (seb), *S. aureus* ATCC 19095 (sec) and *S. aureus* ATCC 23235 (sed).

After amplification, PCR products were analysed using 1.5% agarose gel with 0.125 mg L^{-1} ethidium bromide. A 100 bp ladder (Invitrogen) was run together with PCR products as a molecular weight marker. PCR banding sizes generated with each primer were measured by GelixOne G230 Software (Biostep, Denmark). Only clear, unambiguous and reproducible bands were recorded.

RESULTS AND DISCUSSION

Incidence of MRSA: Out of 1,020 milk samples, comprising milk from 598 mastitis cows, 376 bulk milk tanks and 46 samples of pasteurized milk, 375 (36.8%) were found to contain *S. aureus*. Mastitis milk showed the highest incidence of *S. aureus* contamination (38.3%, 229 of 598 samples) followed by bulk milk tanks (37.5%, 141 of 376 samples) and pasteurized milk (10.9%, 5 of 46 samples). All 375 *S. aureus* isolates were examined for methicillin resistance using 1 μg oxacillin and 30 μg cefoxitin discs. The zones of inhibition generated are shown in Table 2. Seventy four of the isolates (19.7% of 375 *S. aureus* isolates) were identified as MRSA using CLSI guidelines (Clinical and Laboratory Standard Institute, 2009). MRSA isolates exhibited inhibition zone diameters of 6.1±0.4 mm around oxacillin discs. In diffusion tests with 30 μg cefoxitin discs, MRSA isolates exhibited inhibition zone diameters of 11.9±4.9 mm. MRSA isolates from mastitis milk expressed resistance to cefoxitin with small zone size (11.0±5.1 mm) while the cefoxitin zone diameters were higher for 22 isolates from bulk tanks and one isolate from pasteurized milk (13.5±3.5 and 21.0±0.0 mm, respectively). According to Table 3, the incidence of MRSA positive isolates was high in mastitis milk (22.3%, 51 of 229 isolates). The proportion of *S. aureus* isolates obtained from bulk milk and pasteurized milk identified as MRSA was 15.6% (22 of 141 isolates) and 20.0% (1 of 5 isolates), respectively.

Table 2: Zones of inhibition (mm) produced by oxacillin and cefoxitin against MRSA isolates from different sources

Antibiotics	Mastitis cows (n = 51)	Bulk milk tanks (n = 22)	Pasteurized milk (n = 1)	Total (n = 74)
Oxacillin	6.1±0.4	6.0±0.0	6.0±0.0	6.1±0.4
Cefoxitin	11.0±5.1	13.5±3.5	21.0±0.0	11.9±4.9

The incidence of methicillin resistant isolates varied according to the dairy locations (Table 3). No MRSA isolates were present in Loei, Sakon Nakhon or Sara Buri. Interestingly, most of the MRSA (30 of 74 isolates) were found in mastitis milk from just 2 locations: 15 isolates obtained from the Phangthui district of Khon Kaen and 15 isolates obtained from the Srithaat district of Udon Thani. This represents a very high level of MRSA at both locations (42.9 and 41.7%, respectively) (Table 3).

Distribution of SE: According to the results of multiplex PCR analysis of all isolates (Table 3) a total of 61 isolates (82.4%) harbored at least one classical *SE* gene whereas 13 isolates (17.6%) were negative. Thirty isolates possessed only one type of enterotoxin gene and the remaining 31 were found to be positive for more than one toxin gene.

With regard to the 74 MRSA isolates examined, the genes most commonly detected were seb (12 isolates, 16.2%) and sed (12 isolates, 16.2%). The number of sea and sec positive isolates was 6.8 and 1.4%, respectively. The presence of enterotoxin genes was most commonly

Table 3: Incidence of MRSA isolates harboring classical SE encoding genes

Locations (Provinces and districts)		Total samples	*S. aureus* isolates (n)	MRSA (n)	SE positive (n)	*sea*	*seb*	*sec*	*sed*	*sea*, *seb*	*sea*, *sed*	*seb*, *sec*	seb, *sed*	*sec*, *sed*	*sea*, *sec*, *sed*	*sea*, *seb*, *sed*	*seb*, *sec*, *sed*
Mastitis cows (isolated from 598 milk samples)																	
Khon Kaen	Kra nuan	78	22	1	1	-	1	-	-	-	-	-	-	-	-	-	-
	Nam phong	71	35	4	4	-	-	1	3	-	-	-	-	-	-	-	-
	Phangthui	48	35	15	13	2	-	-	1	3	-	-	4	-	-	3	-
Lop Buri	Nong ri	36	7	3	3	-	1	-	-	-	1	-	-	-	1	-	-
	Suanmaduea	24	16	7	6	-	2	-	1	-	1	-	-	-	2	-	-
	Sapkradan	26	9	1	1	-	-	-	-	-	-	-	1	-	-	-	-
Udon Thani	Nongwuaso	21	10	5	5	-	3	-	-	-	-	1	1	-	-	-	-
	Srithaat	79	36	15	14	-	3	-	2	3	-	-	4	-	-	-	2
Other locations		215	59	0	-	-	-	-	-	-	-	-	-	-	-	-	-
Total		598	229	51	47	2	10	1	7	6	2	1	10	-	3	3	2
Bulk milk tanks (isolated from 376 milk samples)																	
Khon Kaen	Kra nuan	17	10	1	1	-	-	-	-	-	-	-	1	-	-	-	-
	Nam phong	15	12	3	3	-	-	-	2	1	-	-	-	-	-	-	-
	Phangthui	22	13	2	1	-	-	-		1	-	-	-	-	-	-	-
	Muang Khon Kaen	17	10	4	3	-	2	-	1	-	-	-	-	-	-	-	-
Maha Sarakham	Kantharawichai	48	16	2	1	1	-	-	-	-	-	-	-	-	-	-	-
	Khoh kho	45	10	4	2	2	-	-	-	-	-	-	-	-	-	-	-
Nakhon Ratchasima	Pak thong chai	4	3	1	1	-	-	-	1	-	-	-	-	-	-	-	-
	Pak chong	9	2	1	0	-	-	-	-	-	-	-	-	-	-	-	-
	Muak lek	14	6	2	0	-	-	-	-	-	-	-	-	-	-	-	-
	Soeng sang	6	1	1	1	-	-	-	-	-	-	-	-	-	-	-	1
Udon Thani Kutchap		9	5	1	0	-	-	-	-	-	-	-	-	-	-	-	-
Other locations		170	53	0		-	-	-	-	-	-	-	-	-	-	-	-
Total		376	141	22	13	3	2	0	4	2	-	-	1	-	-	-	1
Pasteurized milk (isolatedfrom 46 milk samples)																	
Maha Sarakham	Khoh kho	29	4	1	1	-	-	-	1	-	-	-	-	-	-	-	-
Other locations		17	1	0		-	-	-	-	-	-	-	-	-	-	-	-
Total		46	5	1	1	-	-	-	1	-	-	-	-	-	-	-	-
Total number of samples examined MRSA harboring enterotoxin (%)		1,020	375	74	61 (82.4%)	5 (6.8%)	12 (16.2%)	1 (1.4%)	12 (16.2%)	8 (10.8%)	2 (2.7%)	1 (1.4%)	10 (13.5%)	1 (1.4%)	3 (4.1%)	3 (4.1%)	3 (4.1%)

detected in MRSA isolates obtained from mastitis milk. Most mastitis isolates (92.2%, 47 of 51 isolates) were positive for the presence of enterotoxin genes as were bulk milk isolates (59.1%, 13 of 22 isolates). Remarkably, one of the isolates from pasteurized milk was also found to be both methicillin resistant and enterotoxin gene positive.

Among the 47 mastitis isolates investigated, 2 isolates were found to be positive for the presence of *sea* genes, 10 isolates contained the gene for *seb*, 1 isolate contained the gene encoding *sec*, 7 isolates contained the gene for *sed* and the remaining isolates harbored combinations of these. The detection of multiple types of toxin gene was more common among MRSA isolates from mastitis milk than those from bulk milk tanks or pasteurized milk. The results show that *seb* and *sed* are the predominant genes.

On comparing the data relative to the different dairy locations, the isolates taken from Khon Kaen harbored most detected enterotoxin genes. Other locations where enterotoxin genes were frequently detected were the provinces of Udon Thani and Lop Buri. Khon Kaen was the only location where MRSA isolates from both mastitis milk and bulk milk were found haboring enterotoxin genes. Among the 5 *S. aureus* strains isolated from pasteurized milk only one was methicillin resistant and enterotoxin positive. This strain was isolated in Mahasarakham and was positive for the *sed* gene.

Throughout the world, *S. aureus* is a frequent cause of bovine mastitis. According to the investigation, *S. aureus* infection or contamination was present in 38.3% of herds, 37.5% of bulk lots of milk and 10.9% of pasteurized milk samples. This serves as a warning that *S. aureus* is widespread in this region and may pose a health risk to consumers. Inadequate hygiene on farms and during milk transportation may have contributed to the spread of *S. aureus* in these areas. The study sought to assess the virulence of these isolates using tools for MRSA identification and enterotoxin gene detection. A total of 74 isolates were identified as methicillin resistant based on the size of zones of inhibition produced by oxacillin and cefoxitin. This represents the first report of MRSA strains being isolated from mastitis cases, bulk milk tanks and pasteurized milk in Thailand. Most MRSA

isoaltes originated from mastitis cows. This finding correlates well with other studies. For example, Lee (2003) reported that 9 of 12 MRSA isolates of milk origin were from cows with subclinical signs of matitis. Also, in a study by Moon *et al*. (2007) it was found that 2.8% of *S. aureus* from bovine mastitis cases were methicillin resistant. The occurrence and spread of MRSA in dairy farms is concerning because of possible transmission between cows and humans. According to the results of Lee (2003) and Juhasz-Kaszanyitsky *et al*. (2007), MRSA isolates of bovine and human origin are indistinguishable. Furthermore, MRSA of animal origin may contaminate foods and represent a source of MRSA infection or intoxication in humans. Therefore, the direction of genetic transfer warrants further investigation.

The high incidence of MRSA the study detected in specific locations is of interest. The two districts in question, Phangthui district of Khon Kaen and Srithaat district of Udon Thani are approximately 160 km apart and share a common route of milk transportation. The emergence of methicillin resistance in these regions may have been caused by excessive therapeutic use. *S. aureus* can adapt rapidly to the selective pressure of antibiotics and becomes methicillin resistant by the acquisition of the *mecA* gene. This gene encodes a Penicillin Binding Protein (PBP2a) with a low affinity for β-lactams (Ortega *et al*., 2010). The genetic relevance of these isolates should therefore be studied.

The incidence of MRSA isolates encoding classical *SE* genes was high (82.4%). The percentage of isolates that harbored enterotoxin genes increased (92.2%) when only mastitis milk isolates were considered. In a previous study, Omoe *et al*. (2002) found that 15 *S. aureus* isolates (71.4%) originating from cows with mastitis were positive for one or more enterotoxin genes. Sila *et al*. (2009) has indicated that the classical genes, *sea*, *seb* and *sed* are more frequently detected in MRSA than MSSA. The *sec* gene for enterotoxin C was also more frequent in MSSA. Absence of the *see* gene has been noted in several previous studies (Peacock *et al*., 2002; Becker *et al*., 2003; Boerema *et al*., 2006; Sila *et al*., 2009). Previous data indicate that the *sea* gene is most prevalent with SEA being involved in outbreaks of staphylococcal food poisoning worldwide (Gencay *et al*., 2010; Wang *et al*., 2009; Chiang *et al*., 2008).

The present study revealed that some isolates harbored the *sea* gene but that this was not predominant. In the study, *seb* and *sed* were the predominant enterotoxin genes in mastitis and bulk milk isolates. Boerema *et al*. (2006) and Peles *et al*. (2007) also reported that seb was the most commonly detected gene in strains isolated from bulk tank milk. Interestingly, the study also detected an MRSA isolate in pasteurized milk that harbored the *sed* gene. This discovery highlights a potential risk for consumers.

CONCLUSION

Results from this study indicate that MRSA has spread between different milk-producing locations in Thailand. This is of concern because it reduces the therapeutic options for mastitis treatment. Most of the isolates contaminating mastitis milk, bulk milk and pasteurized milk were also shown to be enterotoxigenic.

These findings suggest that further detailed analysis using functional genomics are warranted to gain a better understanding of enterotoxin activity and epidemiology.

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