Effects of Medium and High Discomfort Periods during Dry Environment on either Pathogens Causing Subclinical Mastitis or Antimicrobial Resistance of Environmental *Streptococci* and Coagulase-negative *Staphylococci*

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ABSTRACT

The objectives of this study were firstly to compare the prevalence of subclinical mastitis among pathogens between medium and high discomfort periods of the dry season in Chiang Mai, Thailand. Secondly, as the pathogens most frequently responsible for causing mastitis in Thailand, the resistant patterns of both environmental streptococci and coagulase-negative staphylococci (CNS) were also determined for both discomfort periods. Eighty small-holder dairy farms in Chiang Mai province, Thailand, were involved in the study. All clinically-healthy cows in the enrolled farms were used and tested for subclinical mastitis. Milk samples from subclinical mastitis cows were collected for bacteriological identification. Isolates from environmental streptococci and CNS were tested for their antimicrobial susceptibility. The periods were determined by levels of discomfort from heat and humidity, including December to February as medium discomfort period (MEDIUM) and November, March and April as high discomfort periods (HIGH). From a total of 691 cows, 40.1% of cows were positive to California mastitis tests (n=277). At udder level, most pathogens found in this study were minor pathogens, especially environmental streptococci (13.0%, 138 isolates) and CNS (9.9%, 105 isolates). The prevalence of mastitis with environmental streptococci and Staphylococcus aureus in MEDIUM were more than that in HIGH (P<0.05). In contrast, Enterobacteriaceae spp. in HIGH was higher than in the Medium discomfort period (P<0.05). The majority of the environmental streptococci isolates resisted to the antimicrobial agents (97.3%). No association was found between antimicrobial resistance against environmental streptococci and dry-discomfort environmental periods. For CNS, a total of 56% of CNS isolates were resistant to one or more antimicrobial drugs. During MEDIUM discomfort period, CNS was more resistant to cloxacillin and cephalexin than

during HIGH (P<0.01). In conclusion, there are some variations of pathogens causing mastitis and the antimicrobial resistant pattern of antimicrobial drugs against CNS mastitis between Medium and High discomfort periods.

Key words: Antimicrobial resistance, Coagulase-negative staphylococci, Dairy cattle, Dry discomfort environment, Environmental streptococci, Subclinical mastitis

INTRODUCTION

Mastitis is a costly disease in dairy industries (Bartlett et al., 1990), including clinical and subclinical forms. As the most prevalent type of mastitis, the subclinical form causes more damage in terms of overall economical losses. This is especially due to the fact that subclinical mastitis cows can be reservoirs of infection on most farms.

Bacterial intramammary infection is the most common cause of mastitis, and various bacterial species causing mastitis may be to blame, depending on time, geographic area, management and environmental factors. In general, mastitis occurrences were highest during the wet season in many countries such as Thailand (Rojstien et al., 2004), India (Joshi and Gokhale, 2006), Brazil (Costa et al., 1998) and Israel (Shpigel et al., 1998). In Europe, mastitis occurrences were more prevalent during the summer (Green et al., 2006; Olde Riekerink et al., 2007). The major pathogens associated with causing mastitis also differed among seasons (Waage et al., 1999; Osteras et al., 2006). Increases of mastitis occurrences in summer, however, were always associated with wet environments such as muddy areas (Suriyasathaporn et al., 2002). As a type of environmental streptococci, *Streptococcus uberis* contamination of paddocks and muddy places increased when wet conditions prevailed and when the cows' grazing density was higher (Osteras et al., 2006). In addition, Zadok et al., (2005) showed that the proportion of fecal samples containing *Streptococcus uberis* was highest during the summer grazing season. Therefore, it is controversial whether or not hot environment without any wet environment (or dry season) is related to mastitis occurrences and the type of pathogens causing mastitis.

As a tropical country, Thailand has an average precipitation of a very high level during rainy season (above 100 mm) in comparison to European countries that have year-round averages at about 50 mm, even during their wet summer season (BBC weather, 2007). During dry season in Chiang Mai, Thailand, precipitation averages are very low and range between 0 to 30 mm (BBC Weather, 2007). This dry season can be separated into 2 periods, based on levels of discomfort from heat and humidity as MEDIUM (December-February) and HIGH (November, March and April) discomfort periods (BBC weather, 2007). Climate details during both discomfort periods of year 2007 are described in Table 1. Based on data from Table 1, maximum and minimum temperature averages in MEDIUM (29.7°C and 14.0°C, respectively) were lower than HIGH (33.3°C and 19.3°C, respectively). During the HIGH discomfort period, though not for MEDIUM, the mean temperature-humidity-index (THI) is always above 78 (Suriyasathaporn et al., 2006), exceeding the critical THI point for lactating cows at 72 (Armstrong, 1994). Cows with heat stress were shown to have increased shedding of *Enterobacteriaceae* spp. such as *E. coli* (Edrington et al., 2004). Thus, the HIGH discomfort period might have differences with regard to the pathogens causing subclinical mastitis compared to the MEDIUM period. Therefore, the first goal of this study was to compare the prevalence of subclinical mastitis among pathogens between medium and high discomfort periods of the dry season in Chiang Mai, Thailand.

In Thailand, groups of pathogens such as CNS and environmental streptococci become dominant pathogens for subclinical mastitis (Ajariyakhajorn et al., 2003; Boonyayatra and Chaisri, 2004). Because antimicrobial drugs play an important role for the treatment and control of mastitis, therapy decisions are usually based on previous susceptibility information for the herd. The different susceptibility patterns among various mastitis pathogens have been widely reported, including groups of pathogens like CNS and environmental streptococci (Gentilini et al., 2002; Pikala et al., 2004; Mekonnen et al., 2005; Pol and Ruegg, 2007). However, information with regard to the seasonal differences in susceptibility or resistant patterns of both mastitis pathogens in the small-holder dairy farms in this area is limited. With regard to dry season variations, the second goal of this study was to determine the resistant patterns of antibacterial agents for both mastitis pathogens.

MATERIALS AND METHODS

Animal and sample collection

The study was performed during November 2004 to April 2005, using cows from small-holder dairy farms in Chiang Mai province, Thailand. All farms were members of their local dairy cooperatives, and farmers enrolled to participate into the study. All farms had approximately 5 to 15 milking cows housed in their tied-stall barns. For each farm, all clinically healthy lactating cows were tested, using the California Mastitis Test (CMT). The results were interpreted as follows: score 0 = no reaction; trace = slight slime that disappears with continued swirling; +1 = distinct slime but without gel formation; +2 = immediate formation of gel which moves as a mass during swirling; and +3 = gel develops a convex surface and adheres to the bottom of the paddle. A cow with a CMT score of ≥+1 at least one quarter was identified as a subclinical mastitis cow, and was included in the study. Milk samples from all quarters of the subclinical mastitis cows were separately collected with aseptic techniques in accordance with National Mastitis Council guidelines (NMC, 1999). The samples were kept in cool temperatures and transported to the laboratory immediately for bacterial identification.

Bacterial identification

Bacterial identification was performed according to the standard procedure described by National Mastitis Council's guidelines (NMC, 1999). Ten microliters of an individual quarter milk sample was cultured on either a 5% bovine blood agar plate or a MacConkey agar plate. Plates were incubated at 37°C for 24-48 hours. Bacterial colonies were identified based on gross morphology, number of colonies and hemolytic pattern. Appropriate tests were performed on the isolated colonies to identify pathogens, including Gram staining and a catalase test to identify between streptococci and staphylococci. The hemolytic patterns and coagulase reaction with rabbit plasma were used to identify between *S. aureus* and CNS. Esculin hydrolysis and CAMP reaction were used to differentiate *S. agalactiae* and environmental streptococci. *Arcanobacterium* spp. was identified by using culture characteristic on blood agar, motility and catalase reaction test. Gram-negative bacteria were identified as *Enterobacteriaceae* spp., using culture morphology on MacConkey agar (Merck, Germany), lactose fermentation, motility and reaction in triple sugar iron. Other colony types were grouped as other microorganisms. The degrees of confidence in diagnosing an infection were classified as not significant, questionably significant, probably significant and highly significant, based on the National Mastitis Council's guidelines (NMC, 1999). Samples that contained three or more bacterial species were considered to be contaminated. Isolates of either *S. agalactiae* or *S. aureus*, however, were always defined as intramammary infection (NMC, 1999).

Susceptibility testing

The highly significant isolates were tested for antibiotic susceptibility by the agar disk diffusion method in accordance with the standard procedure set forth by NMC guidelines (NMC, 1999). Firstly, all isolates were checked for purity by subculturing on proper media. Three to five colonies of pure isolated pathogens were picked up and suspended in trypticase soy broth and incubated at 37°C for 2-8 hours to increase amounts of bacteria. The standard turbidity of bacterial suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. The entire surface of agar plates was inoculated by using a sterile cotton swab. Commercially-prepared antimicrobial sensitivity discs, having the following antimicrobial agents and concentrations, were used: ampicillin (10 μg), cloxacillin (30 μg), cephalexin (30 μg), gentamicin (10 μg), erythromycin (10 μg), tetracycline (30 μg) and sulfa-trimethroprim (10 μg). Most of them were in the range of minimal inhibitory concentration (MIC) levels at which an isolate was considered susceptible according to Clinical and Laboratory Standards Institute guidelines (Pol and Ruegg, 2007). Discs were placed onto the agar surface and gently pressed to ensure contact. Plates were then incubated at 37°C for 24 hours. Subsequently, the diameter of the zone of inhibition around the disc was measured. The isolated microorganisms were categorized by susceptibility and resistance according to methods and criteria described by the National Committee for Clinical Laboratory Standards (NCCLS, 2002).

Statistical analyses

Contaminated milk samples were excluded from statistical analysis. Discomfort periods were defined by date during the collection of milk samples.

The periods were determined by levels of discomfort due to heat and humidity (BBC weather, 2007) including December to February as a medium discomfort period (MEDIUM) and November, March and April as a high discomfort period (HIGH). A summary of weather information on Chiang Mai is shown in Table 1. Frequencies of subclinical mastitis among pathogens and the resistant patterns were described as percentage. Effects of dry-discomfort periods on bacterial resistance were analyzed separately for each antimicrobial. The Fisher exact chi-square tests were used to evaluate the association of the dry-discomfort periods with either subclinical mastitis occurrence among pathogens or antimicrobial resistant pattern for both environmental streptococci and CNS. The significant levels were defined as $P<0.05$.

Table 1. Annual average of parameters on climate of Chiang Mai province, Thailand (BBC weather, 2007).

Month	Average Sunlight (hours)	Temperature Average (°C)		Discomfort from heat and humidity	Relative humidity (%)		Average Precipitation (mm)	Wet Days (+0.25 mm)
		Min	Max		am	pm		
Jan	9	13	29	Medium	96	52	0	0.5
Feb	9	14	32	Medium	93	44	10	1
March	9	17	34	High	88	40	8	2
April	9	22	36	High	88	49	36	5
May	8	23	34	Extreme	90	60	122	12
June	6	23	32	High	92	67	112	15
July	5	23	31	High	94	69	213	21
Aug	4	23	31	High	95	73	193	20
Sept	6	23	31	High	63	72	249	17
Oct	7	21	31	High	96	69	94	8
Nov	8	19	30	High	96	63	31	4
Dec	9	15	28	Medium	96	57	13	2

RESULTS

From a total of 691 cows, 40.1 % of cows were positive to CMT tests (n = 277). Because of collecting management and individual cow factors, only 1,085 milk samples from all subclinical mastitis cows were collected and used for bacterial identification. Approximately 1.9% of the samples (n = 21) were excluded because of bacterial contamination. From a total of 277 cows and 1,064 quarter samples, milk samples from 56.3% of cows (n = 180) and 27.8% of quarters (n = 291) had positive results on bacterial identification. At udder level, most pathogens found in this study were minor pathogens, especially environmental streptococci (13.0%, 138 isolates) and CNS (9.9%, 105 isolates). For major pathogens, *S. aureus* was found only 1.2% (13 isolates) and no *S. agalactiae* isolation was found. Percentages of mastitis pathogens isolated from quarter milk samples divided by the discomfort periods are shown in Table 2. The prevalence of mastitis with environmental streptococci and S. aureus in MEDIUM were more than that in HIGH (P<0.05). In contrast, Enterobacteriaceae spp. in HIGH was higher than in the MEDIUM discomfort period (P<0.05).

Table 2. Percentages of mastitis pathogens isolated from quarter milk samples separated by the discomfort periodsa of Chiang Mai province, Thailand.

Discomfort periods[a]						
	Medium		High			
	n	%b	n	%b	χ^2	P-value[c]
No growth	366	70.7	391	71.6	0.39	0.53
Environmental *Streptococcus* spp.	89	17.2	49	9.0	12.17	<0.01
Coagulase-negative staphylococci	44	8.5	61	11.2	1.54	0.25
Arcanobacterium spp.	7	1.4	15	2.7	2.34	0.14
S. aureus	10	1.9	3	0.5	4.17	0.05
Enterobacteriaceae spp.	2	0.3	13	2.4	7.29	<0.01
Other	0	0	14	2.6	12.89	<0.01
Total	518	100	546	100		

[a]The periods were determined by levels of discomfort from heat and humidity as medium (Dec-Feb) (n=518) and high (Nov, March and April) (n=546) discomfort environments.
[b]A percentage of no growth sample was compared to all positive samples. Percentages of specified pathogen were compared to number of no growth samples.
[c]Associations of pathogens with dry-discomfort periods were separately tested by Fisherís Exact test.

From a total of specified isolations, 81.9% and 70.5% for environmental streptococci (n = 113) and CNS (n=74), respectively, were successfully revived for susceptibility testing. The majority of the environmental streptococci isolates were resistant to the antimicrobial agents (97.3%). For environmental streptococci, percentages of resistance for seven antimicrobial agents in different periods are shown in Figure 1. Most antimicrobial drugs had high levels of resistance (ranges between 63% and 89%), except cephalexin and ampicillin, which had resistance levels of 15% and 27 to 34%, respectively. No association between antimicrobial susceptibilities versus environmental streptococci and dry-discomfort environmental periods was found.

For CNS, a total of 56% of CNS isolates were resistant to one or more antimicrobial drugs. Percentages of resistance for seven antimicrobial agents in different periods are shown in Figure 2. The average of resistance percentages for cloxacillin was highest, and the average resistance for cephalexin was lowest. During MEDIUM discomfort period, CNS resisted more to cloxacillin than HIGH (P<0.01). In addition, a resistance percentage of CNS to cephalexin during MEDIUM tended to be higher than during HIGH (P<0.1). No association between antimicrobial resistances and dry-discomfort periods among other antimicrobial drugs was found.

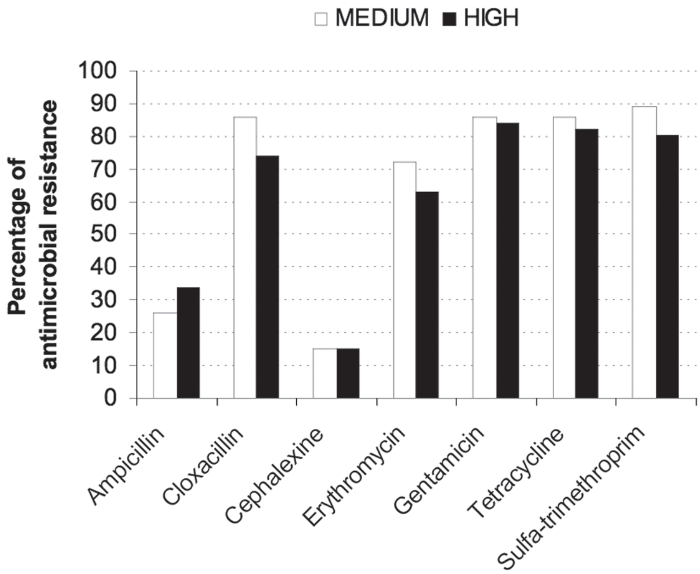

Figure 1. Percentages of antimicrobial resistance for Environmental *Streptococcus* spp. (n=113) separated by dry-discomfort periods. The periods were determined by levels of discomfort from heat and humidity as MEDIUM (Dec-Feb) (n=70) and HIGH (Nov, March and April) (n=43) discomfort environments.

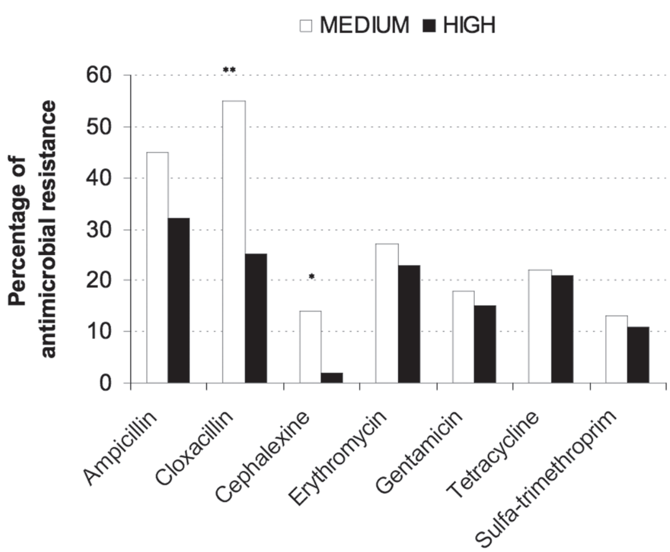

Figure 2. Percentages of antimicrobial resistance for coagulase-negative staphylococci (n=74) separated by dry-discomfort periods. The periods were determined by levels of discomfort from heat and humidity as MEDIUM (Dec-Feb) (n=22) and HIGH (Nov, March and April) (n=52) discomfort environments. *, ** indicated association between dry-discomfort periods and antimicrobial susceptibilities at $P<0.1$ and $P<0.05$, respectively.

DISSCUSSION

The climate of Thailand is influenced by the seasonal monsoon and the local topography. Most areas in Thailand including agricultural areas are recognized as tropical savannah. The tropical savannah climate is characterized by levels of precipitation during three distinct seasons: a hot dry season (March to May), a rainy season (June to October) and cool dry season (November to February). Therefore, the dry discomfort periods defined in this study were in the dry summer and cool seasons. From Table 1, the number of wet days (or precipitation higher than 0.25 mm) during this period are less than or equal to 5 days during the month, indicating that these periods are reasonably dry. Differences between MEDIUM and HIGH discomfort periods are determined by averages of maximum and minimum temperatures and relative humidity of both periods (Table 1). Although November has a low average temperature, the high humidity during the afternoon results in higher discomfort period than other months during the cool dry season in Thailand. To understand the effects of environment on animal production, THI was developed by the US Weather Bureau as a warm-weather discomfort index for evaluating conditions likely to result in livestock stress (Starr, 1981; Johnson, 1991) and is derived from measurements of air temperature and humidity. In this study, THI values during MEDIUM and HIGH using 3-month averages of temperatures and humidity, calculated by using equations from McDowell and colleagues (1979), were 69.3 and 75.9, respectively. This indicates that cows in HIGH were in heat stress status (Armstrong, 1994).

In this study, the prevalence of subclinical mastitis in dry season defined by the CMT test was 40.1%. The cow-prevalence of subclinical mastitis in tropical countries ranged between 40 to 90% including approximately 45% in India (Roman et al., 2000; Joshi and Gokhale, 2006), 38.2% in Ethiopia (Workineh et al., 2002) and ranging between 75.9% (Karimuribo et al., 2006) and 90.3% (Kivaria et al., 2004) in Tanzania. The huge variations among that prevalence might be related to the seasonal variation of the studies. In Chiang Mai, Thailand, Boonyayatra and Chaisri (2004) conducted studies using small-holder dairy farms and found that monthly subclinical mastitis prevalence ranged between 36.4 to 83.3%. By using bulk milk somatic cell count, Rojstien and colleagues (2004) suggested that subclinical mastitis was more severe during the rainy season. In comparison between the medium and high dry discomfort periods, no association was found with the prevalence of subclinical mastitis. In support of a previous study, heat stress did not reduce immune function capacity and did not relate to increased incidence of mastitis during the summer (Elvinger et al., 1991).

The highest prevalence of subclinical mastitis found in this study were environmental streptococci (13.0%, 138 isolates) and CNS (9.9%, 105 isolates), with low prevalence of subclinical mastitis from major pathogens such as *S. aureus* and *S. agalactiae*. This result was in agreement with previous reports in Thailand that both pathogens were the most frequent isolates (Ajariyakhajorn et al., 2003; Boonyayatra and Chaisri, 2004). A mastitis survey in Thailand showed that the most frequently found to cause mastitis during dry period was CNS (Leesirikul et al., 1994). In contrast, *S. aureus* was the most common cause of subclinical mastitis in northeastern Thailand, Ethiopia and Kenya (Aiumlamai et al., 2000; Mekonnen et al., 2003; Shitandi and Kihumbu, 2004, respectively). However, CNS (Waage et al., 1999; Pitkälä et al., 2004; Rajala-Schulz et al., 2004) and environmental streptococci, especially for subclinical cases (Jayarao et al., 1999; Dingwell et al., 2004), have become the predominant pathogens for mastitis in many western countries. In Chiang Mai, the high emphasis on a mastitis control program by its cooperatives and university staffs could result in changing major mastitis pathogens in this area. In addition, the selected dry period in this study might cause the differences in pathogens causing mastitis.

From Table 2, the prevalence of mastitis with environmental streptococci and S. aureus in MEDIUM was more than that in HIGH. It is quite difficult to compare our results with previous studies because our environmental temperatures were relatively high and levels of precipitation were low (Table 1). Regardless of average precipitations, a study in Ohio where the average precipitation is the same year-round showed that the rate of environmental streptococcal intramammary infection (IMI) during a cow's dry period and during lactation was greatest during the summer (Todhunter et al., 1995), when the average maximum and minimum temperatures range between 23 to 28°C and 11 to 16°C, respectively, which is comparable to the temperature range in the MEDIUM discomfort period in this study.

For *S. aureus*, many studies showed that warmer season did not reflect an increased prevalence of intramammary infection (IMI) or/and mastitis. For example, in Louisiana, the prevalence of *S. aureus* intramammary infection in breeding age heifers was much greater in fall than in summer (Fox et al., 1995). Data from Fox and Hancock (1989) showed an increased prevalence of *S. aureus* IMI during acute cold weather, indicating that season influenced the prevalence of IMI. In Thailand, a study using 4-year-old data showed that the overall rate of subclinical mastitis was highest during cooler months (Trisanarom et al., 1994). It is possible that high prevalence of subclinical mastitis in warmer climate might be caused by *Streptococcus* spp. and S. aureus. In contrast, we showed that *Enterobacteriaceae* spp. in HIGH was higher than in the MEDIUM discomfort period ($P < 0.05$). It is quite difficult to compare the prevalence here to other studies because of very high environmental temperatures in this study. However, it is possible that the increase of *Enterobacteriaceae* spp. during HIGH might be caused by increased shedding of *Enterobacteriaceae* spp. such as *E. coli* when cows were experiencing more heat stress (Edrington et al., 2004).

For CNS, the resistant patterns of all antimicrobial drugs were less problematic than environmental streptococci. The majority of the environmental streptococci isolates were resistant to the antimicrobial drugs (97.3%), compared to a lower percentage for CNS (56%) that were resistant to one or more antimicrobial drugs. Differences between resistance levels of these isolates may be caused by the difference of the MIC of antimicrobial for the isolates. Pol and Ruegg (2007) showed higher MIC levels of most antimicrobial versus environmental streptococci than were found in CNS. This resistance percentage of environmental streptococci was higher than those in previous reports (Busato et al., 2000; Erskine et al., 2002;

and Mekonnen et al., 2005). The highest resistance percentage of CNS was to β-lactams antibiotics, which is similar to many previous reports (Owens et al., 1997; Gentilini et al., 2002). Cloxacillin seemed to be the least useful antimicrobial for these isolates, as both isolates were determined to have a high resistance level to it.

In northern Thailand, a limited number of antimicrobial drug groups have been available for intramammary treatment of mastitis, the commercial products such as β-lactams (penicillin, cephalosporin groups, cloxacillin) and aminoglycocides (gentamicin). In addition, cloxacillin represents over 80% of the intramammary drugs available on the market. A wide variety of antimicrobial drugs have been used, often in an indiscriminate and inappropriate manner, impairing the solution of the problem or leading to its aggravation. Moreover, an important problem that arises from this kind of conduct is the increasing occurrence of microbial resistance (Susamo and Ocampo, 1992). Furthermore, the wide use of sulfa-trimethroprim, tetracycline and gentamicin to treat gastro-intestinal and other diseases in cattle has probably aided in developing resistance to these antimicrobial agents.

In this study, we found an association between the discomfort periods and the resistance of subclinical mastitis-causing CNS to the antimicrobial drugs cloxacillin and cephalexin. Resistance levels during the cool season, MEDIUM, in Thailand were higher than in summer, HIGH, for both antimicrobial drugs (Figure 2). Two explanations that might be related to this finding include management factors and biological factors. For the management factors, it is possible that most cows in MEDIUM were just in the early postpartum period when most cows were receiving dry- cow therapy with antimicrobial drugs prior to performing the study. In northern Thailand, most cows are conceived during December to March (Punyapornwithaya et al., 2005). With regard to the biological factors, some studies found some seasonal variations on resistance to antimicrobial drugs. An example might be the huge seasonal association of the prevalence of penicillin-G resistance that was found in both *S. aureus* and CNS (Osteras et al., 2006). Our finding supports this recent finding that CNS is seasonally resistant to cloxacillin and cephalexin, both of which are β-lactams. A higher level of resistance was found more frequently during MEDIUM discomfort period. This finding was in accordance with the study of Osteras et al. (2006), who also found a higher proportional rate of penicillin resistance during the late indoor season. The reason for this is unknown; however, it was so characteristic that it will be important to investigate it in future studies. To our knowledge, there is, at present, no information in the available bovine mastitis literature on the seasonal occurrence of resistant pattern.

In conclusion, environmental streptococci and CNS were the most commonly isolated organisms responsible for subclinical mastitis in this area. During the dry periods (summer and cool-dry season), prevalence of mastitis with environmental streptococci and *S. aureus* in the medium discomfort period, the cool-dry season, was higher than that in the high discomfort period, which refers to the dry part of the summer in Thailand. In contrast, the prevalence of *Enterobacteriaceae* spp.

was higher in the high discomfort period. In Thailand, most of the environmental streptococci isolates were resistant to antimicrobial drugs (97.3%), while this was true for just over half (56%) of CNS isolates. Finally, we found an association between the discomfort periods and the resistance of subclinical mastitis-causing CNS to the antimicrobial drugs cloxacillin and cephalexin. Resistance levels during the cool season, MEDIUM, in Thailand were higher than during the summer, HIGH, for both antimicrobial drugs.

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