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Prevalence and Characterization of Pathogenic Bacteria in Bulk Tank Raw Milk, Thailand

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Abstract

Although there are a number of raw milk collection centers in Nakhon Ratchasima, there is a lack of information with regard to the process of isolation and characterization of foodborne pathogens in raw milk. Therefore, the purpose of this research was to investigate the prevalence and characterization of foodborne pathogens, including *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Salmonella* spp. and *Staphylococcus aureus* from 33 raw milk samples from 9 different raw milk collection centers located in 8 districts in Nakhon Ratchasima, Thailand. This study was conducted from January to March 2016. Results revealed that the contaminations of *L. monocytogenes* and *Salmonella* spp. were not detected in any of the raw milk samples tested. The prevalence of *B. cereus*, *E. coli*, and *S. aureus* in raw milk samples was found to be 9 % (10 - 2.0 $\times 10^4$ CFU/ml), 42.4 and 54 % (85 - 2.7 $\times 10^4$ CFU/ml), respectively. The distribution of virulence genes was tested in *B. cereus* and *S. aureus* using gene specific primers by polymerase chain reaction. Out of the 29 analyzed coagulase-positive *S. aureus* isolates, 27 isolates (93 %) were positive for *eap* gene amplification and 14 isolates (48 %) showed amplicon of *eap* gene and all 5 enterotoxin genes, including *seG*, *seGV*, *seI*, *seIV*, and *seM* genes. All 8 *B. cereus* isolates tested showed positive PCR result with enterotoxin *FM* (ent*FM*) gene but they showed negative with hemolysin gene (*hblA* and *hblD* genes) amplifications. It was inferred from these findings that bulk tank milk is a potential source of *S. aureus* and *B. cereus* in milk.

Keywords: Foodborne pathogens, Raw milk, Raw milk collection center, Virulence genes

Introduction

In recent years, foodborne disease has been a worldwide problem. The safety of food supply has become a focal point of public concern. Foodborne disease outbreak in South East and Central Asia is generally underreported. Typically, the number is extrapolated from the limited data because the records and monitoring of foodborne disease are inadequate [1,2]. Milk and dairy products are basic components of human diet which provide a dietary source of proteins, vitamins and minerals. However, they also serve as a good medium for the growth of many microorganisms. Thus, it is possible that milk and dairy products can be contaminated with a variety of microorganisms from different sources, especially bacterial pathogens, including the families of Enterobacteriaceae, Streptococcaceae, and Bacillaceae [3-5]. The presence of pathogens in raw milk depends on the ingestion of contaminated feed followed by amplification in bovine hosts and fecal dissemination in farm environment. The final outcome of this cycle is a constantly maintained reservoir of foodborne pathogens that can reach humans by direct contact, ingestion of raw contaminated food, or contamination during the milk processing [4,6]. Therefore, even though pasteurization is an effective control method for bacterial pathogens, it is important to maintain high preprocessing standards. The quality of raw milk is important because the contaminated raw milk with pathogens might provide a reservoir for recontamination at milk processing plants. The introduction of raw milk contaminated with foodborne pathogens into milk processing plants and their persistence in biofilms represents an important risk of post-pasteurization contamination that could lead to the exposure of the consumers to pathogenic bacteria [1,6].

It is necessary to investigate and characterize foodborne pathogens in raw milk from local dairy farms and Raw Milk Collection Centers (RMCC) in order to evaluate the risk of foodborne pathogen contamination. Although there are numerous dairy farms and RMCC in Nakhon Ratchasima province, which is located in the northeast of Thailand, the information as to the process of isolation and characterization of foodborne pathogens and food safety indicators from raw milk is still lacking. In fact, there has been limited studies that focused on the detection of major foodborne pathogens in milk, including *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, and food safety indicator, *Escherichia coli*, in the bulk tank milk from RMCC in Nakhon Ratchasima.

The objective of this research was to isolate the dominated foodborne pathogens and microbial food safety indicators, including *B. cereus, L. monocytogenes S. aureus*, *Salmonella* spp. and *E. coli* in raw milk from different RMCC in Nakhon Rathasima, Thailand. The physiological characterization and analysis of virulence genes of each isolate were performed. The prevalence and characterization profiles of pathogenic bacteria from these investigations can be used as an index for dairy farm sanitation in these local areas.

Materials and methods

Sample collection

Raw cow milk samples were collected from RMCC located in 8 districts in Nakhon Ratchasima province, Thailand. The samples were collected 1 - 4 times from 9 RMCC in the mentioned areas from January to March 2016 to obtain a total of 33 samples. Approximately 200 ml raw milk samples aseptically collected in a bulk tank raw milk from each RMCC which were contained in a sterile bottle. Immediately after the collection, all samples were kept at 4 °C and transported to the laboratory for the analysis. The physical properties of raw milk samples, including pH, color, texture of raw milk, and temperature of the bulk tank milk were recorded. The microbiological quality, including total bacterial count and foodborne pathogen contamination were also measured.

Microbiological analysis

In this investigation, the enumeration of total aerobic bacteria, *S. aureus*, and *B. cereus* was performed as described in Bacteriological Analytical Manual [7]. For total bacterial count, the sample was serially diluted into 0.85 % NaCl solution which ranged from 10^0 - 10^{-9}. One hundred µL of each dilution was spread on Plate Count Agar (PCA) (Himedia, Mumbai, India) and incubated at 37 °C for 24 h. For *S. aureus* and *B. cereus* detection, each dilution was spread on Baird-Parker agar (Himedia) and supplemented with egg yolk and 3.5 % potassium tellurite solution (Himedia) and on Mannitol Yolk Polymyxin agar (MYP; Himedia) supplemented with egg yolk and polymyxin B selective supplement (Himedia), respectively. Baird-Parker and MYP agars were incubated at 35 °C for 48 h. and 30 °C for 24 h. to detect *S. aureus* and *B. cereus*, respectively. The presumptive colonies of *S. aureus* were identified using cell morphological and biochemical characteristic, including Gram stain, methyl red (MR)-reactive compound test, Voges Proskauer (VP)-reaction test, catalase test, glucose and mannitol fermentation test, and coagulase test. For coagulase test, Baird-Parker agar containing fibrinogen plasma trypsin inhibitor supplement (Himedia) was used for coagulase positive strain identification. For *B. cereus* identification, biochemical characteristic and cell morphological profiles, including endospore forming, rhizoid growth on NA, MR-reactive compound, VP-reaction, catalase, lysozyme resistant, nitrate reduction, and mannitol utilization tests were performed.

For isolation of *E. coli* and coliform bacteria, 1 ml of the sample was transferred to 9 ml lauryl tryptose broth (LST) (OXIOD, Basingstoke, United Kingdom) and incubated at 37 °C for 24 h. One loopful of gassing LST cultures was inoculated in brilliant green lactose, bile 2 % (BGLB; OXIOD) and incubated at 37 °C for 24 - 48 h. Gassing BGLB cultures were streaked on eosin-methylene blue (EMB) agar (Himedia) and incubated at 37 °C for 24 - 48 h. The typical *E. coli* colonies with metallic green sheen were subcultured on EMB (Himedia) and incubated at 37 °C for 24 h. Single colony was tested for biochemical characteristic, including IMViC (indole production, MR-reactive compound, VP-reaction, Citrate utilization tests), lactose and inositol fermentation tests. The IMViC profiles were used to differentiate *E. coli* from other coliform bacteria.

To detect *L. monocytogenes*, pre-enrichment step was performed by adding 90 ml of Half-Fraser broth (Himedia) into 10 ml of raw milk sample and mixed in sterile flask. The mixtures were incubated at 37 °C for 48 h. Then 100 µl of the pre-enrichment cultures were transferred into 10 ml of Fraser broth (Himedia) and incubated at 37 °C for 24 h. The culture of Fraser broth was streaked on PALCAM agar (Himedia) with antibiotic supplement (Himedia) and incubated at 37 °C for 48 h. The presumptive

colonies were subcultured on PALCAM and incubated at 37 °C for 24 - 48 h and streaked on trypticase soy agar (TSA ;composed of tryptone 15 g/l, proteose peptone 5 g/l, sodium chloride 15 g/l, and agar 15 g/l) for characterization. The characterization was carried out using Gram stain and was identified by biochemical characteristic tests including, oxidase, catalase, urea hydrolysis, motility, carbohydrate utilization, H_2S production, indole production, VP-reaction and MR-reactive compound tests [7,8].

For the isolation of *Salmonella* spp., pre-enrichment culture was performed by adding 90 ml of lactose broth (LB) [7] into 10 ml of sample and mixed in sterile flask. The culture was incubated at 37 °C for 24 h. The enrichment steps initiated by transferring 100 µl of pre-enrichment culture to 10 ml Rappaport-Vassiliadis (RV) broth (Himedia) and 10 ml tetrathionate (TT) broth (Himedia) and were incubated at 42 °C for 24 h. Then the culture broths were streaked on xylose lysine desoxycholate (XLD) agar (OXIOD) and incubated at 37 °C for 24 h. Typical *Salmonella* colonies were subcultured on bismuth sulphite (BS) agar (OXIOD) and incubated at 37 °C for 24 h. For single colony purification, the suspected colonies showing typical-*Salmonella* morphologies were re-streaked on TSA and tested for Gram stain and biochemical characteristics. The biochemical reactions for *Salmonella* identification were oxidase, catalase, urea hydrolysis, motility, gelatin hydrolysis, nitrate reduction, carbohydrate utilization, H_2S production and IMViC tests [7,8].

The isolate of foodborne pathogens was further confirmed using Polymerase Chain Reaction (PCR) with gene specific primers (**Table 1**).

Identification of bacterial isolates using PCR

The prevalence of bacterial isolates in local area containing the virulence genes were measured by using PCR technique. The genomic DNA (gDNA) of target bacterial isolates was extracted from 16-24 h grown in pure cultures on TSA using phenol-chloroform methods [9]. The gDNA template of each isolate was amplified by PCR technique using gene specific primers as shown in **Table 1**. For amplification of each target gene, the PCR reactions were performed individually in a total volume of 25 µl containing 1x GoTaq Flexi buffer (Promega, Madison, WI USA), 1 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.4 µM forward and reverse primers (**Table 1**), 0.5 U GoTaq Flexi DNA polymerase (Promega), and 10 - 100 ng DNA templates. The PCR reactions were heated at 95 ^{o}C for 3 min and then, 35 cycles of 95 °C for 30 s, 52 °C for 45 s, and 72 °C for 60 s followed by a final step of 5 min incubation at 72 °C. The PCR products were analyzed by 1 % agarose gel electrophoresis.

Results and discussion

Quality of raw milk from RMCC in Nakhon Ratchasima province

The quality of 33 raw milk samples from 9 RMCC located in 8 districts of Nakhon Ratchasima province, northeastern of Thailand, were determined. The physical, chemical, and biological characteristics of all samples were investigated. The physical and chemical properties, including the temperature of raw milk storage condition, the color appearance, raw milk texture, odor, and pH values were recorded. The results were summarized in **Table 2**. The temperature of raw milk storage condition which recorded from the bulk tank milk from RMCC was found to be similar in the range of 2 - 4 °C. The appearance of all raw milk samples was in white color, pH 6.4 - 8.0, normal liquid texture with no smell. These results indicated that the temperature of raw milk storage condition was low enough to maintain the high quality of raw milk.

Table 1 Primer for target bacteria identification.

Target bacteria	Target genes	Primer name	Sequences	PCR product (bp)	References
S. aureus	*seI*	SA_Ent_I_F191	TGATTATATAGATTTAAAAGGCGTCACA	515	[10]
		SA_Ent_I_R705	GCAGTCCATCTCCTGTATAAAACAA		
	seGV	SA_Ent_GV_F340	AGGTTAAAACTGAATTAGAAAATAC	312	[10]
		SA_Ent_GV_R651	CTTTAGTGAGCCAGTGTCTTGC		
	seM	SA_Ent_M_F34	CAATCATAACTTAGTAAAGGAAATGC	430	[10]
		SA_Ent_M_R463	CAGTAGAAATTGTTTTATGTTTGCC		
	seIV	SA_Ent_IV_F269	TGGATATTTTTGGCATTGATTA	265	[10]
		SA_Ent_IV_R533	TCTTTACCTTTACCATTGTTATTA		
	seG	SA_Ent_G_F_35	AGACTGAATAAGTTAGAGGAGGTTTTA	700	[10]
		SA_Ent_G_R_752	GGAACAAAAGGTACTAGTTCTTTTTTA		

Target bacteria	Target genes	Primer name	Sequences	PCR product (bp)	References
B. cereus	*eap*	SA_*eap*_F1	TTAAATCGATATCACTAATACCTC	230	[11]
		SA_*eap*_R1	TACTAACGAAGCATCTGCC		
	hblD	BC_*hblD*_F227	GGTTAGATACAGCGAAGCCACAG	409	[10]
		BC_*hblD*_R638	GCTCCCAATCCACCACCAAT		
	hblA	BC_*hblA*_F181	ATTTGCAAAATCTATGAATGCC	672	[10]
		BC_*hblA*_R852	GCAACTCCAACTACACGATTTAA		
	Enterotoxin *FM*	BC_ent*FM*_F200	TGCTGATGTATTAAATGTTCGTTC	513	[10]
		BC_ent*FM*_R713	GCGTTGTATGTAGCTGGGCCT		
E. coli	*uspA*	EC_*uspA*_F	CCGATACGCTGCCAATCAGT	884	[12]
		EC_*uspA*_R	ACGCAGACCGTAGGCCAGAT		
L. monocytogenes	*prfA*	LM_*prfA*_F	CACAAGAATATTGTATTTTTCTATATGAT	398	[9]
		LM_*prfA*_R	CAGTGTAATCTTGATGCCATCA		
Salmonella spp.	*fimY*	SM_*fimY*_F_410	GCCTCAATACAGGAGACAGGTAGCG	315	This works
		SM_*fimY*_R	AAATGCTAAAGACTGCGCCTGCCG		
All bacteria	16S rRNA	16S_F	AGACTCCTACGGGAGGC	625-655	[9]
		16S_R	GGTAAGGTTCTTCGCGT		

Microbiological quality

The microbiological quality of raw milk samples was tested. The results of the cultural enumerations for total aerobic bacteria of all samples ranged from 2.5×10^4 to 1.5×10^8 CFU/ml (**Table 2**). The similar levels of total bacterial count have been reported in various products in other countries. In Malaysia (2004), the mean count per ml of total plate counts from raw milk (360 dairy farms) was 1.2×10^8 CFU/ml [3]. In USA, 48 % of 861 bulk tank milk samples had total bacteria within 10^3 CFU/ml and 40 % of the samples had > 5×10^3 CFU/ml but the higher level of contamination (ranged from 2.7×10^4 to 2.1×10^8 CFU/g) was found in raw milk cheese samples (41 samples) [1,13]. In Tanzania (2006), the mean total bacterial count from 18 milk samples collected from milk collection center was 9.19×10^6 CFU/ml [14]. Possible reasons for the high counts could be due to infected udders of the cows in the local farm, unhygienic milking procedures or equipment, and/or inferior microbiological quality of water used for cleaning utensils and animals or the bulk tank of milk storage [3]. As demonstrated by [14], water microbial quality, frequency of cleansing milk containers, frequency of milk supply, milk storage time and type of containers, and mixing of fresh and previous milk were significantly associated with milk total bacterial count. The use of soap and good-quality water for cleaning the equipment could be expected to remove milk remains, including microorganisms, thereby affecting the microbial quality of the milk [14].

Table 2 Physical and microbiological qualities of raw milk from RMCC in Nakhon Ratchasima province, Thailand.

Raw milk collection centers	Sample code	pH	Temperature (°C)	Total plate count (CFU/ml)	Target bacterial detection[a]				
					SA (CFU/ml)	BC (CFU/ml)	LM (+/-)	SM (+/-)	EC (+/-)
A	1A	6.58	2	1.1×10^6	-	10	-	-	+
	2A	6.72	4	6.1×10^7	-	-	-	-	+
	3A	6.68	4	1.7×10^6	7.0×10^3	-	-	-	-
	4A	7.96	4	1.5×10^8	-	-	-	-	-
B	1B_1	6.76	3	2.0×10^7	-	-	-	-	+
	1B_2	6.68	3	1.6×10^6	9.0×10^3	-	-	-	-
	1B_3	6.72	4	1.5×10^6	-	-	-	-	-
	2B	6.74	4	2.5×10^4	2.0×10^2	-	-	-	-
	3B	6.76	4	4.0×10^7	2.0×10^3	-	-	-	-
	4B	6.71	4	1.5×10^7	7.0×10^2	-	-	-	+

Raw milk collection centers	Sample code	pH	Temperature (°C)	Total plate count (CFU/ml)	Target bacterial detection[a]				
					SA (CFU/ml)	BC (CFU/ml)	LM (+/-)	SM (+/-)	EC (+/-)
C	1C	6.67	4	2.0×10^6	-	20	-	-	-
	2C	6.69	4	8.7×10^5	-	-	-	-	+
	3C	6.78	4	2.3×10^7	-	-	-	-	-
	4C	6.74	4	7.7×10^6	-	-	-	-	+
D	1D	6.48	4	1.1×10^5	2.5×10^2	-	-	-	+
	2D	6.76	4	2.5×10^4	3.4×10^3	-	-	-	-
	3D	6.72	4	1.9×10^5	85	-	-	-	-
	4D	8.01	4	1.6×10^7	2.7×10^4	-	-	-	+
E	1E	6.68	3	1.8×10^6	1.8×10^3	-	-	-	+
	2E	6.68	4	1.9×10^7	-	-	-	-	-
	3E	6.76	4	3.1×10^7	-	-	-	-	-
	4E	6.70	4	1.3×10^8	2.0×10^2	-	-	-	-
F	2F	6.78	4	2.5×10^5	1.3×10^3	-	-	-	-
	3F	6.68	4	8.5×10^5	5.0×10^3	-	-	-	+
	4F	6.70	4	7.1×10^4	2.6×10^3	-	-	-	+
G	2G	6.76	4	2.0×10^5	1.2×10^3	-	-	-	-
	3G	6.76	4	1.8×10^5	5.1×10^3	-	-	-	-
	4G	7.60	4	2.1×10^7	4.5×10^3	-	-	-	-
H	1H	6.72	3	2.5×10^4	-	2.0×10^4	-	-	+
	2H	6.73	4	4.0×10^5	7.0×10^2	-	-	-	-
	3H	6.79	4	4.2×10^4	-	-	-	-	+
	4H	6.74	4	5.3×10^4	-	-	-	-	+
I	1I	6.68	4	1.9×10^5	-	-	-	-	-

[a] BC: *B. cereus* ; SA : *S. aureus* ; LM: *L. monocytogenes*; SM: *Salmonella* spp.; EC: *E. coli*

Prevalence of *L. monocytogenes* and *Salmonella* spp.

Of all the 33 raw milk samples, neither *L. monocytogenes* nor *Salmonella* spp. was detected on PALCAM and XLD agars, respectively (**Table 2**). These results indicated that 0 % *L. monocytogenes* and *Salmonella* spp. contamination was observed in raw milk from RMCC in this local area. The results in this study agree with the reports of other researches. The incidence of *Salmonella* spp. and *L. monocytogenes* in local raw milk in Malaysia (2004) was only 1.4 and 4.4 % of 930 raw milk samples, respectively [3]. In United States (2004), only 2.6 % (22 samples) and 6.5 % (56 samples) of 861 raw milk samples from bulk tank milk were contaminated with *Salmonella* and *L. monocytogenes*, respectively [1]. In Chiang Mai and in Lampoon, the northern provinces of Thailand (2005), the prevalence of *Salmonella* spp. in dairy cow during 2000- 2003 was only 3 % (of 225 analyzed samples) [15]. In Turkey (2006), *L. monocytogenes* was not detected from all raw milk samples (157 raw milk and dairy products) [16]. In Iran (2010), the prevalence of *L. monocytogenes* in 100 bulk tank milk samples that were delivered to Pegah pasteurization factory ranged from 0 to 4 % [17]. In Italy (2013), of the 618 raw milk samples tested from 112 dairy, only 0.3 % was positive for *Salmonella* spp. and 1.6 % for *L. monocytogenes* [18]. In Iran (2015), no *L. monocytogenes* was detected from 60 individual raw milk samples from 4 dairy bovine and ovine herds [19]. All available data indicated that the prevalence of *Salmonella* spp. and *L. monocytogenes* in raw milk was low at a level of 0.3 - 3 % for *Salmonella* spp. and 0 - 6.5 % for *L. monocytogenes*.

Although the occurrence of *Salmonella* spp. in local milk is low, there are still health risk that threatens the life of the consumers if milk is consumed without any heat treatment. This study supports other research studies that cattle have relatively low *Salmonella* in comparison to other sources [1,15,18]. With *Listeria* contamination, *Listeria* species are widespread in nature and live naturally in plants and soil environments. The sources of *Listeria* spp. in raw milk have been reported to be fecal which is contaminated in the environmental or probably due to insufficient hygiene during milking, storage and

transport. This can also be from infected cows in dairy farms and from poor silage quality. The variation in incidence rates of *Listeria* spp. in raw milk may also be related to other factors such as sample size taken, level of *Listeria* contamination, geographic location, seasonal variation, milk quality, sampling, and detection techniques [1,3,17]. Therefore, a number of samples analyzed and the sample collection period should be extended in order to investigate the trend of *Salmonella* spp. and *L. monocytogenes* contamination in raw milk in these local areas.

Prevalence of *E. coli*

For food safety indicator detection, presumptive colonies of *E. coli* were detected from 14 samples (42.4 %) collected from 7 RMCC in Nakhon Ratchasima (**Table 2**). Fifteen isolates of the presumptive *E. coli* colonies were randomly chosen and tested for their biochemical characteristic and *uspA* gene amplification. The results revealed that almost all biochemical characteristic properties and specific gene (*uspA* gene) amplification of *E. coli* isolates were similar to the reference strains (data not shown). The contamination level of *E. coli* from this research was lower than that reported by [3] and [5] ranging from 64.5 to 76.4 %. The presence of *E. coli* in milk and milk products is an important indicator of fecal contamination and hygienic practices. Thus, farmers and workers must be educated in safe handling and in hand washing. The water supply in the farm and in RMCC must be safe and free from any bacterial contamination that may affect milk quality [3,5,6].

Prevalence of *S. aureus*

The detection of *S. aureus* from raw milk samples was performed using Baird-Parker agar. The black colonies with opaque zone of *S. aureus* were found 54.5 % (18 samples) from 7 RMCC with concentration ranging from 85 to 2.7×10^4 CFU/ml (**Table 2**). Twenty nine typical colonies from 18 positive-samples were randomly picked from Baird-Parker agar and identified using biochemical reaction tests. All 29 analyzed isolates were coagulase-positive *Staphylococcus* isolates. The distribution of *S. aureus* (**Figure 1**) indicated that 61 % of the *S. aureus* contaminated samples (11 of 18 *S. aureus* positive-samples) had *S. aureus* population between 10^3 and 10^4 CFU/ml. Of the remainder, only 5.5 % (1 of 18 samples) had $>10^4$ CFU/ml. These results demonstrated that the level of *S. aureus* contamination in 61 % of raw milk samples was similar as reported by other investigations [3,20]. In other countries, the prevalence of *S. aureus* was 12.4 - 94 % with a concentration level ranging from 1.2×10^4 to 8.9×10^5 CFU/ml [3,20-22]. In Thailand, the prevalence of *S. aureus* in milk has been reported by other investigators. In 2014, the qualities of 60 raw goat's milk samples from 5 farms in Nongchok district, Bangkok, were investigated by [23]. The results showed that there was no *S. aureus* found in all raw goat milk samples [23].

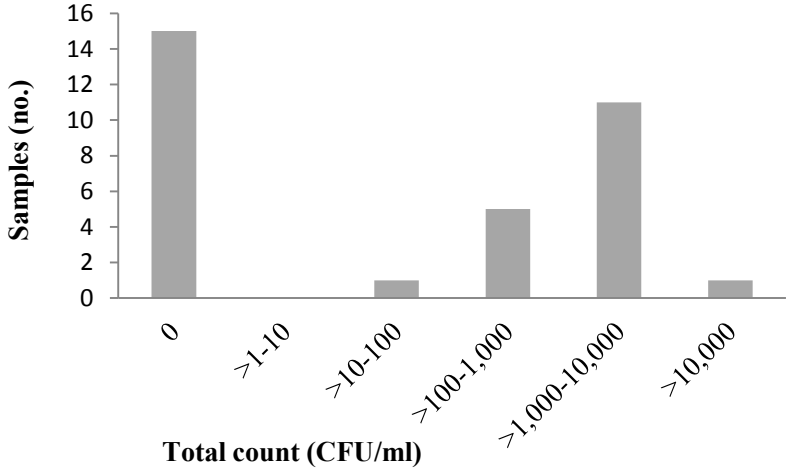

Figure 1 Frequency distribution of *S. aureus* concentration for bulk raw milk samples (n = 33) from 9 RMCC in Nakhon Ratchasima, Thailand. The monitoring was performed from January to March 2016.

Coagulase-positive *S. aureus* high prevalence among tested raw milk, milk products and hands swabs highlighted the necessity of enforcement of hygienic implementations and practices within dairy facilities [20]. In this research, high frequencies of *S. aureus* were detected from RMCC B and D by observing these pathogens in all 4 sample collections. Thus, the sanitation of dairy farm and RMCC in those areas should be improved to increase the qualities of raw milk. All equipment, working area and workers should be cleaned during milking or milk collection process to reduce the *S. aureus* contamination. As suggested by [3], post-milking disinfection has been suggested as an effective procedure to reduce the number of contagious mastitis pathogens on skin immediately after milking.

Prevalence of *B. cereus*

To enumerate number of *B. cereus*, MYP agar was used. The presumptive red colonies with opaque zone were found from 3 (9 % contamination) of 33 analyzed samples at its concentration ranged from 1.0×10^1 to 2.0×10^4 CFU/ml (**Table 2**). The prevalence of *B. cereus* in raw milk and dairy products ranged from 15 to 100 % at concentration level of 3×10^1 to 1.7×10^3 CFU/ml [24-26]. The results obtained from this research indicated that the prevalence of *B. cereus* from RMCC in Nakhon Ratchasima was lower (9 %) than those reported by other investigators [24-26]. In most *B. cereus* outbreaks, the number of *B. cereus* associated with diarrhea ranged from 10^5 to 10^8 CFU/g or CFU/ml of food [24]. This indicated that level of *B. cereus* occurring in raw milk samples in this study was not high enough to cause illnesses. However, the number of *B. cereus* could increase to an infectious level during storage and distribution.

B. cereus is considered to be a common contaminant of raw milk. Most of the *B. cereus* contamination results from the raw milk in which the organism is partly present as spores which are able to survive during pasteurization [27]. Soil, feed, and bedding material are the major sources of *B. cereus* contamination of raw milk [28]. High levels of *Bacillus* spores have also been detected in the surface layers of grass and maize silage. The spores can be transferred to the milk via the faeces. Subsequently, spores present in raw milk can survive during food processing, and after germination and outgrowth to high levels, it may cause spoilage and outbreaks of foodborne illness [29]. Moreover, milking equipment can also be a source of *B. cereus* contamination for raw milk. *B. cereus* is the microbial flora presenting in silo tanks, pasteurizers and filling machines that contribute to post-pasteurization contamination of milk. The contamination of *B. cereus* may cause aggregation of the creamy layer of pasteurized milk because of the lecithinase activity of bacterium [27]. *B. cereus* spores can survive high temperature exposure; therefore, insufficient cooling or storage of food can support their growth in food. As demonstrated by [30], all *B. cereus* isolates incubated at 30 °C produced the HBL enterotoxin. Thus, the recommended temperature for storage of refrigerated dairy products is 7 °C or less [30]. This information indicate that the storage temperature is the most important factor in keeping *B. cereus* numbers to a minimum [27,31]. To evaluate the virulence of *B. cereus* isolates in local area, all 8 isolates of *B. cereus* and 3 other *Bacillus* sp. isolates were tested for virulence gene amplification using PCR methods.

***S. aureus* virulence gene analysis**

The distribution of virulence genes in all 29 coagulase-positive *S. aureus* was evaluated using gene specific primers by PCR technique (**Table 1**). Enterotoxin genes, including Enterotoxin G (*seG*), Enterotoxin GV (*seGV*), Enterotoxin I (*seI*), Enterotoxin IV (*seIV*) Enterotoxin M (*seM*), and anchorless extracellular adherence protein (*eap* gene) were used as targets for amplification from all isolates. Out of the 29 analyzed coagulase-positive *S. aureus* isolates, the amplicon of *eap* gene was found from 27 coagulase-positive *S. aureus* isolates (93 %) (**Figure 2a, Table 3**). These results indicated that the *eap* gene could be detected from most of coagulase-positive *S. aureus* isolates. For enterotoxin gene detections, out of the 29 coagulase-positive *S. aureus* isolates, only 14 isolates (48 % of coagulase-positive *S. aureus* isolates) showed all 5 amplicons of enterotoxin genes including *seG*, *seGV*, *seI*, *seIV*, *seM* genes which are similar to *S. aureus* TISTR 517 (**Figure 2b**). The results of virulence gene distributions (**Table 3**) indicated that not all coagulase-positive *S. aureus* produce toxin as reported by [32]. Enterotoxins produced by enterotoxigenic strains of *S. aureus* were classified according to serotypes into A–H groups and toxic shock syndrome toxin (TSST). The frequency of enterotoxigenicity among staphylococcal strains was highly variable. Studies on *S. aureus* isolated from cows showed enterotoxigenicity that ranged from 0 to 56.5 %. Enterotoxigenic strains of *S. aureus* have been reported to cause a number of diseases or food poisoning outbreaks because of the ingestion of contaminated dairy products or milk [6,32]. The gene coding for enterotoxin A, *seA*, was the most frequent (41 %) found from *S. aureus* isolates from raw or pasteurized bovine milk in Brazil [21]. In Ireland (2012), only 1 isolate from 51 bulk raw milk samples tested was the SEC-producing isolate [32]. In Iran (2014), 28 (53.8 %) out of the 52 *S. aureus* isolates collected from 246 cow milk samples were positive for at least

one enterotoxin genes. The most frequently observed gene was *seA* [33]. The variation of new Staphylococcal enterotoxin gene types found in foods can be explained with the epidemiology in each region and the type of foods in which it was detected [34]. In this research, 48 % (14 of 29 isolates) of *S. aureus* isolates that were collected from 3 RMCC including A, B, and D contained *seG*, *seGV*, *seI*, *seIV*, and *seM* genes. The *seG* and *seI* are in tandem orientation on the same 3.2 kb DNA fragment [35]. The results from this research demonstrated that some virulence strains were found in the local area of Nakhon Ratchasima. The virulence *S. aureus* isolates that maintain all 5 enterotoxin genes were observed from RMCC D in 4 collections (**Table 3**). It was inferred from these studies that bulk tank milk was a potential source of enterotoxigenic *S. aureus* in raw milk and may constitute a health hazard to consumers. In this research, the maximum *S. aureus* concentration level was 2.0×10^4 CFU/ml (**Table 2**) while the concentration level of *S. aureus* needed to sufficiently produce the toxin to cause illness was reached to 10^5 - 10^8 CFU/ml [32]. However, molecular methods were only able to demonstrate the existence of the SE genes in the microorganism but could not prove that the production of SE protein occurred [35].

***B. cereus* virulence gene analysis**

For detection of the virulence genes in *B. cereus*, hemolysis genes including *hblA*, *hblD*, and enterotoxin *FM* gene (ent*FM*) were amplified. The expected sizes of ent*FM*, *hblA*, and *hblD* PCR products were observed in *B. cereus* TISTR 1474. Only the PCR products of ent*FM* were found from all analyzed *B. cereus* isolates from 3 RMCC including, A, C, and H (**Figures 2c - 2e** and **Table 3**). These results indicated that the prevalence of *B. cereus* harboring the hemolysis genes in this local area was low. In *B. cereus*, diarrheal type syndrome caused by enterotoxin (s), resulted in diarrhea and the emetic type induces nausea and vomiting [36]. Hemolysin is a three-component enterotoxin produced by *B. cereus* group which consists of two lytic components (L1 and L2) and a binding component B. The B component is a 37.8 kDa product of the *hblA* gene and may function as the binding subunit of hemolysin BL, while the 38.5 kDa L1 and 43.5 kDa L2 components (products of *hblD* and *hblC*, respectively) are hemolytic. Maximal expression of all HBL activities required all three-protein components [26,37]. In this research, all 8 *B. cereus* isolates lacked *hblA* and *hblD* genes (**Figures 2d - 2e** and **Table 3**). The variation of *B. cereus* isolates containing hemolysin gene has been reported by other researchers [4,27]. The different frequencies of distribution of enterotoxin genes that are presently available may explain the varying contribution of *B. cereus* to foodborne illness in different countries and different food sources [24].

However, the outbreaks associated with strains lacking hemolysis toxin have occurred [27]. In this research, most of *B. cereus* isolates harbored ent*FM* gene (**Figure 2c**). Similar results have been reported by other investigators. Chitov *et al*. [24] reported that 60 % of 125 *B. cereus* isolates originating from different food sources was positive for the ent*FM* gene while *hblA* and *hblD* genes were detected in 64 % of isolates [24]. The ent*FM* gene was the common enterotoxin gene found in 27 (93 %) *B. cereus* isolates [36]. Kovac *et al*. [31] investigated that all 7 *B. cereus* isolates analyzed from raw milk were positive for ent*FM* gene [31]. Enterotoxin FM is a protein. This toxin was first isolated from the *B. cereus* FM1 strain. It was suspected to cause fluid accumulation in rabbit and mouse ligated intestinal loop tests at high doses. The product of this gene was a putative virulence factor reported to be involved in bacterial shape, motility, adhesion to epithelial cells, biofilm formation, and vacuolization of macrophages [31]. The ent*FM* gene was located on the chromosome and appeared to be common to *B. thuringiensis* and *B. cereus* strains. Prevalence studies revealed that ent*FM* is detected in most outbreak-associated strains [27].

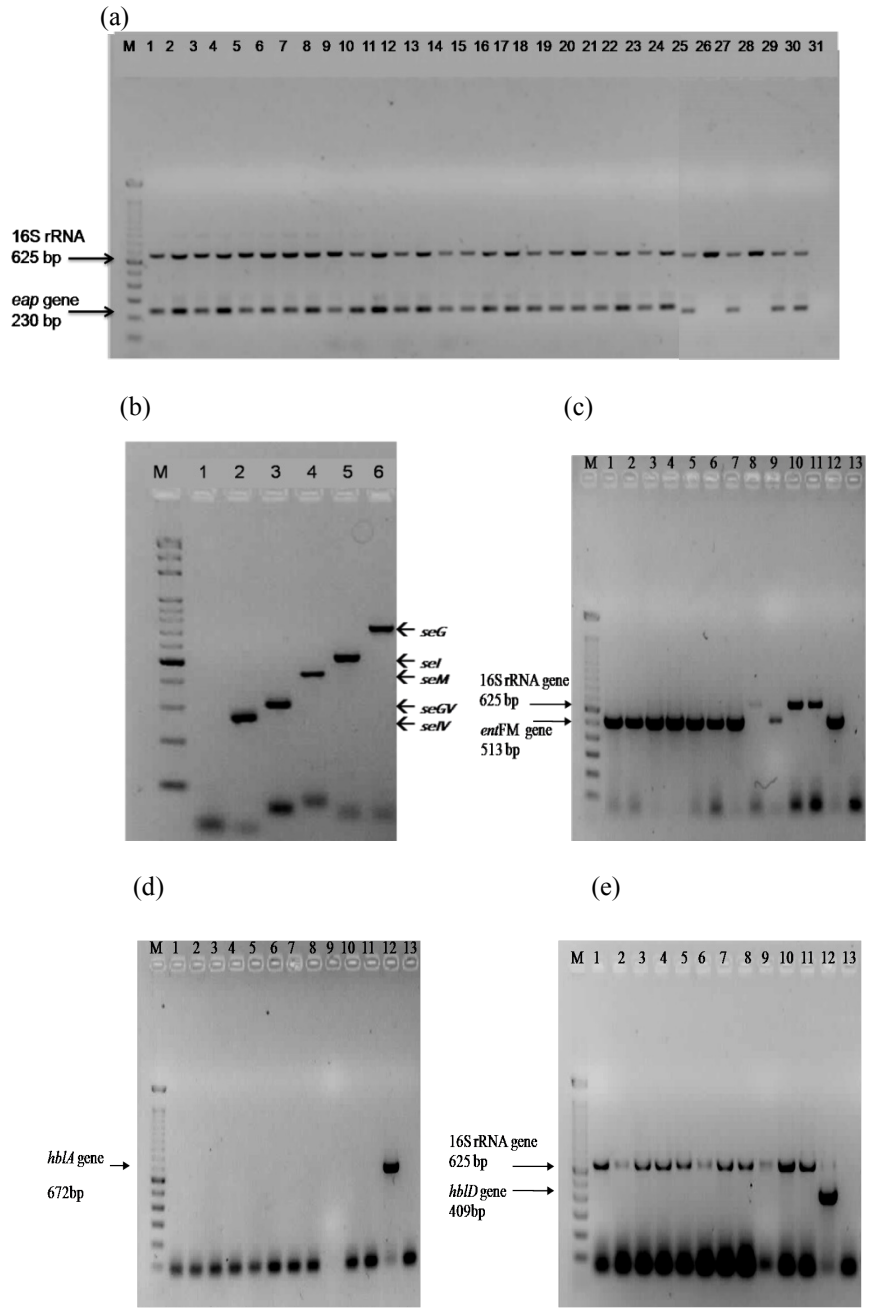

Figure 2 Virulence gene amplifications from gDNA extracted from *S. aureus* and *B. cereus* using PCR method. (a) *eap* gene amplification from gDNA extracted from *S. aureus* isolates using SA_*eap* and 16S rRNA primers. Lane M: Molecular weight marker (100 bp ladder, Invitrogen); Lane 1 - 31, *S. aureus* TISRT 517, 3A_SA1, 3A_SA5, 2B_SA1, 3B_SA2, 4B_SA1, 4B_SA2, 4B_SA3, 1D_SA5, 2D_SA2, 2D_SA8, 3D_SA1, 3D_SA5, 4D_SA1, 4D_SA2, 4D_SA4, 4D_SA5, 1E_SA1, 4E_SA1, 4E_SA2, 4E_SA3, 2F_SA3, 3F_SA1, 4F_SA1, 2G_SA4, 3G_SA7, 3G_SA8, 4G_SA2, 4G_SA6, 2H_SA4 and H_2O, respectively. (b) Enterotoxin gene amplification from gDNA extracted from *S. aureus* TISTR 517. Lane 1, H_2O; lanes; 2 - 6, amplicon of *se*IV, *se*GV, *se*M, *se*I, and *se*G, respectively. (c-e) Enterotoxin *FM* (ent*FM*), *hblA* and *hblD* gene amplifications from gDNA extracted from *B. cereus* isolates and non-*B. cereus* bacteria using (c) BC_Ent_*FM* primers and 16S rRNA primers, (d) BC_*hblA*_primers, and (e) BC_*hblD*_primers and 16S rRNA primers. Lane M: Molecular weight marker (100 bp ladder, Invitrogen); Lane 1 - 13: 1H_BC03; 1H _BC04; 1H _BC06; 1H _BC08; 1H _BC09; 1H _BC10; 1C_BC1; 4A_Y3 (non-*B. cereus* bacteria); 1A_BC1; 2B_BC2 (non-*B. cereus* bacteria); 3B_BC4 (non-*B. cereus* bacteria); *B. cereus* TISTR 1474 and H_2O, respectively.

Table 3 Presence of enterotoxin-encoding genes in *B. cereus* and *S. aureus* isolates.

Raw milk collection center	Bacterial isolates[a]	Presence of virulence genes[b]									
		16S rRNA	*eap*	*seG*	*seGV*	*seI*	*seIV*	*seM*	*hblD*	Ent*FM*	*hblA*
-	*S. aureus* TISTR 517	+	+	+	+	+	+	+	ND	ND	ND
	S. aureus TISTR 746	+	+	-	-	-	-	-	ND	ND	ND
-	*Bacillus subtilis* TISTR 1248	+	ND	ND	ND	ND	ND	ND	-	-	-
-	*B. subtilis* TISTR 1528	+	ND	ND	ND	ND	ND	ND	-	-	-
-	*B. amyloliquefacieus* TISTR 1045	+	ND	ND	ND	ND	ND	ND	-	-	-
-	*B. cereus* TISTR 687	+	ND	ND	ND	ND	ND	ND	-	+	-
-	*B. cereus* TISTR 1449	+	ND	ND	ND	ND	ND	ND	-	+	-
-	*B. cereus* TISTR 1453	+	ND	ND	ND	ND	ND	ND	-	+	-
-	*B. cereus* TISTR 1474	+	ND	ND	ND	ND	ND	ND	+	+	+
-	*B. cereus* TISTR 1527	+	ND	ND	ND	ND	ND	ND	-	+	-
A	1A_BC1	+	ND	ND	ND	ND	ND	ND	-	+	-
	3A_SA1	+	+	-	-	-	-	-	ND	ND	ND
	3A_SA5	+	+	+	+	+	+	+	ND	ND	ND
B	2B_SA1	+	+	-	-	-	-	-	ND	ND	ND
	3B_SA2	+	+	+	+	+	+	+	ND	ND	ND
	4B_SA1, 2, 3	+	+	+	+	+	+	+	ND	ND	ND
C	1C_BC1	+	ND	ND	ND	ND	ND	ND	-	+	-
D	1D_SA5	+	+	+	+	+	+	+	ND	ND	ND
	2D_SA2, 8	+	+	+	+	+	+	+	ND	ND	ND
	3D_SA1, 5	+	+	+	+	+	+	+	ND	ND	ND
	4D_SA1, 2, 4, 5	+	+	+	+	+	+	+	ND	ND	ND
E	1E_SA1	+	+	-	-	-	-	-	ND	ND	ND
	4E_SA1, 2,3	+	+	-	-	-	-	-	ND	ND	ND
F	2F_SA3	+	+	-	-	-	-	-	ND	ND	ND
	3F_SA1	+	+	-	-	-	-	-	ND	ND	ND
	4F_SA1	+	+	-	-	-	-	-	ND	ND	ND
G	2G_SA4	+	+	-	-	-	-	-	ND	ND	ND
	3G_SA7	+	-	-	-	-	-	-	ND	ND	ND
	3G_SA8	+	+	-	-	-	-	-	ND	ND	ND
	4G_SA2	+	-	-	-	-	-	-	ND	ND	ND
	4G_SA6	+	+	-	-	-	-	-	ND	ND	ND
H	1H_BC3, 4, 6, 8, 9, 10	ND	ND	ND	ND	ND	ND	ND	-	+	-
	2H_SA4	+	+	-	-	-	-	-	ND	ND	ND

[a] BC: *B. cereus* isolates; SA : *S. aureus* isolates
[b] PCR amplification results, + : positive PCR result; - : negative PCR result; ND : not determine

Conclusions

The quality of raw milk is very important for dairy industry. The entry of foodborne pathogens via contaminated raw milk into dairy food processing plants can lead to contamination of processed milk products [6]. Thus, foodborne pathogen contamination should be reduced or eliminated from the food chain production. The investigation of foodborne pathogens from 33 raw milk samples of 9 RMCC located in 8 districts, in Nakhon Ratchasima, showed that neither *L. monocytogenes* nor *Salmonella* spp. was detected. Only *B. cereus* (9 %), *E. coli* (42.4 %), and *S. aureus* (54.5 %) were detected from raw milk samples in the local area. The distribution of virulence genes was investigated in *B. cereus* and *S. aureus* isolates. All 8 *B. cereus* isolates harbored the ent*FM* gene but the hemolysin genes, *hblA* and *hblD*, could not be detected. For *S. aureus*, 48 % of coagulase-positive *S. aureus* isolates carried the enterotoxin

genes, including *seG*, *seGV*, *seI*, *seIV*, *seM* genes and 93 % harbored *eap* gene in their genome. The genotype and incidence of enterotoxin genes may vary in different geographical locations and sources of origin. However, since pooled milk samples from RMCC were studied, the findings do not directly reflect the status of individual cows or herds. Thus, more investigation and characterization of foodborne pathogens from the dairy farms or RMCC in high frequency infected areas are needed to evaluate the trends in the occurrence of pathogens and to measure the efficiency of farm managements.

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