Research Article

Prevalence and Genetic Profiles of *Staphylococcus aureus* Isolated from Milk in Mastitic Dairy Cows Based on Conventional and Multiplex PCR Assays in Khon Kaen Province, Thailand

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Abstract

The objective of this study was to investigate the prevalence and genetic profiles of *Staphylococcus aureus* isolated from mastitic milk in dairy cows by conventional methods and multiplex polymerase chain reaction (m-PCR). Four hundred and thirty-five quarter milk samples were recruited from 14 dairy herds in Khon Kaen Province, Thailand, and determined as subclinical cases by California Mastitis test (CMT). Seventy-Six *S. aureus* isolates were then bacteriologically identified according to culture and biochemical tests. The prevalence of *S. aureus*, coagulase negative staphylococci (CNS) and other bacteria were 17.5%, 32.4% and 12.4%, respectively. Genetic profiles of *S. aureus* were carried out by m-PCR using 3 specific primers targeting 16s rDNA gene (420 bp) for detection of the *Staphylococcus* spp., gene encoding coagulase (*coa*: 159, 240 bp) and factors essential for methicillin-resistance (*femA*: 344 bp) for detecting species level of *S. aureus*. Genetic profiles were assigned to 6 different patterns based on specific genes detection out of the 76 *S. aureus* isolates. All of the isolates yielded an amplicon with a size of approximately 420 bp of the 16s-rDNA. Amplification of the *coa* gene yielded two distinct products of 159-bp and 240-bp amplicons in 28 (36.8%) and 29 (38.1%) isolates, respectively. The *fem A* gene (344 bp) was highly expressed in 69 isolates (92.1%).On the basis of genetic variation, DNA band patterns were divided into 6 patterns (pattern I-VI) and recognized as follows: pattern I (420-344-240-159 bp), pattern II (420-344 bp, pattern III (800-600-420-344-240 bp), pattern IV (800-600-420-344 bp), pattern V (600-500-420-240 bp) and pattern VI (800-600-420-240-159 bp), accounted for 25% (19/76), 43.4% (33/76), 5.26% (4/76), 10.52% (8/76), 3.94% (3/76) and 5.25% (4/76), respectively. The results of this study showed that the prevalence of *S. aureus* is relatively high and distributed in several farms. m-PCR assay with 3 specific genes can be successfully applied to assess the genetic characteristics of *S. aureus* isolates from different herds. Furthermore, the genetic diversity of specific genes indicates the same or different clonal distribution of in several dairy herds in the nearby area.

Keywords: Prevalence, Genetic profile, Multiplex PCR, Subclinical mastitis and Dairy cow

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ความชุกและรูปแบบทางพันธุกรรมของ *Staphylococcus aureus* จากตัวอย่างน้ำนมของโคนมที่เป็นโรคเต้านมอักเสบด้วยเทคนิคการตรวจวินิจฉัยแบบมาตรฐาน และ multiplex PCR ในจังหวัดขอนแก่น ประเทศไทย

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บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้เพื่อหาความชุกและรูปแบบทางพันธุกรรมของเชื้อ *Staphylococcus aureus* จากตัวอย่างน้ำนมที่เป็นเต้านมอักเสบ ด้วยเทคนิคการตรวจวินิจฉัยแบบมาตรฐานและ muiltiplex PCR (m-PCR)เก็บตัวอย่างน้ำนมจากแม่โครีดนมที่เป็นเต้านมอักเสบแบบไม่แสดงอาการ โดยคัดกรองเบื้องต้นด้วยเทคนิค CMT จากทั้งหมด 14 ฟาร์ม จำนวน 435 ต่อมนมในพื้นที่จังหวัดขอนแก่น ประเทศไทย แล้วเพาะและแยกเชื้อ *S. aureus* ด้วยเทคนิคทางจุลชีววิทยาและทดสอบทางชีวเคมี ได้ทั้งหมด 76 เชื้อ พบว่าความชุกของเชื้อ *S. aureus*, coagulase negative staphylococci (CNS) และเชื้อกลุ่มอื่น คิดเป็นร้อยละ 17.5, 32.4 และ 12.4 ตามลำดับ รูปแบบทางพันธุกรรมของเชื้อ *S. aureus* จากการตรวจด้วยเทคนิค m-PCR โดยใช้ไพรเมอร์ที่จำเพาะต่อ 3 ยีนเป้าหมาย คือยีน 16s-rDNA (420 bp) ใช้จำแนกในระดับจีนัสของ *Staphylococcus* spp., *coa* (159, 240 bp) และ *femA* (344 bp) ใช้สำหรับจำแนกในระดับสปีชีส์ของ *S. aureus* พบว่าเชื้อทั้งหมดมียีน 16s-rDNA (420 bp) ส่วนยีน *coa* นั้นที่มีขนาดแตกต่างกัน 2 ขนาด คือ ขนาด 159 และ 240 bp คิดเป็นสัดส่วนร้อยละ 36.8 (28/76) และร้อยละ 38.1 (29/76) ตามลำดับ และสามารถจำแนกรูปแบบทางพันธุกรรมของ *S. aureus* ได้เป็น 6 รูปแบบที่แตกต่างกัน คือ (รูปแบบ I-VI) ดังนี้ รูปแบบที่ I (420-344-240-159 bp) รูปแบบที่ II (420-344 bp), รูปแบบที่ III (800-600-420-344-240 bp), รูปแบบที่ IV (800-600-420-344 bp), รูปแบบที่ V (600-500-420-240 bp) และรูปแบบที่ VI (800-600-420-240-159 bp) โดยมีสัดส่วนที่พบคิดเป็นร้อยละ 25 (19/76), 43.4 (33/76), 5.26 (4/76), 10.52 (8/76), 3.94 (3/76) และ 5.25(4/76) ตามลำดับ จากผลการศึกษานี้แสดงให้เห็นว่าความชุกของเชื้อ *S. aureus* พบค่อนข้างสูงและมีการกระจายอยู่ในหลายฟาร์มของพื้นที่ทำการศึกษาการตรวจวินิจฉัยเชื้อ *S. aureus* ด้วยเทคนิค m-PCR โดยการใช้ยีนจำเพาะ 3 ชนิดนี้ สามารถใช้ในการประเมินคุณลักษณะทางพันธุกรรมของเชื้อนี้ในฝูงโคนมแต่ละฝูงได้ โดยเฉพาะอย่างยิ่งความหลากหลายทางพันธุกรรมของเชื้อ บ่งชี้ถึงการกระจายของเชื้อสายพันธุ์เดียวกันและต่างกันในโคนมหลายฝูงที่อยู่ในพื้นที่ใกล้เคียงกัน

คำสำคัญ: ความชุก รูปแบบพันธุกรรม Multiplex PCR โรคเต้านมอักเสบแบบไม่แสดงอาการ และ โคนม

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Introduction

Staphylococcus aureus is known as a major cause of contagious bovine mastitis in Thailand. Although, *S. aureus* is isolated low prevalent from cows than CNS in Thailand (Kampa *et al*., 2010) and some countries in Europe (Schukken *et al*. 2009; Tenhagen *et al*. 2009), *S. aureus* is capably contagious to other cows within a herd and occasionally to other herds, causing clinical and mainly subclinical mastitis (Sindhu *et al*., 2010). Currently, the mastitis pathogen identification is based on a conventional culture procedure. This method is also given a name of "gold standard", where the main clinically relevant factor is the differentiation between the contagious *S. aureus* and other Staphylococci (Karima *et al*., 2004). Recently, molecular techniques such as PCR methods (Shome *et al*., 2011; Anjali and Kasshyap, 2017), highly sensitive and specific approaches for reliably nucleic acid detection, have been used to identify and compare *S. aureus* genotypes (Saei *et al*., 2009). The identification of the bacteria using PCR at the species level is based on amplification of a target mostly rRNA gene, which is highly conserved in bacteria (Anjali and Kasshyap, 2017). To improve the diagnosis, a rapid, sensitive and specific multiplex polymerase chain reaction (m-PCR) assay has been developed for early detection of more than one mastitis-causing pathogen in single PCR reaction. In m-PCR, multiple pairs of primers specific for different DNA segments are included in the same reaction to enable amplification of multiple targeted sequences in one assay (Henegariu *et al*., 1997). This produces amplicons of variable sizes, allowing the identification of targeted bacterial pathogens in a milk sample. However, information regarding the prevalence and genetic profiles of *S. aureus* causing mastitis in the small dairy farm in this area is limited. Due to the limitations of conventional culture methods, time consuming, routinely requiring 48 hours to complete. Compared with the conventional method and m-PCR assays are less time consuming, rapid, sensitive and hence reliable for the detection major pathogens causing bovine mastitis from milk. Therefore, the objectives of this study were to investigate the prevalence and genetic profiles of *S. aureus* from milk in mastitic dairy cows based on conventional and multiplex PCR methods in small dairy farms in Muang District, Khon Kaen Province, Thailand.

Materials and Methods

1. Milk sampling, Isolation and Identification of *S. aureus*

The study was conducted on smallholder dairy farmers in Khon Kaen Province, Thailand (16° 34′ N, 102° 46′ E), from October to December 2014. One hundred and sixty bulk milk samples were initially screened for subclinical mastitis by CMT (Kivaria *et al*. 2007). CMT scores $\geq$ 1 were used as the basis for further quarter milk sampling at the farm level and resulted in 16 positive dairy farms (10%) Second phase, at the farm level, quarter milk samples (QM) were aseptically collected according to the National Mastitis Council (NMC) guidelines from 16 dairy farms (233 cows, 909 QMs). In order to ensure *S. aureus* isolation, primary culture of milk samples was performed on Braid Parker agar (BPA, Himedia®, India), a selective media for coagulase-positive Staphylococci. Black shiny colonies with clear haloon BPA were suspected as *S. aureus*, picked up for subcultures on 5% sheep blood agar to

obtain pure culture, observe colony characteristics and hemolysis types. Gram staining, catalase test and coagulase test were used for the presumptive identification of all isolates (NMC, 1999). *S. aureus* isolates were then kept frozen at-20 °C in tryptic soy broth (TSB, Himedia®, India) containing 15% glycerol for further molecular processes.

2. DNA extraction

S. aureus DNA was extracted from overnight cultures in 10 ml of brain heart infusion broth (Himedia®, India) by using High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) according to manufacturer instruction with some modifications.

3. Oligonucleotide Primers

PCR primers were designed from species specific regions of the DNA 16s-rDNA gene-based identification, *coa* and *femA* genes reference to previously published sequence entries available in the NCBI-GenBank sequence database for the detection of *Staphylococcus* spp. (16s-rDNA) and *S. aureus* (*coa* and *femA* genes)availably. The lyophilized powder of synthesized primers (Sigma-Aldrich PTE Ltd., Singapore) were reconstituted in Tris-EDTA buffer to a final concentration of 1000 pm/µl and stored at -70 °C as a stock solution. The working concentration of 20 pm/µl was prepared with sterile RNase free water, aliquoted and stored at -20 °C. The sequences of 3 different specific primers included 16s-rDNA (420 bp; Strommenger *et al*., 2003) to specifically detect *Staphylococcus* spp. (Forward: 5′ CAG CTC GTG TCG TGA GAT GT 3′ and reverse: 5′ AAT CAT TTG TCC CAC CTT CG 3′); coagulase gene (*coa*) (240, 159 bp; this study) to detect *S. aureus*-specific (Forward: 5′ GCC CAA CAC AAA ACA AGC CA 3′ and reverse: 5′ GCC CAT ATG TCG CAG TAC CA 3′) and factor A essential for methicillin resistance (*femA*344 bp; this study) to detected *S. aureus*-specific (Forward: 5′ CAA AGA GCG TGT TGG CCA CTA TG 3′ and reverse: 5′ AGG GCA CTG CAT AAC TTC CG 3′).

4. Multiplex PCR Assays

The PCR reaction was conducted in a CFX96™ Real Time System, C-1000 Touch Thermal Cycler (CFX96™ Optics Module, USA). Each 25 µl PCR reaction volume containing 12.5 µl of 2X PCR Master Mix (Thermo Scientific, Lithunia), 4 mM $MgCl_2$, 0.4 µM of each primer and up to 25 µl of nuclease free water were totally mixed in a PCR tube. DNA amplification was carried out as follows: initial denaturation at 94°C for 4 min, followed by 40 cycles of amplification (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min), and ending with a final extension at 72°C for 5 min. PCR products were separated by electrophoresis on 1% agarose gel mixed with SYBR SAFE (1:10000; Invitrogen, USA) containing 0.5X Tris-borate-EDTA (TBE) buffer at 100 volts for 30 min and visualized under ChemiDoc XRS+ (Bio-RAD, USA). To ensure that the amplified products were the expected size, a 100 bp DNA ladder (100-1000 bp, Solis Biodye, Estonia) was run simultaneously as a marker.

5. Statistical analysis

Data on classification criteria included the prevalence of *S. aureus* and the molecular and genetic profiles. Descriptive statistics were used to analyze the results and reported as percentage (%) by SPSS version 17.0 (Cary, NC, USA).

Results and Discussion

1. Prevalence of *S. aureus* using conventional method

A total of 435 CMT-positive quarter milk samples from 187 cows in 16 dairy farms were tested and *S. aureus* was isolated from 76 samples based on the cultural and biochemical properties. All the 76 isolates showed β-hemolysis on 5% sheep blood agar media. Gram-stained smears of the pure cultures exhibited clusters of Gram-positive cocci. The isolates also grew on BPA media with shiny black with narrow white margins surrounding by clear zone extending into the opaque medium. The isolates were identified as *S. aureus* by coagulase test positive and then confirmed by the formation of curd like clotting as compared to negative control (data not shown). These colonies were found to be Gram positive cocci in clusters in accordance with a report of El-Hadedy and El-Nour (2012). Earlier reports in Thailand have been conducted to assess the prevalence of *S. aureus* microorganisms in the infected mammary gland and transmitted from mastitic milk attained from small-holder dairy farms. The prevalence of *S. aureus* in this study was 17.5% (76/435) of 14 small-holder dairy farms, which was similar and moderately high as in Aiumlamai *et al*. (2000), carried out in neighboring populations and areas, (17.5% and 17%, respectively) but was higher than that reported by Kampa *et al*. (2010) (only 3%). However, the present finding was lower than that reported by Hashemi *et al*. (2011) (19.56%). The lowest & highest *S. aureus* prevalence individaul farm was 4.4 and 62.5 % respectively (data shown in Table 1). Meanwhile, 141 bacterial isolates were identified as CNS, with the highest prevalence of 32.4% in 14 dairy farms. Contagious bacteria are including *S. aureus* and *Streptococcus agalactiae*, both types of bacteria are infected by cow to cow during milking (Ajariyakhajorn *et al*., 2003). The high prevalence of *S. aureus* is indicative of poorhygienic measures during production, handling and distribution (Zakary *et al*., 2011). According to our investigation, indicated that the management of milking hygiene in many dairy farms these areas were still a problem. However, the prevalence of *S. aureus* and others contagious bacteria in this study was close to the previous 17-year (Aiumlamai *et al*., 2000). This situation could be explained by no improvement of milking hygiene in the studied dairy herds, i.e. milking management inappropriate. Therefore, dairy farmers should be aware of the problem and improve the hygienic milking process.

2. Detection & genetic profiles of genes by multiplex PCR

A total 76 *S. aureus* isolates were genetically determined using the multiplex PCR method including 3 specific primers. To substantiate the m-PCR method, all *S. aureus* strains were also primarily screened for the presence of individual genes by singleplex PCRs. The results of the singleplex PCRs corresponded precisely with those from multiplex PCR (Fig.1), demonstrating the reliability of the multiplex PCR method. In the m-PCR, the detection of *S. aureus* at the genus and species levels was accomplished through the amplification of the genes encoding 16s-rDNA, coagulase and *femA*, respectively. As demonstrated by our m-PCR, the primer pair performed in the specific amplification of the 16s-rDNA gene from species of *Staphylococcus*. In addition, the *coa* and *femA* primers designed for

this study were specific for *S. aureus* strains. The 16s-rDNA gene (420 bp) was detected in all sample of isolates (100%, 76/76), indicating that all strains were classified as genus Staphylococcus. Earlier reports, Chotar *et al*. (2006) and Sindhu *et al*. (2010) have also used 16S-23S rRNA spacer-based PCR assays for specific detection of staphylococci. The identification of *S. aureus* based on their 16s-rRNA is preferred because 16s rRNA genes are highly conserved throughout bacterial evolution. They consist of common regions of all eubacteria and other regions, which are extremely species specific and have been used for species identification of *S. aureus* by many workers (Lange *et al*., 2015; Zhang *et al*., 2004; Fan *et al*., 2008). PCR amplification of *coa* gene from 76 isolates generated five different types in relation to sizes, ranging from 159-800 bp, which were recognized as follows: *coa* type I (159 bp), *coa* type II (240 bp), *coa* type III (500 bp), *coa* type IV (600 bp) and *coa* type V (800 bp) (Fig.1, 2 and 3) and were found at 36.8 and 38.1%, respectively (remaining data not shown). The majority of the isolates exhibit *coa* gene type II with 240 bp in size. The predominance of a particular strain of *S. aureus* might be the increased resistance to the host immune response as compared to those with the rare genotypes which could have a lower resistance (Mullarky *et al*., 2001 cited by Lateef *et al*., 2015). The result was compatible with the studies of Abbas *et al*. (2014) and Lateef *et al*. (2015)

Table 1 Bacteriological finding in milk samples from infected quarters by mastitis in dairy farms of Khon Kaen Province, Thailand

Farm ID.	Dairy cow sample (cow)	Quarter milk sample (QM)	*S. aureus* (No. isolate) (n, %)	Coagulase-negative staphylococci (n, %)	Other bacteria (n, %)	No Growth Bacteria (n, %)
F288	11	36	2 (5.6)	3 (8.3)	0	31 (86.1)
F631	12	25	8 (32)	7 (28)	1 (4)	12 (48)
F674	3	6	0	6 (100)	0	2 (33.3)
F049	23	72	8 (11.1)	23 (31.9)	2 (2.8)	40 (55.6)
F557	17	46	2 (4.4)	27 (58.7)	2 (4.3)	19 (41.3)
F041	22	49	10 (20.4)	13 (26.5)	3 (6.1)	30 (61.2)
F653	5	14	1 (7.1)	0	0	12 (85.7)
F386	10	30	13 (43.3)	1 (3.3)	0	15 (50)
F101	10	29	3 (10.3)	15 (51.7)	16 (55.2)	5 (17.2)
F043	12	26	2 (7.7)	9 (34.6)	9 (34.6)	7 (26.9)
F680	9	9	1 (11.1)	7 (77.8)	1 (12.5)	2 (22.2)
F602	25	33	6 (18.2)	8 (24.2)	9 (27.3)	11 (33.3)
F163	7	16	10 (62.5)	1 (6.3)	0	5 (31.3)
F570	5	13	4 (30.7)	0	7 (53.8)	3 (23)
F621	6	9	0	5 (55.6)	2 (22.2)	2 (22.2)
F598	10	22	6 (27.3)	16 (72.7)	2 (9.1)	2 (9.1)
	n=187	n=435	76 (17.5)	141 (32.4)	54 (12.4)	198 (45.5)

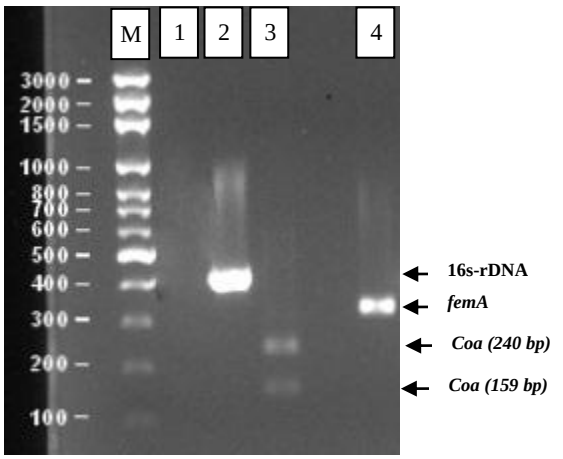

Fig. 1 PCR amplification of 3 specific primers *S. aureus* analyzed by electrophoresis on a 1% agarose gel. Lane M: 100 bp DNA Ladder, Lane (1) Negative Control, Lane (2-4): PCR amplified products of Reference Positive control (*S. aureus* ATCC 25923), Lane (2) 420 bp of 16s-rDNA, Lane (3) 159, 240 bp of *coa* gene and Lane (4) 344 bp of *femA* gene

The reason for this polymorphism in the *coa* gene among *S. aureus* isolates could be due to this gene which consists of three distinct regions: (i) the N-terminus containing the prothrombin-binding site, (ii) a central region which is highly conserved, and (iii) a C-terminal region composed of 81-bp tandem repeated units, which each encode-27-amino acid residue (Janwitha-yanuchit *et al*., 2006). Therefore, the size of 3' region of the *coa* gene is variable in *S. aureus* strains (Shopsin *et al*., 2000). In the present study, almost all isolated strains were relevant to 344-bp band of *fem A* gene (92.1%; 69/76). The *fem A* genes were suggested to be specific for *S. aureus*, encodes a factor which is essential for methicillin resistance and is universally present in all MRSA isolates (Singh *et al*., 2014). Therefore, it is possible that some strains found in this study may not be resistant strains. On the basis of genetic variation, DNA band patterns were divided into 6 patterns (pattern I-VI) (shown in Table 2) were recognized as follows: pattern I (420-344-240-159 bp), pattern II (420-344 bp), pattern III (800-600-420-344-240 bp), pattern IV (800-600-420-344 bp), pattern V (600-500-420-240 bp) and pattern VI (800-600-420-240-159 bp) (Fig, 2,3 and Table 2) and were found 25% (19/76), 43.4% (33/76), 5.26% (4/76), 10.52% (8/76), 3.94% (3/76) and 5.25% (4/76), respectively. Pattern II (420-344 bp) was most predominantly distributed (43.4%) in 33 isolates of 8 dairy farms in Muang, Khon Kaen area. Therefore, this pattern of *S. aureus* has the potential to spread to other farms in the neighborhood, if there is no adequate protection and control of farm sanitation. Furthermore, the minor predominant pattern was found a pattern I (420-344-240-159), distributed in 19 isolates of 6 dairy farms. It was found that one farm (Farm ID. 163) distributed in both patterns, indicating that it was spread from one farm to another. It is possible that cows are infected during milking, including from the milking machine with contamination from the animal's skin, milk, or hand of the operator (Bava *et al*., 2009). The results of this study are consistent with many reports by Abdulghany *et al*. (2014), Gao *et al*. (2010), Fan *et al*. (2008) and Abbas *et al*. (2014). In addition, the results of this study show the genetic diversity of *S. aureus* in mastitic dairy cows in Khon Kaen Province, Thailand.

Conclusion

In conclusion, the prevalence of mastitis-causing *S. aureus* in milking cow of small holder dairy farms in Khon Kaen Province, Thailand, was relatively high. m-PCR assays can be used as a rapid diagnostic method with high sensitivity to

diagnose and identify the genetic diversity of mastitis-causing *S. aureus.* In addition, genetic variation of specific genes indicated a distribution of different clonal in the nearby area where the most common genetic patterns were distributed in several neighboring herds. Simple dairy hygiene such as dipping the milking clusters in sterilizing solution between each cow milked would be a very effective (tried and tested) control measure.

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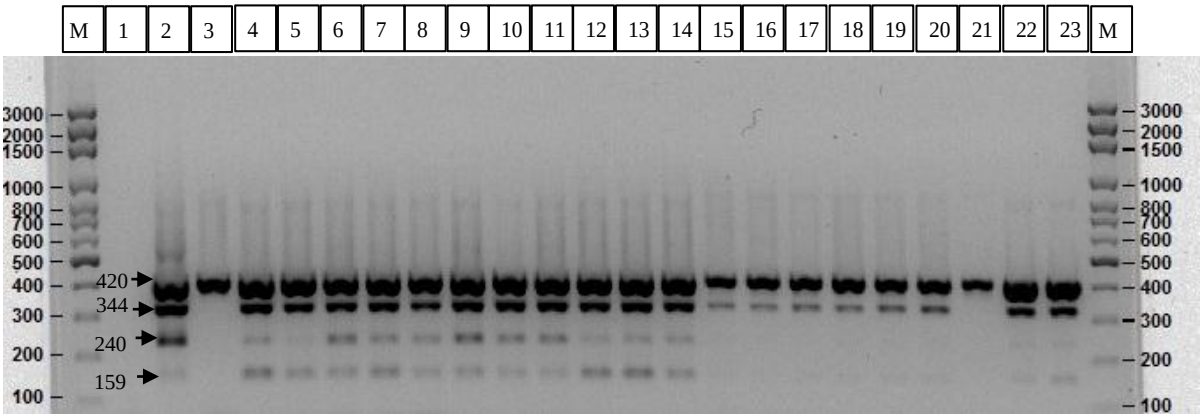

Fig. 2 m-PCR amplification products of the 3 specific primers (16s-rDNA; (420 bp), *coa*; (159, 240 bp) and *femA*; (344 bp) gene) *S. aureus* combinations were analyzed by electrophoresis on a 1% agarose gel. Lane M = 100 bp DNA Ladder, Lane 1 NTC = Negative control (NTC), Lane 2 = *S. aureus* ATCC25923; Lane 3 = *S. epidermidis* ATCC29990, Lane 4-23 = *S. aureus* sample

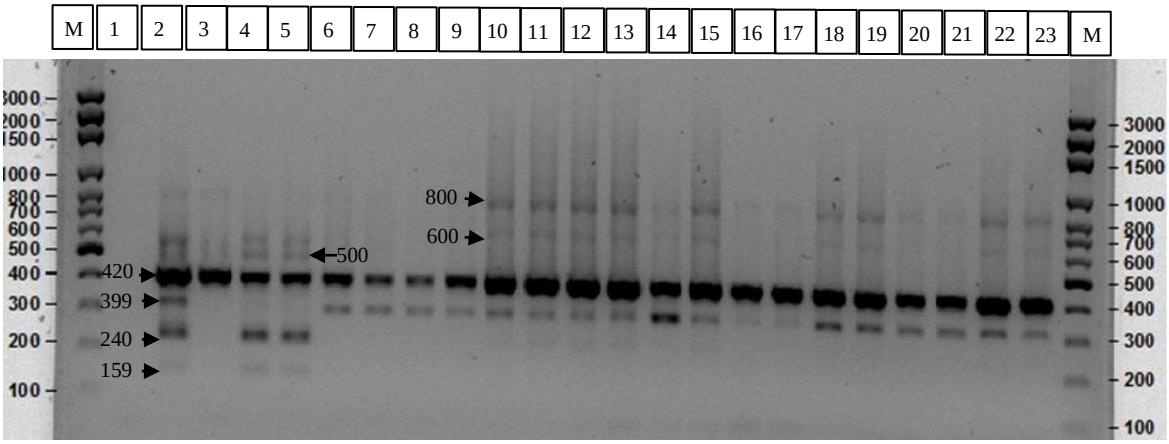

Fig. 3 m-PCR amplification products of the 3 specific primers (16s-rDNA; 420 bp), (*coa*; 159, 240 bp) and(*femA*; 344 bp) genes). *S. aureus* primers combinations were analyzed by electrophoresis on a 1% agarose gel. Lane M = 100 bp DNA Ladder, Lane 1 NTC = negative control (NTC), Lane 2 = *S. aureus* ATCC25923; Lane 3 = *S. epidermidis* ATCC29990, Lane 4-23 = *S. aureus* sample

Table 2 *S. aureus* genotypes profiles distribution in small-holder dairy farms in Khon Kaen, Thailand using m-PCR through specific 3 genes (16s-rDNA, *coa* and *femA*)

Pattern	PCR product (bp)	% (n)	Farm ID
I	420-344-240-159	25 (19/76)	F288, F631, F049, F55, F101, F163
II	420-344	43.4 (33/76)	F631, F041, F386, F101, F602, F163, F570, F598
III	800-600-420-344-240	5.26 (4/76)	F386
IV	800-600-420-344	10.52 (8/76)	F386, F043, F602, F163
V	600-500-420-240	3.94 (3/76)	F653, F598
VI	800-600-420-240-159	5.25 (4/76)	F101, F570, F598

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