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*Original Article*

# Existing drug resistance among *Staphylococcus* spp. from raw milk samples in Khon Kaen province, Northeastern Thailand by direct quadriplex PCR

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**Abstract**

To describe the proportion of methicillin resistant *Staphylococcus aureus*, and mupirocin resistant among the isolates from milk, three hundred and eighty-one samples were collected in Khon Kaen province, Thailand, during January to March, 2014. Quadriplex PCR was a method of choice. The occurrence of *S. aureus* and other *Staphylococcus* spp. were 21.26 and 34.12%, respectively. Among the 81 *S. aureus* isolates, 82.72 (67/81), 11.11 (9/81), and 6.17% (5/81) were *S. aureus*, *S. aureus* carrying *mec*A, and *S. aureus* harboring *mup*A genes, respectively. These two mutant genes may possibly be transferred to other bacteria in milk. Therefore, good hygienic practices and strict control may limit the spread.

**Keywords:** drug resistance, fresh raw milk, methicillin, mupirocin, *Staphylococcus aureus*

## 1. Introduction

Conventional dairy farming was first introduced in 1962 to Thailand by the assistance from the Danish government. From the promotion of dairy farming, the number of cows and milk production output were gradually increasing. It was noted that during 1999-2000, the yearly average increase of the number of cows and milk production were 12.75 and 14.78% (Office of Agricultural Economic). The Ministry of Public Health (MOPH) encouraged people to consume more milk on the occasion of the World Milk Day (June 1, 2015). Current data showed that Thai youths consumed 4-7 times less milk than the global average i.e. only 14 liters of milk/person/year (Thai Visa News, 2015). Global milk consumptions varied from 57 to 330 liters/person/year. The country that produced largest amount of milk is India, followed by USA, Pakistan, China, and Germany. India and Pakistan produced milk only enough for their populations. In addition, New Zealand, Germany, The Netherlands, France, and Belgium were sufficient for their populations, as well as export to other countries. In Thailand, people consumed 13.02 liters/person/year or 1 glass of milk per week. The recommendation for milk consumption in Thailand was 2-3 glasses per day for children, and 1-2 glasses per day for adult. The Food and Agriculture Organization (FAO) assigned June 1, every year as the World Milk Day (Thai Dairy Fact Book, 2009).

In recent years, milk consumption has been increasing in Thailand. Farmers who raised their dairy cow experienced mastitis from *S. aureus* since they are frequently reported in dairy cows in northeastern areas of Thailand, of which is also a suitable area for milk industries. Globally, member of

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genus *Staphylococcus* spp. in particular *S. aureus* is a major human pathogen of public health concerns (Diekema *et al.*, 2001). Milk-borne staphylococcosis is one of the important zoonotic diseases encountered in human from consumption of fresh raw or under-cooked milk (Dhanashekar *et al.*, 2012). In Thailand, the normal practice was that fresh raw milk was boiled, and used in coffee and other hot and cold drinks by majorities of food venders.

This study was to assess the microbiological quality of fresh raw milk collected from individual tank at milk collecting center, and to look into drug resistant genes among *S. aureus* isolates using specific quadriplex PCR primers to detect the resistant genes followed that of Zhang *et al.* (2004) with minor modifications.

## 2. Materials and Methods

Sample collections: Fresh raw milk samples were taken from 381 individual milk tanks in milk collecting center during January to March, 2014 at Nampong and Ubonratana districts, Khon Kaen Province, northeastern region of Thailand.

Identification of the resistant organism: Conventional plating procedure was performed on Blood Agar plates (Columbia Blood Agar, Oxoid, Hamsphere, United Kingdom). Typical colony characteristics for *Staphylococcus aureus* (SA) were picked up, and streaked for single colony on Nutrient Agar (Oxoid) plates. After that, direct quadriplex PCR procedure for *Staphylococcus* spp. was performed (Zhang *et al.*, 2004). The oligonucleotide primers employed in this study were synthesized, and purchased from Thermo Scientific (Thermoscience, Singapore). The primers included the designed primer Staph 756F (5'-AAC TCT GTT ATT AGG GAA GAA CA-3'), and the previously published 3' primer Staph750R (5'-CCA CCT TCC TCC GGT TTG TCA CC-3') (Jaffe, 2000) for *Staphylococcus* genus-specific 16S rRNA, Nuc1 (5'-GCG ATT GAT GGT GAT ACG GTT-3'), and Nuc2 (5'-AGC CAA GCC TTG ACG AAC TAA AGC-3') for *nuc* (Shortle, 1983), MupA (5'-TAT ATT ATG CGA TGG AAG GTT GG-3') and MupB (5'-AAT AAA ATC AGC TGG AAA GTG TTG-3') for *mup*A (Anthony, 1999), and MecA1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3'), and MecA2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3') for *mec*A (Ryffel, 1990). PCR amplification, a quadriplex PCR assay targeting 16S rRNA (*Staphylococcus* genus specific), *nuc* (*S. aureus* species specific), *mup*A (a determinant of MUP resistance), and *mecA* (a determinant of MET resistance) were used followed the instruction of Zhang *et al.* (2004) with minor modifications.

Direct hot start multiplex PCR (M-PCR) assays were performed using direct colony of bacteria from the agar plate. Colony was added to PCR master mix for hot start PCR protocol according to the manufacturer instruction (Thermo Science, Singapore). The primer pairs for each specific region were 0.12 M each 16S rRNA, and *mec*A primers, 0.05 M each *mup*A primer, 0.04 M each *nuc* primer, and 1.0 U of Taq DNA polymerase (Thermo Science) were added into the reaction mixture. Amplification was performed using an EsCo Swift MiniPro thermal cycler (Esco Micro Pte Ltd, SWT-MIP-0.2-2, Singapore). Amplification was carried out as follows: an initial denaturation step at 94°C for 5 min; 10 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 1 min; 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. For increased specificity, strict PCR conditions were used as follows: an initial denaturation step at 94°C for 5 min; 10 cycles of 94°C for 40 s, 68°C for 40 s, and 72°C for 1 min; 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. For single-target amplification, PCR was carried out with a 20 µl PCR mixture containing 0.2 M each primer and with the following cycling parameters: an initial denaturation step at 94°C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. All quadriplex PCR procedures were conducted using both negative control (without DNA template), and *S. aureus* positive control (*S. aureus* ATCC 29213). The PCR amplicons were visualized using a gel documentation system (Syngene®, Cambridge, UK) after gel electrophoresis on a 1.50% (w/v) agarose gel containing 0.50 g of ethidium bromide/ml. In addition, *S. aureus* MECA, and *S. aureus* MUPA were designated as *S. aureus* with resistant gene to methicillin (MET), and *S. aureus* with resistant gene to mupirocin (MUP), respectively.

Statistical analysis: Descriptive statistic such as mean, standard deviation of mean, was used for describing the isolated strains.

## 3. Results and Discussion

Fresh raw milk in Khon Kaen province contains important foodborne pathogen in this study. It was evident from quadriplex PCR test that the occurrence of SA was 21.26% (81/381) (Table 1). However, majority of the isolates from fresh raw milk were identified as *S. aureus* (67/81, 82.72%). Among these *S. aureus* isolates, the prevalence of *S. aureus* MECA was 11.11% (9 out of 81), and *S. aureus* MUPA was 6.17% (5 out of 81) as shown in Table 2. Resistant determinant may transfer to humans who consumed under-processed milk. Strict control of heat treatment may decrease the carry-over of the resistant bacteria in milk to consumers. Nevertheless, raw milk consumption was not practiced among Thai folks. In fact, undercooked or improper boiled milk may pose some illnesses among Thai people.

Table 1. Direct quadriplex PCR for specific species identification of isolates from raw fresh milk samples.

| Organisms | No. of isolates | Occurrence % |
|---|---|---|
| *S. aureus* | 81 | 21.26 |
| *Other Staphylococcus* spp. | 130 | 34.12 |
| Non-*Staphylococcus* spp. | 170 | 44.62 |
| Total | 381 | 100.00 |

Table 2. Proportion of *Staphylcoccus aureus*, *S. aureus* MECA, and *S. aureus* MUPA strains identified by direct quadriplex PCR.

| | Total no. (N = 81) | Prevalence % |
|---|---|---|
| *S. aureus* | 67 | 82.72 |
| *S. aureus* MECA | 9 | 11.11 |
| *S. aureus* MUPA | 5 | 6.17 |
| Total | 81 | 100.00 |

Note: *S. aureus* MECA stands for *S. aureus* with resistant gene to methicillin (MET), and *S. aureus* MUPA means *S. aureus* with resistant gene to mupirocin (MUP).

In terms of the method of choice, quadriplex PCR had been validated, and demonstrated the 100.00% sensitivity, specificity, and accuracy for detecting the methicillin- and mupirocin resistant *S. aureus* as well as other *Staphylococcus* species (Zhang *et al.*, 2004). It provided the high discriminatory power for *Staphylococcus* spp., particularly resistant determinant. In addition, antibiotic resistant were measured in just one PCR reaction. The present findings confirmed that isolates from raw milk carried *mec*A gene. Compared to Europe, *S. aureus* carrying *mec*C is a new MRSA variant *Staphylococcus* species that present in Europe, and it was first isolated from bulk milk, and humans (Garcia-Alvarez *et al.*, 2011; Gongora *et al.*, 2015). *Staphylococcus* species found in the aforementioned studies are the different variant *Staphylococcus* species compared to the present study where *mec*A was detected. *S. aureus* MECA has been identified in Thailand in particular areas. However, in northeastern Thailand like Khon Kaen province, limited study was conducted using direct colony hot start PCR to detect both *S. aureus* MECA, and *S. aureus* MUPA. This is the first report of *S. aureus* MECA, and *S. aureus* MUPA contamination in raw milk in northeastern Thailand using quadriplex PCR.

## 4. Conclusions

The occurrence of *S. aureus* was 21.26% (81/381). Approximately 1/10 (11.11%) of them were *S. aureus* MECA, and the prevalence of resistance genes for mupirocin (*S. aureus* MUP) were 6.17%. The resistance to methicillin and mupirocin antibiotics needs to be closely monitored in Thailand due to the fact that this resistance may be transferred to other bacterial strains in the near future.

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