Introduction to the research problem and its significance

Bovine mastitis is an important animal health disease leading to significant economic losses to the dairy industry throughout the world (Seegers et al., 2003). These losses can be attributed to a reduction in milk production, the associated costs of treatment and the culling of persistently infected and repeatedly infected cows. Bovine mastitis is characterized by an inflammation of the mammary gland caused by a wide variety of microorganisms which are commonly divided into those that show a contagious route of transmission and those that also frequently infect the udder from an environmental reservoir. Current mastitis control programs based on improved milking hygiene, culling of chronically infected cows, and antibiotic therapy at drying off have a significant impact in controlling contagious mastitis pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae* (Bradley, 2002; Oliver, 1988; Oliver and Mitchell, 1984; Smith et al., 1985). However, these implementations are not significantly effective for controlling mastitis caused by environmental pathogens such as *Streptococcus uberis* and *Escherichia coli* (Leigh et al., 1999). Consequently, the prevalence of intramammary infections (IMI) caused by environmental mastitis pathogens have been increasing, especially in well-managed dairy herds with low somatic cell counts (Bradley, 2002; Hogan et al., 1989). A similar phenomenon has been observed in Thailand. Many studies have shown that environmental pathogens, particularly environmental streptococci, are one of the major causes of bovine mastitis in many regions of the country (Ajariyakhajorn et al., 2003; Boonyayatra and Chaisri, 2004). Therefore, there has been as increasing interesting in developing effective control strategies for environmental mastitis pathogens.

Environmental streptococci are characterized as *Streptococcus* species other than *Streptococcus agalactiae* which is a well-defined contagious pathogen for bovine mastitis. Among all species in this group, IMI caused by *S. uberis* has been reported to be a major cause of bovine mastitis in many countries throughout the word, including Australia (Phuektes et al., 2001), Brazil (Costa et al., 1998), Canada (Sargeant et al., 1998), the Netherlands (Barkema et al., 1999; Poelarends et al., 2001), New Zealand (Douglas et al, 2000), the United Kingdom (Leigh, 1999), the United States (Wilson et al, 1997) and Thailand (Boonyayatra et al., 2007). *Streptococcus uberis* is an important pathogen implicated in bovine mastitis and is predominantly associated with subclinical and clinical IMIs at any period of lactation cycles including the stage of mammary gland involution in the dry period (Hill, 1988). *Streptococcus uberis* can even caused IMI in

pregnant heifers (Oliver et al., 2005) which emphasis the transmission route to be originated from environment, not cow-to-cow transmission. Sources of *S. uberis* in the environment include body sites, manure, pasture and bedding material (Bramley, 1982; Cullen and Little, 1969; Harmon et al., 1992); therefore, the eradication of this species is particularly problematic (Bramley & Dodd, 1984).

In spite of its environmental nature of transmission, a number of observations indicating that contagious or cow-to-cow transmission of *S. uberis* may be important have been reported based on molecular and mathematical epidemiological approaches. Molecular studies have shown evidences for direct transmission and the predominant strains in some herds (Phuektes et al., 2001; Baseggio et al., 1997). Mathematical studies applying a concept of the standard Susceptible-Infectious-Recovered (SIR) model for infectious diseases (De Jong, 1995) explained the prevalence of *S. uberis* infections to be a predictor for the incidence of new infections; therefore, this particular study is another evidence supporting the hypothesis that some strains of *S. uberis* can be transmitted from cow-to-cow (Zadoks et al., 2001). These observations suggest variations among strains of *S. uberis* and an existing of some strains that are hypervirulent and hypertransmissible between cows.

Understanding in pathogenesis and virulence factors required for IMI by *S. uberis* is not well established. Intramammary infections (IMI) caused by *S. uberis* may be clinical or subclinical, and can vary in duration. Various virulence factors such as hyaluronic acid capsule; HasA,B,C (Ward et al., 2001), plasminogen activator proteins; PauA (Rosey et al., 1999) PauB (Ward and Leigh, 2002) and streptokinase (Johnsen et al., 1999), lactoferrin binding proteins; Lbp (Moshynskyy et al., 2003), adhesion protein; SUAM (Almeida et al., 2006), CAMP factor (Jiang et al., 1996), a surface dehydrogenase protein; GapC (Pancholi et al., 1993), protein involved in the active transport of solutes essential for growth in milk; OppF (Smith et al., 2002), and a lipoprotein receptor antigen responsible for acquisition of manganese during growth in milk; MtuA (Smith et al., 2003) have been suggested to be associated with pathogenesis of IMI caused by *S. uberis*. In Thailand, even though a high frequency of *S. uberis* has been reported to cause both clinical and subclinical mastitis in dairy cattle (Boonyayatra et al., 2007), the occurrence of virulence-associated genes among *S. uberis* isolates from milk samples has never been reported. Moreover, it is interesting to determine if there is any association between the presences of virulence-associated genes to the clinical outcomes. This knowledge can fulfill the understanding of the

pathogenesis of IMI caused by *S. uberis* which is a major basis for establishing an effective control strategy for this microorganism.

Literature review

Nature of *S. uberis*

Streptococcus uberis is a gram positive bacterium in the family streptococcaceaea which is a distinct bacterial family containing species that are commensal and/or pathogenic organisms in humans and animals. *Streptococcus uberis* can be isolated from various body sites including tonsils, intestines, skins and epithelium of genital track of healthy cattle (Buddle et al., 1988; Cullen and Little, 1969; Razavi-Rohani and Bramley, 1981). *Streptococus uberis* is often detected in feces and can also be isolated from the environment such as pasture and bedding materials (Bramley, 1982; Kruze and Bramley, 1982). Based on an analysis of the genomic sequence of *S. uberis*, this organism has a great variety of metabolic capabilities and nutritional flexibility but relatively few classical streptococcal virulence factors (Ward et al., 2009). Thus, *S. uberis* is well capable of adapting to survive and colonize in different environments, such as bovine intestine, environmental materials, and the mammary gland.

Epidemiology of *S. uberis* associated with bovine mastitis

Streptococcus uberis is responsible for about 20-30% of cases of clinical mastitis in dairy herds in North America, Europe, and Australia (Compton et al., 2007; Olde Riekerink et al., 2008; Sorensen et al., 2008). In Thailand, particularly in northern Thailand, the prevalence of *S. uberis* ranged from 17% to 32% of mastitis cases in cattle (Boonyayatra et al., 2007; Chaisri et al., 2010). Several molecular epidemiological studies of *S. uberis* strains have revealed a divergent distribution of PFGE profiles (Baseggio et al., 1997; Douglas et al., 2000; Wang et al., 1999), multilocus sequence typing (MLST) profiles (Zadoks et al., 2005) and protein profiles between and within infected herds (Groschup et al., 1992). However, persisting infections in individual cows are usually caused by a single genotype as determined by either PFGE or MLST (McDougall et al., 2004; Phuektes et al., 2001; Pullinger et al., 2007; Wang et al., 1999; Zadoks et al., 2003). A couple studies have demonstrated an association of MLST profiles of *S. uberis* isolates with disease status (Tomita et al., 2008) and evolution of certain virulence genes (Zadoks et al., 2005). Taken all together, these evidences

strongly support an existing of some dominant strains of *S. uberis* which are highly virulent among a diverse population of this pathogen isolated from dairy cattle.

Pathogenesis of *S. uberis* intramammary infection and virulence factors

Followed entry through the teat canal, the organism attaches and proliferates on the mammary epithelium inside the udder inducing an influx of neutrophils into the secretory acini that is evident in 24 hours. This is followed by septal edema, vacuolation of secretory cells, necrosis of alveoli, and infiltration of septa by lymphocytes. As the disease progresses, there is hypertrophy of ductular epithelium, involution of glandular tissue, and early stage fibrosis. Streptococci can be located both extracellularly and intracellularly of alveolar epithelial cells, macrophages and neutrophils. The organism is also present in lymphatic vessels and lymph nodes and attaches to ductular epithelium (Thomas et al., 1994; Pedersen et al., 2003). The severity of the mastitis varies greatly from chronic infection with only slight inflammatory response to changes of udders and milk (Leigh, 1999). This variation is determined by strain virulence, number of infecting organisms, season, immune status of the cow, parity, and stage of lactation. Infections are more common during the nonlactating period, especially during the early nonlactating period and near parturition (Oliver, 1988).

Several possible *S. uberis* virulence factors have been postulated. The plasminogen activator is considered to be one of the most well characterized virulence factors (Johnsen et al., 1999; Leigh, 1993; Rosey et al., 1999; Ward et al., 2003). Additional virulence factors such as hyaluronic acid (Ward et al., 2001), CAMP factor (Hassan et al., 2000; Jiang et al., 1996; Skalka et al., 1980), hyaluronidase (Khan et al., 2003; Schaufuss et al., 1989), lactoferrin binding protein (Lbp) (Moshynskyy et al., 2003) and *S. uberis* adhesion molecule (SUAM) (Almeida et al., 2006) have also been proposed.

The acquisition of nutrients and the growth of *S. uberis* in the mammary gland

Plasminogen activator (PauA)

Streptococcus uberis is highly auxotrophic and depends on acquisition of several amino acids, vitamins, trace elements and a carbohydrate source successfully to grow (Leigh, 1994a). Because lactating mammary gland is deficient in free and peptide-associated amino acids (Aston, 1975), *S. uberis* must possess a mechanism to utilize proteins found in mammary gland as its sources of amino acids required for growth.

Casein is the most abundant protein found in milk secretion. Plasmin is a proteolytic enzyme capable to digest casein into amino acids found in animals (Leigh and Lincoln, 1997). Several bacteria included *S. uberis* can convert plasminogen to plasmin via the activity of a plasminogen activator known as PauA (Leigh, 2000; Leigh et al., 1999). However, in a more recent study, mutational analysis of pauA gene has demonstrated that the activation of plasminogen by PauA does not play a major role in the ability of *S. uberis* to either grow in milk or infect the bovine mammary gland (Ward et al., 2003). Nevertheless, vaccination with PauA not only induced a neutralizing antibody response but also reduced the rate of colonization and resulted in a decreased incidence of disease following experimental challenge with homologous and heterologous strains of *S. uberis* (Leigh, 1999).

Glyceraldehyde-3-phosphate dehydrogenase (GapC)

To initiate the reaction between the bacteria and plasmin, the bacteria need to be close in proximity and attach to the molecule of plasmin. This reaction may be facilitated via a surface protein presented in various gram positive bacteria called GapC which is responsible for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity (Goji et al., 2004; Perez-Casal et al., 2004). Even though the ability to bind plasmin by GapC has never been demonstrated with *S. uberis*, a study showing a protection after vaccination with the S. uberis GapC protein (Fontaine et al., 2002) supports the hypothesis that GapC may play a role in pathogenesis of *S. uberis* infection.

Oligopeptide permease (OppF)

The ability of bacteria to grow in mastitic milk is enhanced by the presence of the caseinolytic enzyme, plasmin (Marshall and Bramley, 1984), which is transformed from plasminogen in the host tissue and requires the presence of bacterial plasminogen activators as described earlier. Following the digestion of casein, amino acids derived from milk casein are uptaken into bacterial cells via some specific transport systems. Smith et al. (2002) successfully isolated and characterized a mutant strain of *S. uberis* lacking ability to utilize a plasmin derived β-casein peptide for the acquisition of methionine. Their findings indicated a role of an oligopeptide permease which is necessary for the utilization of amino acids and essential for the bacterium to achieve an optimal growth in milk (Smith et al., 2002). Even though the oligopeptide permease

gene has never been considered as a virulence factor, its product may be a good candidate for targeting to prevent the growth of *S. uberis* in the mammary gland.

Metal transporter uberis A (MtuA)

Trace elements are another group of nutrients that bacteria require to achieve an optimal growth in the mammary gland, and consequently cause mastitis. Smith et al. (2003) successfully demonstrated an importance for intramammary infection of a lipoprotein receptor antigen, called metal transporter uberis A (MtuA), from *S. uberis*. This protein is responsible for Manganeses uptaking. A lack of ability to grow in raw milk and a failure to infect mammary glands was demonstrated with an mtuA mutant strain of *S. uberis* (Smith et al., 2003). Because the expression of this protein is crucial for infection, it may be considered a virulence factor for infection of *S. uberis*.

Resistant to phagocytosis and the bactericidal activity of neutrophils

Hyaluronic acid capsules (HasA,B,C)

Avoidance of phagocytosis is a hallmark of pathogenesis mechanisms by *S. uberis*. One of important antiphagocytic factors of a bacterium is its capsule. About 50% of *S. uberis* isolates were encapsulated (Mathews et al., 1994b,c) which is primarily composed of hyaluronic acid (Almeida and Oliver, 1993a). The production of hyaluronic acid is controlled by the *hasA, hasB* and *hasC* encoded hyaluronate synthase, UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase respectively (Ward et al.,2001). These gene-products catalyze the reaction to produce hyaluronic acid from N-acetylglucosamine and glucuronic acid (Scott et al., 2003). A mutagenesis study of *S. uberis* has suggested that the presence of both *hasA* and *hasC* are essential for the production of a hyaluronic acid capsule with isolates possessing *hasC*, but lacking *hasA*, unable to produce a capsule (Ward et al., 2001). Almeida and Oliver (1993b) demonstrated significant lower ingesting and killing rates of encapsulated *S. uberis* by macrophages compared to those of non-encapsulated *S. uberis*. In addition, the presence of *hasA* was more prevalent in *S. uberis* associated with clinical mastitis compared to *S. uberis* isolated from environmental sources (Field et al., 2003). The antiphagocytic activity by hyaluronic capsule may be facilitated by blocking Fc receptors on the surface of phagocytic cells (McNeil and Wiebkin, 1989) and by electrostatic repulsion between electronegative materials on both bacterial capsules and phagocyte membranes (Almeida and Oliver, 1993a). Moreover, the hyaluronic acid present in the

streptococcal capsule contains glycans with ß-glycosidic linkages (Almeida and Oliver, 1993), which are similar to those found in animal tissue and undigested by mammalian enzymes (Scott et al., 2003). Therefore, hyaluronic acid capsules can stabilize chains of bacteria and protect cells from being engulfed by phagocytes.

Lactoferrin binding protein (Lbp)

Followed entry of the bacteria into the mammary gland, a rise of lactoferrin, an iron-binding glycoprotein, in mammary secretion is a typical response by the cow host (Moshynskyy et al., 2003; Smith and Oliver, 1981). The biological roles of bovine lactoferrin include amplification of the inflammatory response by promoting adhesion and aggregation of polymorphonuclear leukocytes to the endothelial surface and stimulation of macrophages (Moshynskyy et al., 2003). Moreover, lactoferrin formation restricts the availability of iron for bacterial growth (Hagivara et al., 2003). Thus, the presence of a lactoferrin-specific receptor, termed lactoferrin binding protein (Lbp), is considered to be essential for bacterial pathogenesis and has been investigated as a potential virulence factor (Moshynskyy et al., 2003).

Attachment and invasion of bovine tissues by *S. uberis*

S. uberis adhesion molecule (SUAM)

A possible new virulence factor with an affinity for bovine lactoferrin called *S. uberis* adhesion molecule (SUAM) has been identified (Almeida et al., 2006). This protein is involved in the adherence of *S. uberis* to a bovine mammary epithelial cell line in vitro. Moreover, inhibition of cell adherence and internalization of *S. uberis* by antibodies against SUAM (Almeida et al., 2006), and by antibodies produced from vaccinated animals using newly developed recombinant SUAM vaccine (Prado et al., 2011) suggesting a role for SUAM during the initial stages of infection and colonization of bovine mammary epithelial cells.

Other potential virulence factor

CAMP factor

Another potential virulence determinant produced by some strains of *S. uberis* is the co-haemolysin or CAMP factor (Skalka, 1980). The production of this activity is restricted to a small proportion of isolates (Khan et al., 2003; Reinoso et al., 2011). The role of this activity in the pathogenesis of bovine mastitis has never been demonstrated.

However, because its protein analog, *S. agalactiae* CAMP factor, is capable of binding IgG and IgM involving in resistant to phagocytosis (Fontaine et al., 2002; Jurgens et al., 1987), CAMP factor is still considered to be a potential virulence factor for *S. uberis*.

Investigation of *S. uberis* virulence factors

Although some antigens derived from potential *S uberis* virulence associated gene products such as PauA and GapC have been used in vaccine trials, such vaccines did not fully protect cows from *S. uberis* challenge (Moshynskyy et al., 2003) which strongly suggests that key *S uberis* virulence factors remain to be determined. A recent study has reported a distribution of virulence patterns of *S. uberis* strains isolated from bovine mastitis cases in Argentina (Reinoso et al., 2011). Although a dominant virulence pattern was found, they could not reveal some classical virulence factors. Distribution of virulence-associated genes among *S. uberis* genomic subtypes and its contribution to the severity of intramammary infection in a cattle population has never been extensively investigated. This knowledge can improve the understanding in epidemiology and pathogenesis of bovine mastitis caused by *S. uberis* which is a major mastitis causing pathogen in Thailand. Moreover, the information from this study may be able to reveal some good candidates for treatment and/or vaccination targeting, leading to an effective control strategies of the disease.

Objectives

1. To investigate the distribution of *S. uberis* subtypes using PFGE technique.
2. To investigate the distribution of previously proposed *S. uberis* virulence-associated factors in the Thailand isolates, primarily from northern Thailand, using PCR. The virulence-associated genes investigated were as follows: *pauA, gapC, oppF, mtuA, hasA, hasB, hasC, lbp, sua,* and *cfu* genes.
3. To determine the association of the PFGE profiles and the presence of virulence-associated genes with severity of infection: clinical, subclinical and latent infection.

Methodology

1. Sample size calculation

Based on the prevalence reported by Boonyayatra et al. (2007), the prevalence of 30% was used for sample size calculation. The population of milking cows in the area proposed to study was 2,000 cows. Accepted error is set at 5% with a 95% confidence level. The sample size was calculated based on the following formula:

$$n = \left(\frac{t \times SD}{L} \right)^2$$

Where:

- t is Student's t-value which was 1.96 based on the 95% confidence interval.
- L was the accepted absolute error or precision which is 5%.

However, the calculated sample size equaled 323, which is larger than 10% of the total population size. Therefore, the correct sample size should be used instead of the original sample size. The correct sample size was estimated using the following formula:

$$n(c) = \frac{n}{1 + f}$$

Where:

- n(c) is the corrected sample size
- n is the originally calculated sample size
- f is the sampling fraction (n/the population size)

The estimated correct sample size was 278, giving the expected of 83 *S. uberis* to be isolated. However, a higher prevalence of *S. uberis* was observed in the study. The expected number of *S. uberis* isolates was met at the lower sample size of milking cows, therefore; only 181 milking cows were enrolled in the study. These cows were from 55 farms in Chiang Mai province, Thailand.

2. Milk sample collection for somatic cell count and microbiological culture.

Quarter milk samples, 645 samples, were aseptically collected from 181 milking cows and divided into 2 fractions; one for somatic cell counting and the other for microbiological culture. Milk somatic cell counts (SCC) were analyzed using Somacount®150 (Bentley Instrument, USA). Ten microliters of milk were cultured on

blood agar with 5% bovine blood. Streptococcal isolates were identified based on colonial appearance, Gram stain reaction and catalase test (National Mastitis Council, 2004). The selected colonies were frozen at -20^{o}C in brain heart infusion broth (BHIB) containing 20% glycerol for further characterization.

3. Severity of infection

All quarter milk samples were classified for severity of infection using the following criteria:

- Clinical infection: abnormal milk appearance with/without other clinical signs such as udder swelling, firmness or fever.
- Subclinical infection: normal milk appearance with somatic cell count $\geq$250,000 cells/ml
- Latent infection: normal milk appearance with somatic cell count < 250,000 cells/ml

4. Identification of *S. uberis*

Genomic DNA was extracted from all streptococcal isolates. All streptococcal isolates were identified using conventional PCR which is specific to amplify a region in the 16S rRNA gene of *S. uberis* (Hassan et al., 2001) using primers as shown in Table 1. For PCR, the reaction mixture (30 µL) contained 1 µL of primers (10 pmol/liter), 0.6 µL of deoxynucleotide triphosphate (10 mMol), 3 µL of PCR buffer with 1.8 µL of $MgCl_2$ (25 mMol), 0.1 µL of Taq DNA polymerase (5 U/µL), and 20.0 µL of distilled water. Finally, 2.5 mL of DNA preparation was added to each reaction tube. The tubes were placed in a thermal cycler with the program as followed; initial denaturation at 94 °C for 60 seconds, 30 cycles of denaturation at 94°C for 60 seconds and annealing at 58°C for 90 seconds and extension at 72°C for 90 seconds. The presence of PCR products were determined by electrophoresis of 12 mL of the reaction product in a 2% agarose gel and stained with ethidium bromide to visualize the PCR products.

5. PCR amplifications of virulence-associated genes

Regions in virulence-associated genes including *pauA, gapC, oppF, mtuA, hasA, hasB, hasC, lbp, sua,* and *cfu* genes were amplified using 3 multiplex PCRs: 1) hasA, hasB, hasC and sua, 2) gapC, lbp, pauA, and 3) oppF, mtuA and cfu. The multiplex PCRs were standardized for the detection of each set of genes. Details of the primer

sequences were shown in Table 1. To amplify the genes, 25 µL of reaction mixture was made containing 20 ng of template DNA, 1 µM of oligonucleotide primers, 0.4 µM of each dNTPs, 1.5 U of Taq polymerase and 1.5 mM $MgCl_2$. The reactions were carried out in a thermal cycler and a negative control was included in each run. The PCR products were revealed on 2% agarose gel at 110 V for 1.5 h.

Table 1. PCR primers, annealing temperature and expected PCR for *Streptococcus uberis* strains

Target gene	Primer sequence (5'-3')	Annealing temperature (^{o}C)	Product size (bp)	References
16S rRNA	CGCATGACAATAGGGTACA GCCTTTAACTTCAGACTTATCA	58	445	Hassan et al. (2001)
hasA	GAAAGGTCTGATGCTGATG TCATCCCCTATGCTTACAG	58	319	Field et al. (2003)
hasB	TCTAGACGCCGATCAAGC TGAATTCCTATGCGTCGATC	58	532	Field et al. (2003)
hasC	TGCTTGGTGACGATTTGATG GTCCAATGATAGCAAGGTCAC	58	225	Field et al. (2003)
gapC	GCTCCTGGTGGAGATGATGT GTCACCAGTGTAAGCGTGGA	55	200	Reinoso et al. (2011)
lbp	TGACCGAAGAACGTGATG AGAGACCACTTGCCACTA	55	570	Tomita (2008)
pauA	TTCACTGCTGTTACATAACTTTGTG CCTTTGAAAGTGATGCTCGTG	55	976	Tomita (2008)
oppF	GGCCTAACCAAAACGAAACA GGCTCTGGAATTGCTGAAAG	54	419	Smith et al. (2002)
sua	ACGCAAGGTGCTCAAGAGTT TGAACAAGCGATTCGTCAAG	58	776	Reinoso et al. (2011)
mtuA	GAACCGCTACCTGAGGATGT ATTTGATCCGGTGTTCCTTC	54	500	Accession number AJ539135.1
cfu	ATCCATTAAAGGGCAAGTCG TTGGTCAACTTGTGCAACTG	54	259	Accession number U34322

6. PFGE analysis

Pulsed-field gel electrophoresis based on the methods previously described (Douglas et al., 2000; Tomita et al., 2008) was performed with all *S. uberis* isolates. All *S. uberis* isolates were incubated in 5 mL of BHIB for 16 h at 37°C with gentle shaking. Two hundred microliters of the broth culture were transferred into a sterile microcentrifuge tube and centrifuged at 13,000 x g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 0.5 ml of Pett IV buffer (10 mM Tris-HCl, pH 7.6, 1M NaCl). The resuspension was mixed with an equal volume of 1% pulse-field certified agarose in Pett IV buffer and carefully loaded into the plug molds. The plug molds were incubated at 37°C overnight in lysis solution (0.2% sodium deoxycholate, 0.5% N-lauryl sarcosine, 1 mg/ml lysozyme, 6 mM Tris-HCl [pH 7.6], 1 M NaCl, 0.1 M EDTA [pH 7.6]). The plugs were subsequently incubated in ESP buffer (1% N-lauryl sarcosine, 1 mg/ml proteinase K, 0.5 M EDTA [pH 8.6]) for 16 hours at 50 °C and then incubated in TE buffer (10 mM Tris-HCl, 10 mM EDTA) with 1 mM phenylmethylsulfonyl fluoride (PMSF) for 2 hours at 37 °C. The plugs werre washed 4 times, 15 min for each time, using TE buffer. The plugs containing *S. uberis* DNA were digested with 40 U of SmaI at 30 °C overnight. After digestion, the plugs were loaded into a 1% agarose gel. The DNA fragments were separated using a contour-clamped homogenous electric field device (CHEF DRII) (Biorad, Hercules, CA) with pulse times of 5 sec to 15 s for 7.7 h and 15 s to 45 s for 9.5 h at 6 V/cm. Gels were stained with ethidium bromide (0.5 μg/ml), and illuminated with an UV light.

7. Analysis of PFGE patterns

All PFGE patterns were interpreted according to the criteria described by Tenover et al. (1995) as shown in Table 2. Isolates with PFGE patterns showing the same number of assigned band-sizes were considered indistinguishable isolates (Tenover et al., 1995). Alll PFGE patterns were analyzed using the Bionumerics® version 7.1 software (Applied Maths BVBA, Kortrijk, Belgium). The gel images were normalized by aligning the bands of the size marker in each gel. The optimization and band position tolerance setting was 0.5%. Similarity of the band patterns was calculated using Pearson's correlation coefficient and then clustered using dendrogram generated by unweighted pair group of arithmetic mean (UPGMA) method.

Table 2. Interpretation of genetic relationship based on numbers of PFGE bands that were different between *S. uberis* isolates (adapted from Tenover et al., 1995).

Interpretation of genetic relationship	Numbers of band differences
Indistinguishable	0
Closely related	2-3
Possibly related	4-6
Different	≥7

8. Statistical analysis

Simpson's index of diversity (D) with 95% confidence intervals (95% CI) of the PFGE techniques were determined as described previously (Grundmann et al., 2001; Hunter and Gaston , 1988). Distributions of virulence-associated genes were presented as percentages (%). Associations of the PFGE profiles and the patterns of virulence-associated genes to severity of *S. uberis* infection were determined using Chi-squared test in R statistical software (R Core Team, 2013) at the statistically significance of $P<0.05$.

Results

A total of 88 *Streptococcus uberis* isolates were cultured from 88 quarter milk samples of 62 milking cows from 24 farms. Twenty-seven percents of isolates (24/88) were from Farm B (Table 3) and 9% (8/88) of isolates were from Farm U. Most *S. uberis* were isolated from subclinical cases of IMI (73%, 64/88). Only 14 *S. uberis* were isolated from clinical IMI, and 10 isolates from latent infection (Table3 and 4).

Distribution of virulence genes

Multiplex PCRs, including 1) for *hasA, hasB, hasC* and *sua* detection, 2) for *gapC, lbp* and *pauA* detection, and 3) for *oppF, mtuA* and *cfu* detection, could amplify the targeted region in each gene of *S. uberis*. PCR products of the three multiplex PCRs were shown in Figure 1 and 2. All isolates carried at least 1 virulence gene. Most *S. uberis* isolates carried 9 virulence-associated genes (30/88), followed by 8 virulence-associated genes (20/88) and 7 virulence-associated genes (14/88) (Table 4). The prevalence of virulence genes varied among clinical, subclinical and latent IMI. The most prevalent virulence-associated gene was *oppF* (94.32%) followed by *hasC* (92.05%) and *mtuA* (92.05%) as shown in Table 3. The *cfu* gene was the least

presented gene among the *S. uberis* isolates (35.23%). The *hasA* and *hasB* genes were always co-presented. The most common virulence pattern was *hasA+hasB+ hasC+sua+ gapC+ lbp+ pauA+ oppF+ mtuA* (34.1%, 30/88) followed by *hasC+sua+gapC+lbp+pauA+ oppF+mtuA+cfu* (20.5%, 18/88), and *hasA+hasB+hasC+sua+gapC+lbp+pauA+oppF+ mtuA+cfu* (9.1%, 8/88).There was no significant association between the presence of each virulence gene and the severity of infection.

Table 3. Patterns of virulence genes of *Streptococcus uberis* isolates associated with clinical, subclinical and latent intramammary infection in dairy cows in Chiang Mai.

Isolate	Severity of infection	Farm	Putative virulence genes detected by PCRs
SB1-005	clinical	O	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-007	subclinical	A	*sua gapC*
SB1-008	clinical	B	*hasC sua gapC lbp pauA oppF mtuA*
SB1-009	subclinical	B	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB1-010	subclinical	B	*hasC sua gapC lbp oppF*
SB1-011	subclinical	B	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB1-012	subclinical	B	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB1-015	subclinical	B	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB1-016	subclinical	B	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*
SB1-017	subclinical	B	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB1-018	subclinical	B	*hasC sua gapC lbp oppF mtuA cfu*
SB1-032	clinical	B	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-033	clinical	Q	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-035	subclinical	N	*gapC*
SB1-039	latent	N	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-040	subclinical	N	*gapC*
SB1-044	subclinical	N	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-045	subclinical	N	*gapC*
SB1-068	subclinical	C	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-070	subclinical	C	*hasA hasB hasC sua gapC pauA oppF mtuA*
SB1-075	subclinical	N	*hasA hasB hasC sua gapC oppF mtuA*

Table 3 (continued). Patterns of virulence genes of *Streptococcus uberis* isolates associated with clinical, subclinical and latent intramammary infection in dairy cows in Chiang Mai.

Isolate	Severity of infection	Farm	Putative virulence genes detected by PCRs
SB1-082	clinical	R	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-088	clinical	N	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-090	subclinical	D	*hasA hasB hasC gapC oppF mtuA*
SB1-091	subclinical	S	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-093	subclinical	T	*hasA hasB hasC sua gapC oppF mtuA*
SB1-095	subclinical	T	*sua*
SB1-096	clinical	E	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-097	subclinical	E	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB1-099	clinical	Y	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-002	subclinical	E	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-004	clinical	U	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-008	clinical	Z	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-020	subclinical	V	*hasC sua gapC lbp oppF mtuA*
SB2-025	subclinical	V	*hasC sua gapC lbp oppF mtuA cfu*
SB2-027	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-028	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-030	latent	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-031	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-032	clinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-033	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-034	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-035	clinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-036	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA*
SB2-038	clinical	P	*hasA hasB hasC gapC oppF mtuA cfu*
SB2-045	clinical	AA	*hasA hasB hasC gapC lbp pauA oppF*
SB2-058	subclinical	G	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-064	subclinical	H	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-065	latent	H	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*

Table 3 (continued). Patterns of virulence genes of *Streptococcus uberis* isolates associated with clinical, subclinical and latent intramammary infection in dairy cows in Chiang Mai.

Isolate	Severity of infection	Farm	Putative virulence genes detected by PCRs
SB2-066	subclinical	H	*hasA hasB hasCgapC lbp pauA oppF mtuA*
SB2-067	subclinical	H	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*
SB2-069	subclinical	H	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-072	latent	H	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-090	subclinical	I	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB2-093	subclinical	I	*hasC sua gapC oppF mtuA*
SB3-005	subclinical	W	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-019	latent	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-020	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-021	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-022	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-023	subclinical	F	*hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-035	latent	S	*oppF mtuA*
SB3-045	latent	S	*hasC sua gapC lbp pauA oppF mtuA*
SB3-049	subclinical	S	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-051	subclinical	J	*hasC sua oppF mtuA*
SB3-056	subclinical	J	*hasA hasB hasC sua oppF mtuA cfu*
SB3-058	subclinical	J	*sua gapC lbp pauA oppF mtuA cfu*
SB3-059	subclinical	J	*hasC sua gapC lbp pauA oppF mtuA*
SB3-061	latent	K	*hasC sua oppF mtuA*
SB3-062	latent	K	*hasC sua gapC oppF mtuA*
SB3-073	subclinical	K	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB3-074	subclinical	K	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB3-076	subclinical	K	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*
SB3-080	subclinical	K	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB3-084	subclinical	K	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB3-089	latent	K	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB4-005	subclinical	L	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*
SB4-006	subclinical	L	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*

Table 3 (continued). Patterns of virulence genes of *Streptococcus uberis* isolates associated with clinical, subclinical and latent intramammary infection in dairy cows in Chiang Mai.

Isolate	Severity of infection	Farm	Putative virulence genes detected by PCRs
SB4-007	subclinical	L	*hasC sua gapC lbp pauA oppF mtuA*
SB4-010	subclinical	L	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB4-012	subclinical	L	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB4-013	subclinical	L	*hasA hasB hasC sua gapC lbp pauA oppF mtuA cfu*
SB4-014	subclinical	L	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB4-022	subclinical	M	*hasA hasB hasC sua oppF mtuA*
SB4-027	subclinical	M	*hasA hasB hasC sua oppF mtuA*
SB4-028	subclinical	M	*hasA hasB hasC sua oppF mtuA*
SB4-036	subclinical	X	*hasA hasB hasC sua gapC lbp pauA oppF mtuA*
SB4-041	subclinical	X	*hasC sua gapC lbp pauA oppF mtuA*

Table 4. Proportions of individual virulence gene of *Streptococcus uberis* isolates associated with clinical, subclinical and latent intramammary infection in dairy cows in Chiang Mai.

Virulence genes	Positive proportion (%)	Severity of infection		
		Clinical infection (14)*	Subclinical infection (64)*	Latent infection (10)*
hasA	49/88 (55.68%)	11	34	4
hasB	49/88 (55.68%)	11	34	4
hasC	81/88 (92.05%)	14	58	9
sua	80/88 (90.91%)	12	59	9
gapC	80/88 (90.91%)	14	58	8
lbp	69/88 (78.41%)	13	49	7
pauA	66/88 (75.00%)	13	46	7
oppF	83/88 (94.32%)	14	59	10
mtuA	81/88 (92.05%)	13	58	10
cfu	31/83 (35.23%)	3	25	3

Table 5. Numbers of *Streptococcus uberis* isolates containing different numbers of virulence genes in their virulence gene profiles.

Numbers of virulence-associated genes	1	2	4	5	6	7	8	9	10
Numbers of isolates	4	2	2	3	5	14	20	30	8

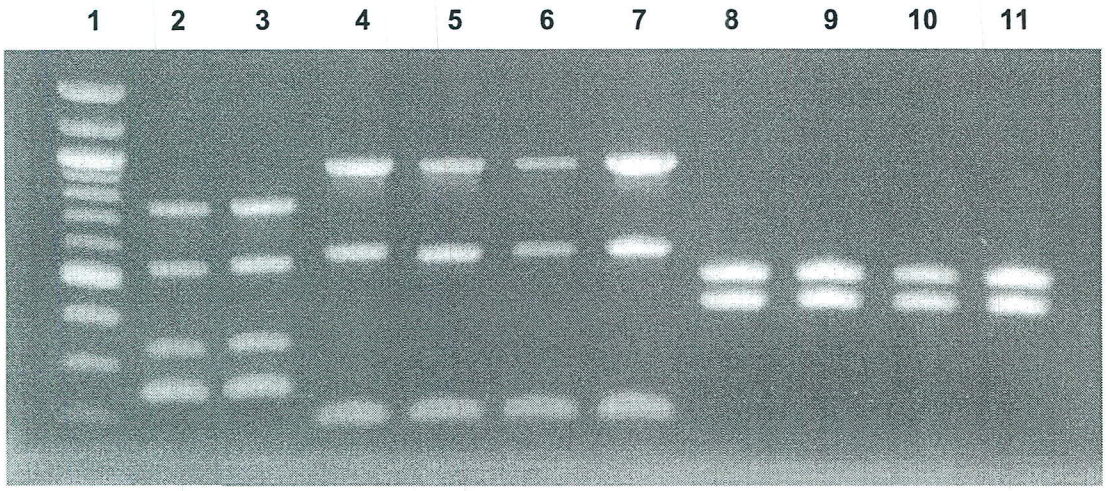


Figure 1. PCR products of virulence genes of *Streptococcus uberis* using three multiplex PCRs. Lane 1; 100 bp molecular ruler, Lane2 and 3; PCR products of *sua* (776 bp), *hasB* (532 bp), *hasA* (319 bp) and *hasC* (225 bp), Lane 4 to 7; PCR products of *pauA* (976 bp), *lbp* (570 bp) and *gapC* (200 bp), Lane 8 to 11; PCR products of *mtuA* (500 bp) and *oppF* (419 bp).

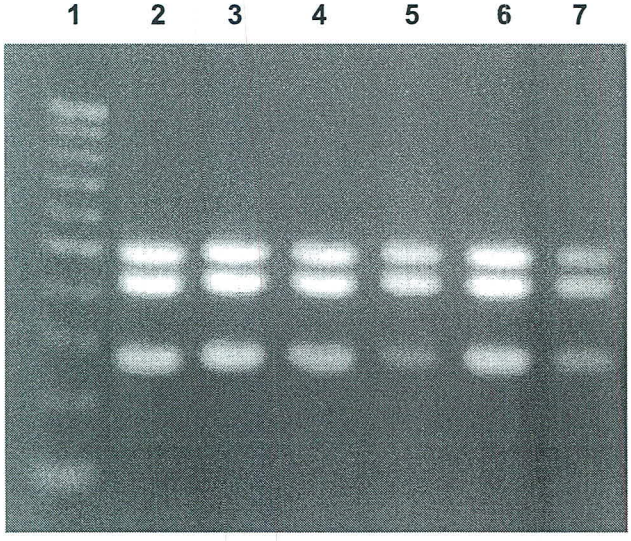
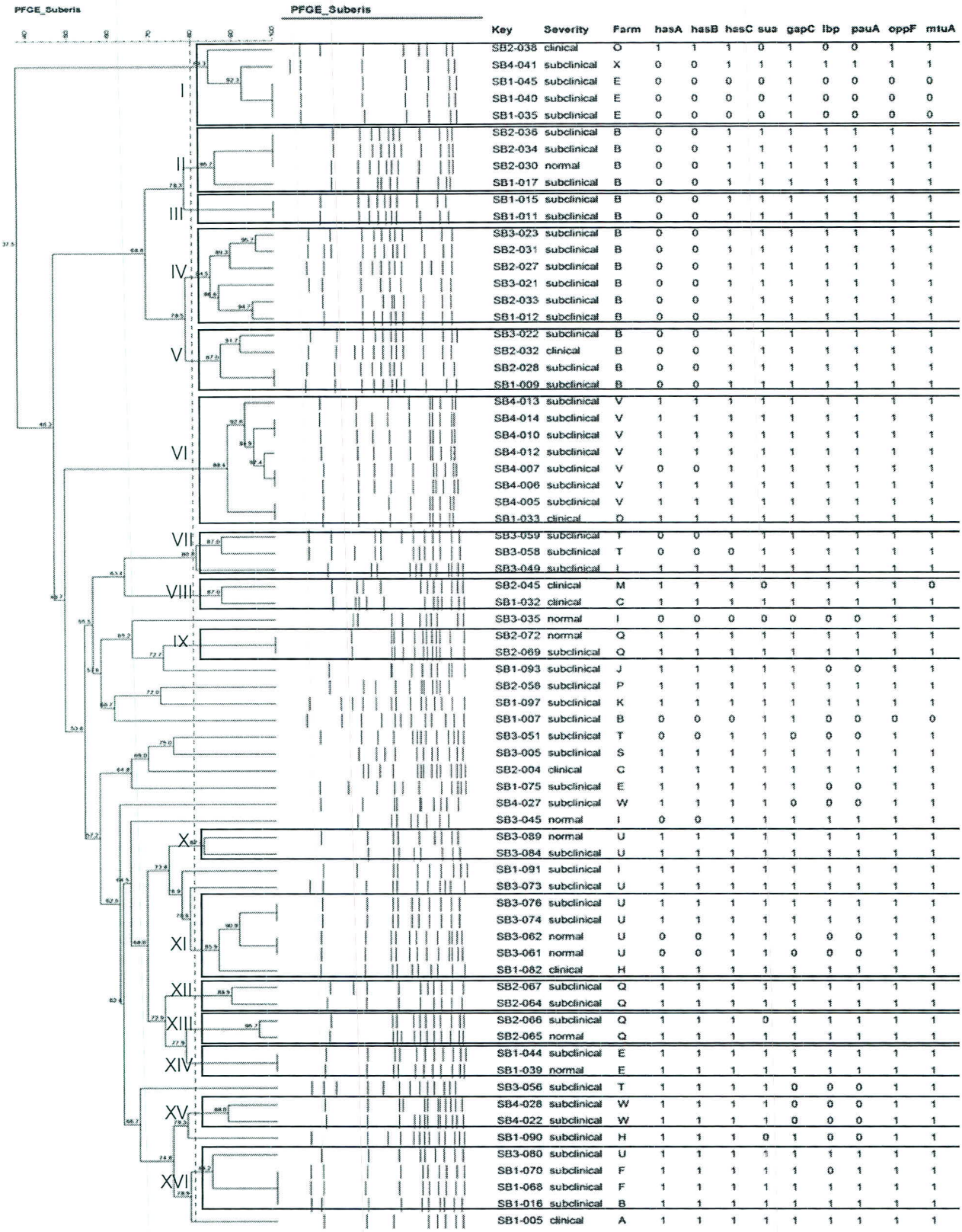


Figure 2. PCR products of virulence genes of *Streptococcus uberis* using the multiplex PCR to amplify *mtuA, oppF* and *cfu* genes. Lane 1; 100 bp molecular ruler, Lane 2 to 7; PCR products of *mtuA* (500 bp), *oppF* (419 bp) and *cfu* (259 bp).

PFGE

PFGEs were performed with only 71 *S. uberis* isolates due to no-growth of 17 isolates when frozen isolates were re-grown in BHIB. PFGE analysis using *SmaI* endonuclease generated 56 distinct electrophoretic profiles composed of 6-14 polymorphic bands ranging from 50 to 580 kb. PFGE typing revealed a genetically diverse population with a Dice similarity index ranging from 40 to 100 %. Simpson's index of diversity (D) of the performed PFGE was 0.993 (0.988-0.998). When the threshold of the Dice similarity index at 80% was used to cluster similar PFGE band patterns, 32 groups of similar-PFGE patterns were generated. Groups of two or more *S. uberis* isolates with similar PFGE patterns were clustered into 16 clusters; cluster I to XVI (Figure 3). Eleven clusters containing 2 or more *S. uberis* isolates from 1 farm included cluster II, III, IV, V, VII, IX, X, XII, XIII, XIV, and XV. There were 5 clusters consisting of *S. uberis* isolates from mixed farms, including cluster I, VI, VIII, XI, and XVI. Regarding the Tenover's criteria as shown in Table 2 (Tenover et al., 1995), PFGE patterns of *S. uberis* isolates in cluster III, VI, IX, XIII, XIV and XVI were considered indistinguishable, whereas those of isolates in cluster I, II, V, X, XII and XV were considered closely related, and those of isolates in cluster IV, VII, VIII and XI were considered possibly related. There were 7 clusters of *S. uberis* isolates that shared identical patterns of virulence genes within each cluster, including cluster III, IV, V, IX, X, XIV and XV.



Key	Severity	Farm	hasA	hasB	hasC	sua	gapC	Ibp	pauA	oppF	mtuA
SB2-038	clinical	O	1	1	1	0	1	0	0	1	1
SB4-041	subclinical	X	0	0	1	1	1	1	1	1	1
SB1-045	subclinical	E	0	0	0	0	1	0	0	0	0
SB1-040	subclinical	E	0	0	0	0	1	0	0	0	0
SB1-035	subclinical	E	0	0	0	0	1	0	0	0	0
SB2-036	subclinical	B	0	0	1	1	1	1	1	1	1
SB2-034	subclinical	B	0	0	1	1	1	1	1	1	1
SB2-030	normal	B	0	0	1	1	1	1	1	1	1
SB1-017	subclinical	B	0	0	1	1	1	1	1	1	1
SB1-015	subclinical	B	0	0	1	1	1	1	1	1	1
SB1-011	subclinical	B	0	0	1	1	1	1	1	1	1
SB3-023	subclinical	B	0	0	1	1	1	1	1	1	1
SB2-031	subclinical	B	0	0	1	1	1	1	1	1	1
SB2-027	subclinical	B	0	0	1	1	1	1	1	1	1
SB3-021	subclinical	B	0	0	1	1	1	1	1	1	1
SB2-033	subclinical	B	0	0	1	1	1	1	1	1	1
SB1-012	subclinical	B	0	0	1	1	1	1	1	1	1
SB3-022	subclinical	B	0	0	1	1	1	1	1	1	1
SB2-032	clinical	B	0	0	1	1	1	1	1	1	1
SB2-028	subclinical	B	0	0	1	1	1	1	1	1	1
SB1-009	subclinical	B	0	0	1	1	1	1	1	1	1
SB4-013	subclinical	V	1	1	1	1	1	1	1	1	1
SB4-014	subclinical	V	1	1	1	1	1	1	1	1	1
SB4-010	subclinical	V	1	1	1	1	1	1	1	1	1
SB4-012	subclinical	V	1	1	1	1	1	1	1	1	1
SB4-007	subclinical	V	0	0	1	1	1	1	1	1	1
SB4-006	subclinical	V	1	1	1	1	1	1	1	1	1
SB4-005	subclinical	V	1	1	1	1	1	1	1	1	1
SB1-033	clinical	D	1	1	1	1	1	1	1	1	1
SB3-059	subclinical	T	0	0	1	1	1	1	1	1	1
SB3-058	subclinical	T	0	0	0	1	1	1	1	1	1
SB3-049	subclinical	I	1	1	1	1	1	1	1	1	1
SB2-045	clinical	M	1	1	1	0	1	1	1	1	0
SB1-032	clinical	C	1	1	1	1	1	1	1	1	1
SB3-035	normal	I	0	0	0	0	0	0	0	1	1
SB2-072	normal	Q	1	1	1	1	1	1	1	1	1
SB2-069	subclinical	Q	1	1	1	1	1	1	1	1	1
SB1-093	subclinical	J	1	1	1	1	1	0	0	1	1
SB2-058	subclinical	P	1	1	1	1	1	1	1	1	1
SB1-097	subclinical	K	1	1	1	1	1	1	1	1	1
SB1-007	subclinical	B	0	0	0	1	1	0	0	0	0
SB3-051	subclinical	T	0	0	1	1	0	0	0	1	1
SB3-005	subclinical	S	1	1	1	1	1	1	1	1	1
SB2-004	clinical	C	1	1	1	1	1	1	1	1	1
SB1-075	subclinical	E	1	1	1	1	1	0	0	1	1
SB4-027	subclinical	W	1	1	1	1	0	0	0	1	1
SB3-045	normal	I	0	0	1	1	1	1	1	1	1
SB3-089	normal	U	1	1	1	1	1	1	1	1	1
SB3-084	subclinical	U	1	1	1	1	1	1	1	1	1
SB1-091	subclinical	I	1	1	1	1	1	1	1	1	1
SB3-073	subclinical	U	1	1	1	1	1	1	1	1	1
SB3-076	subclinical	U	1	1	1	1	1	1	1	1	1
SB3-074	subclinical	U	1	1	1	1	1	1	1	1	1
SB3-062	normal	U	0	0	1	1	1	0	0	1	1
SB3-061	normal	U	0	0	1	1	0	0	0	1	1
SB1-082	clinical	H	1	1	1	1	1	1	1	1	1
SB2-067	subclinical	Q	1	1	1	1	1	1	1	1	1
SB2-064	subclinical	Q	1	1	1	1	1	1	1	1	1
SB2-066	subclinical	Q	1	1	1	0	1	1	1	1	1
SB2-065	normal	Q	1	1	1	1	1	1	1	1	1
SB1-044	subclinical	E	1	1	1	1	1	1	1	1	1
SB1-039	normal	E	1	1	1	1	1	1	1	1	1
SB3-056	subclinical	T	1	1	1	1	0	0	0	1	1
SB4-028	subclinical	W	1	1	1	1	0	0	0	1	1
SB4-022	subclinical	W	1	1	1	1	0	0	0	1	1
SB1-090	subclinical	H	1	1	1	0	1	0	0	1	1
SB3-060	subclinical	U	1	1	1	1	1	1	1	1	1
SB1-070	subclinical	F	1	1	1	1	1	0	1	1	1
SB1-068	subclinical	F	1	1	1	1	1	1	1	1	1
SB1-016	subclinical	B	1	1	1	1	1	1	1	1	1
SB1-005	clinical	A	1	1	1	1	1	1	1	1	1

Figure 3. Dendrogram showing genetic relatedness of the 71 *S. uberis* isolates determined by the unweighted pair group of arithmetic mean (UPGMA) of PFGE patterns. The dash line represents the threshold of 80% Dice similarity index. Roman numerals and boxes denote clusters of isolates sharing band similarities above the threshold.

Discussion

In the present study, the majority of *S. uberis* were isolated from subclinical mastitis cases. Only 16% of isolates were from clinical mastitis cases. These findings strongly agree with a number of previous reports, which described that *S. uberis* IMIs are usually chronic and subclinical (Lerondelle, 1985; Khan et al., 2003). Subclinical mastitis caused by *S. uberis* can be mostly found prior the drying-off period and around parturition, whereas the clinical mastitis cases of *S. uberis* IMI are frequently observed in the first months of lactation (Bramley, 1980; Jayarao et al., 1999). Without any diagnosis and treatment, long term of subclinical infection of *S. uberis* can cause significant losses of milk production to the dairy farms in this region. Identification of *S. uberis* is, therefore, crucial in improving milk production of dairy herds in northern Thailand.

S. uberis is generally identified on the basis of colony morphology on blood agar and esculin hydrolysis. However, additional subsequent technique is usually required to confirm its identification. We use species-specific PCR technique targeting 16S rRNA gene developed by Hassan et al. (2001) for *S. uberis* identification. This technique can rapidly provide unambiguous results to detect *S. uberis*, and can distinguish *S. uberis* from another phenotypically identical species called *S. parauberis* (Hassan et al., 2001). This PCR protocol has been widely used to identify *S. uberis* isolated from mastitis cases prior to further genotyping methods (Gilbert et al., 2006; Tomita et al., 2008).

In Chiang Mai, Thailand, we found that the virulence gene-pattern of *hasA+ hasB+hasC+sua+gapC+lbp+pauA+oppF+mtuA* were the most prevalent pattern among our *S. uberis* collection. These genes encode virulence factors that promote resistance to phagocytosis of neutrophils, attachment and invasion of bovine tissue, the acquisition of nutrients and the growth of *S. uberis* in the mammary gland.

Oligopeptide permease is essential for the utilization of amino acids for bacterial growth and multiplication in milk (Smith et al., 2002). The gene *oppF*, encoding for an oligopeptide permease, mostly presented among the examined isolates (94.32% of the studied isolates). Frequencies of *oppF* detected from *S. uberis* associated with bovine mastitis had been differently reported. A study in 2005 reported an absolute absence of *oppF* gene from 50 *S. uberis* isolated from bulk tank milk samples in New York State, USA, and quarter milk samples from the Netherlands (Zadoks et al., 2005), whereas a higher prevalence of *oppF* gene (64.1%) among 78 *S. uberis* isolates from Argentina was described in 2011 (Reinoso et al., 2011). To our knowledge, we report the highest

prevalence of *oppF* gene among the highest number of examined isolates of *S. uberis* (88 isolates).

The has operon, consisting of *hasAB* gene cluster and *hasC* gene, is responsible for the production of hyaluronic acid capsule of *S. uberis* (Ward et al., 2001). The *hasA* and *hasB* genes were always co-presented, and the *hasABC* was more common genotype compared to the presence of the single *hasC* genotype. However, considering the presence of both *hasABC* and *hasC* alone, *hasC* gene is the second most common virulence gene detected from the studied *S. uberis* isolates. These findings strongly agree with previous reports (Field et al., 2003; Ward et al., 2009). Ward et al. (2009) suggested that *hasC* gene is a common virulence gene of *S. uberis*, and it does not associate with capsule production of *S. uberis*. In addition, they discussed that the hyaluronic acid capsule of *S. uberis* plays only a minor role in the early stages of IMI. Field et al. (2003) also discussed that non-capsulated strains of *S. uberis* is still able to resist to phagocytosis by neutrophils and cause mastitis in dairy cows. However, its role in the pathogenesis of intramammary infection and the reason of highly frequent detection has not clearly been described.

Metal transporter uberis A (MtuA); a lipoprotein receptor antigen (LraI), has been identified to be necessary for growth of *S. uberis* in milk and for IMI in dairy cows (Smith et al., 2003). Without the function of *mtuA*, *S. uberis* is not able to grow in milk (Smith et al., 2003). To our knowledge, we firstly describe the distribution of *mtuA* gene from field isolates of *S. uberis*. A high frequency of *mtuA* detection (92.05%) was observed from our *S. uberis* collection. Our findings confirm that most *S. uberis* associated with bovine mastitis carry *mtuA* gene which may play a crucial role in the pathogenesis.

In the current study, *cfu* gene encoding for CAMP factor are reported to be the least prevalent gene found in our *S. uberis* collection. The distributions of *cfu* gene of *S. uberis* isolates were varying from very low (3.8%) (Khan et al., 2003) to very high (76.9%) (Reinoso et al., 2011). We are reporting a low-to-moderate prevalence of *cfu* gene (35.23%) among the *S. uberis* isolates in Thailand. Therefore, the CAMP factor may be not a major virulence factor contributing to IMI in dairy cows in this region.

We previously reported that *S. uberis* is one of the major pathogens causing bovine mastitis in northern Thailand (Boonyayatra et al., 2007). Since then, *S. uberis* has been continuously isolated from many chronic mastitis cases in this region. However, molecular epidemiological study of this pathogen in this region has never been assessed. We used PFGE to illustrate genotypic patterns of *S. uberis* associated

with bovine mastitis in Chiang Mai, Thailand. Our *S. uberis* collection demonstrated a considerable diversity of genotypes determined by PFGE method. In spite of the variety of PFGE profiles, *S. uberis* included in the study tended to be clustered by farms and were considered to be indistinguishable, closely and possibly genetic related within each farm regarding the Tenover's criteria (Tenover et al., 1995). We decided to use the threshold of 80% of the Dice similarity index to cluster our *S. uberis* isolates because all isolates within the clusters with ≥80% similarity had less than 7 different PFGE bands, and were consequently interpreted to be genetically related based on the Tenover's criteria (Tenover et al., 1995). Most isolates from farm B (16/18) shared high similarity ranged from 78 to 100%, and were clustered into 4 clusters regarding to the 80% threshold; cluster II, III, IV and V. Even though isolates were not identical, they were related and might be genetically mutated from a predominant clone within the farm.

Twelve identical PFGE patterns were detected among the examined isolates in the present study (Figure 3). Most identical PFGE patterns were shared between isolates from the same farms, such as isolate SB1-045, SB1-040 and SB1-035 from farm E in cluster I, and isolate SB2-036, SB2-034, and SB2-030 from farm B in cluster II (Figure 3). These findings suggest that spreading of *S. uberis* from cow-to-cow or from a common environmental source within the farm might have been the mode of transmission. This hypothesis agrees with a number of previous reports demonstrating the persistent infection of *S. uberis* of the same and/or different cows within the same dairy herd (Phuektes et al., 2001; Zadoks et al., 2003, Rato et al., 2008).

In addition to similarities of PFGE patterns within the farm, some common PFGE patterns were observed from *S. uberis* isolates from cows in different farms, such as isolate SB1-070 and SB1-068 from farm F, and SB1-016 from farm B (Figure 3). The explanation of this finding is unclear, but it is probably due to the importation of mastitis cows from one farm to another farm. The infected cows might carry and introduce some different clones of *S. uberis* into the new herd. However, this finding needs to be further investigated.

From our PFGE results, we are able to demonstrate that dominant clones of *S. uberis* exist in some farms in Chiang Mai. These clones may be hypervirulent and be able to be transmitted from cow-to-cow within a farm. However, due to the limitation of variation in severity, we could not identify any PFGE patterns that might be associated with either clinical or subclinical IMI in dairy cattle.