The occurrence of MRSA, MSSA and antibiotic resistance, related factors in area of dairy farming of Mahasarakham province, Thailand

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Abstract The occurrence of *Staphylococcus aureus* isolates, especially MRSA and MSSA isolates contaminated in raw milk, can cause endemic chronic bovine mastitis in the area of dairy farming, Maha Sarakham province of Thailand. A total of 165 milk samples were collected and *S. aureus* isolation was conducted by using a conventional method and PCR reaction of 994 bp of coagulase gene fragment. The results revealed 25 isolates of *S. aureus* that showed a positive result of coagulase gene. All 25 isolates were tested for MRSA and MSSA identification by *mec*A gene that demonstrated 2 *mec*A-positive MRSA isolates (n=2) and 23 *mec*A-negative MSSA isolates (n=23). Two isolates of MRSA indicated that the antibiotic resistance was a continuing problem in this area. All isolates have also been found to be isolates having enterotoxin producing genes including *sea*, *seb*, *sec*, and *sed* but not found any enterotoxin genes in MRSA and MSSA. To investigate factors related to MRSA, a survey research was conducted and showed that all farms used both of injecting drug and intramammary infusion antibiotics for their bovine mastitis treatment. The case-control study was divided into farms using oxytetracycline and penicillin as the first choice. The study found that oxytetracycline has been used routinely to treat mastitis in 2 and 18 farms having MRSA and MSSA, respectively. The odds of MRSA among farms using oxytetracycline was more than the odds of MSSA among farms using oxytetracycline. This study found that the often use of oxytetracycline in this area might create new properties of bacteria to become resistant to antibiotics. However, it may have other factors affecting bacterial resistances that need to study in further research to more understanding and to introduce the result of risk factors investigation to farmers.

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Introduction

Staphylococcus aureus (*S. aureus*) is a major microorganism pathogen in humans and an animal can produce a wide variety of diseases. It is present the most frequent cause on the skin and soft tissue infection and it is also an opportunistic pathogen that can cause many types of infectious and syndromes of diverse severity (Lozano *et al*., 2016). *S. aureus* can frequently be isolated from milk from mastitis cows and can produce many important virulence factors such as staphylococcal enterotoxins (SE). The staphylococcal enterotoxins are the cause of gastroenteritis resulting from the consumption of contaminated food. Milk from unhealthy cows may contain sufficient amounts of preformed enterotoxin (Argudń *et al*., 2010; Le Loir *et al*., 2003). The severity of the illness depends on the amount of enterotoxin in contaminated food (Smyth *et al*., 2004) It causes clinical sign include nausea, vomiting, abdominal cramping, with or without diarrhea (Balaban and Rasooly, 2000; Pinchuck *et al*., 2010). Staphylococcal enterotoxins are classified by the different serologically distinct extracellular proteins with specific immunological activity. Worldwide recognized five classic enterotoxins are SEA, SEB, SEC, SED, and SEE as the most frequently implicated in food contamination and disease outbreaks (Hunt *et al*., 2014) and known to be responsible for 95% of staphylococcal food poisoning cases. SEA and SED are most common enterotoxins recovered from food poisoning out-breaks (De buyser *et al*., 2001).

Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-susceptible *Staphylococcus aureus* (MSSA) are a strain of *S. aureus*. Some epidemiologic studies and a meta-analysis from nosocomial MRSA strain found MRSA is more virulent than MSSA because increased morbidity and/or mortality, when compared with those from MSSA (Gordon and Lowy, 2008) MRSA is remaining a major concern public health problem it challenging to treat. Methicillin was developed which stemmed from penicillin. A methicillin-resistant strain was detected in the UK after one year later methicillin was used in the clinic in 1959 (Jevons *et al.*, 1963; Xia *et al*., 2013). By practice towards antibiotic use, MRSA is resistant to a large group of antibiotics called β-lactams through a production of beta-lactamases*,* including oxacillin, nafcillin, dicloxacillin, and cefazolin penicillins and cephalosporins (Chang *et al*., 2015). Resistance is a great concern in medical treatment makes because it challenging to treat. In addition, it has acquired resistance to multiple antibiotics, such as ciprofloxacin, clindamycin, tetracycline, erythromycin in many MRSA strains. Mechanisms of MRSA is have developed resistance to virtually all non-experimental antibiotics. They are intrinsically resistant to β-

lactams by mutation of the normal penicillin-binding protein and change to newly acquired low-affinity penicillin-binding protein 2A (PBP2A) by encoded of *mec*A gene (Pinho *et al*., 2001). *mec*A gene encodes an additional 78 kDa present in a chromosomal mobile genetic element called Staphylococcal cassette chromosome mec (SCCmec) (Adhikari *et al*., 2017). Resistance mechanisms are PBP2A can build the wall when other PBPs are blocked by β-lactams which has a low affinity for β-lactam antibiotics, designing β-lactams capable of blocking this additional target should help solve the issue. Older molecules including penicillin G, amoxicillin and ampicillin (Guignard *et al*., 2005; Peacock and Paterson, 2015) It can be used *mec*A gene as a biomarker gene for detection of resistant with methicillin. Several methods are available for detection of *S*. aureus. The phenotype identification of the *S*. aureus isolates was performed by mannitol salt aga (MSA) is used as a selective and differential medium for the isolation and identification of pathogenic staphylococci. In the presence of high salt concentration it can grow on mannitol salt agar, *S*. *aureus* fermented mannitol producing acid which changes the pH turning phenol red to yellow (Jasuja *et al*., 2013). The confirmed genotype identification using PCR. coagulase gene *(*coa*)* was conducted to discriminate *S*. *aureus*. *mec*A gene for the detection and differentiation of MSSA and MRSA, *mec*A gene is a positive result of MRSA and negative result of MSSA. This study also aims to reveal isolates that contain enterotoxin producing genes by *sea*, *seb*, *sec*, and *sed* gene. This is very important to do research with MRSA, MRSA is becoming a major veterinary and public health problem. Evidently, *mec*A can be transmitted only once by the weakest strain of Methicillin (MSSA) or the strain of *S*. aureus and able to spread to the environment and to survive in a virulent state (Cimolai, 2008; Berglund and S öderquist, 2008). This study conducted the case-control study to find factors of antibiotics used that related to the occurrence of methicillin-resistant *S*. *aureus* (MRSA). It may have other factors affecting bacterial resistances that need to study in further research to more understanding and to introduce the result of risk factors investigation to farmers.

Materials and methods

Sample collection and identification

A total of 165 specimens were collected from raw milk in dairy farms at Mahasarakham, Thailand. The phenotype identification of the *S*. *aureus* isolates was performed by brain heart infusion broth (BHI), mannitol salt agar (MSA) and catalase test. The confirmed genotype identification using PCR. Isolates

of *S. aureus* were confirmed by *coagulase* gene. MRSA and MSSA identification by *mec*A gene. enterotoxin producing genes by *sea*, *seb*, *sec*, and *sed* gene.

DNA isolation and DNA amplification by PCR

The DNA of isolates was prepared by the GF-1 bacterial DNA extraction kit (vivantis, Germany) as described by the manufacturer. The sequences of the primers, PCR product sizes, and the references are summarized (see Table 1). For PCR amplification, the reaction mixture (25 µl) contained 2 µl DNA template, 1 µl of primer F (10nM/µl), 1 µl of primer R (10 nM/µl), 2.5 µl of PCR buffer (vivantis), 0.5 µl dNTP (0.2 mM, vivantis), 0.75 µl Mgcl$_2$ (1.5 mM, vivantis), 0.2 µl Taq DNA polymerase (5u/µl, vivantis) and distilled water to the final volume of 25 µl.

DNA amplification of *S. aureus* was performed in a thermal cycler (Biometra, Germany) with initial denaturation at 94 ℃ for 5 min annealing 58 ℃ for 45 s and extension at 72 ℃ for 2 min.

The amplified PCR products were electrophoresed in a 1% agarose gel (Fermentas, Lithuania) containing Nucleic Acid Gel Stain (GelStar™) and visualized by transillumination under UV. Molecular size marker (vivantis, Malaysia) was included in each agarose gel. The *S. aureus* reference strains ATCC were used as positive controls. The reference strain *Staphylococcus epidermidis* were used as negative control.

Table 1. Primers for the detection of *S. aureus*, MRSA and enterotoxin A-D by PCR method

Gene	Primers	Sequence (5ʹ- 3ʹ)	Base pairs	Annealing temp. (℃)	References
coa	Coa-1	gcatcgaacaatcggaagc	994	58	-
	Coa-2	acgatggaggcattagcca			
mecA	mecA	gtagaaatgactgaacgtccgataa	341	59	Sanjiv *et al*.
		ctttggaacgatgcctaatctcat			(2008)
sea	SEA-1	ttggaaacggttaaaacgaa	120	50	Johnson *et*
	SEA-2	gaaccttcccatcaaaaaca			*al*. (1991)
seb	SEB-1	tcgcatcaaactgacaaacg	478	50	Johnson *et*
	SEB-2	gcaggtactctataagtgcc			*al*. (1991)
sec	SEC-1	gacataaaagctaggaattt	257	50	Johnson *et*
	SEC-2	aaatcggattaacattatcc			*al*. (1991)
sed	SED-1	ctagtttggtaatatctcct	317	50	Johnson *et*
	SED-2	taatgctatatcttataggg			*al*. (1991)

Results

Phenotype identification including growth on mannitol salt agar and catalase were done. The positive result was observed in 75 samples (45.45%) of 165 raw milk samples. According to the results of PCR assay by amplification of the coagulase gene specific to *S. aureus*, all 25 isolates contained 994 bp DNA fragments bands and showed positive PCR assay. Figure 1 showed the results of molecular tests for the detection of coagulase gene. Of the 25 isolates of *S. aureus* tested, 2 (8%) were positive for *mec*A gene (MRSA), 23 (9%) were negative for mecA gene (MSSA), and not found enterotoxin producing genes including *sea*, *seb*, *sec*, and *sed* in MRSA and MSSA. The case-control study was conducted by the study of farms using oxytetracycline and penicillin as the first choice. The result found that oxytetracycline has been used routinely to treat mastitis in 2 and 18 farms having MRSA and MSSA, respectively. The result of odds ratio (OR) 0.025 of MRSA among farms using oxytetracycline.
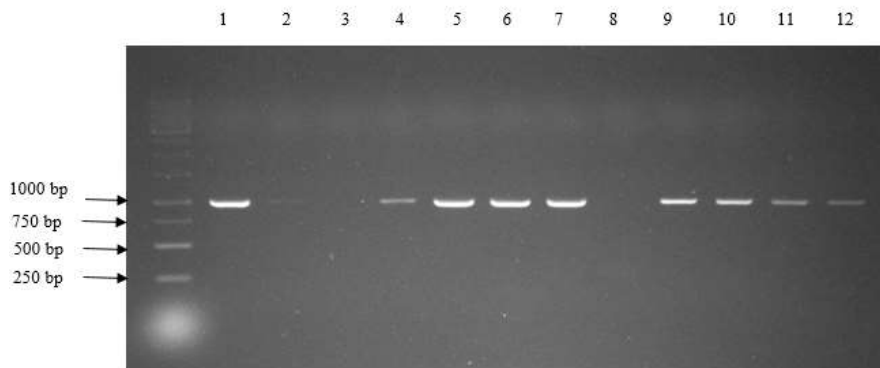

Figure 1. PCR amplification for the detection of *staphylococcus aureus* coagulase genes. 1000 bp ladder; Lane 8, *S. epidermidis* negative control; Lane 9, *S. aureus* positive control; Lanes 1–7, 10-12, PCR products

Discussion

The positive test of isolates that growth on mannitol salt agar and catalase test has shown the phenotype characteristics. The positive results were observed in 75 samples (45.45%) of 165 raw milk samples. According to the results of PCR assay by amplification of the coagulase gene specific to *S. aureus*, all 25 isolates contained 994 bp DNA fragment bands and showed positive PCR assay. The results were accuracy phenotype identification of 33.33% genotype detection. Many laboratories screened for

presumptive *S. aureus* based on growth on mannitol salt agar (Kateete *et al*., 2010). This result showed that low accuracy phenotype identification was the result of the growth of other bacteria (non-*S. aureus*) in the mannitol salt agar.

By using the PCR technique, the results showed that all of 25 samples were not found any enterotoxin genes: *sea*, *seb*, *sec*, and *sed* in MRSA and MSSA. This showed the absence of any classical enterotoxin genes in these sample groups. Classical enterotoxin genes detection was a preliminary test before further other molecular biological techniques such as a PCR, RFLP, AFLP, and PFGE (Lee *et al*., 2007) and gene expressions analysis by a reverse transcription real-time PCR (Berdal *et al*., 1981).

This survey research using the case-control study for investigating factors related to MRSA *mec*A gene has been conducted in all sources of the MRSA isolates. Surveillance results showed that antibiotics administration of all farms was interesting. Both antibiotic injection and intramammary infusion were used for their bovine mastitis treatment. The case-control study was demonstrated that oxytetracycline has been used routinely to treat mastitis in 2 and 18 farms having MRSA and MSSA, respectively. The odds of MRSA among farms using oxytetracycline was more than the odds of MSSA among farms using oxytetracycline. This often used oxytetracycline in this area might create new properties of bacteria to become resistant to antibiotics. Detection of MRSA in dairy farms may also be helpful in explaining the severity of the bacterial infection through early diagnosis. The appropriate mastitis treatment can significantly limit the duration and outcome of infection. This study revealed investigated factors related to MRSA use for appropriate antimicrobial therapy and the reduce spread of MRSA strains. However, it may have other factors affecting bacterial resistances that need to study in further research to more understanding and to introduce the result of risk factors investigation to farmers.

Acknowledgment

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