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Epidemiology of Subclinical Mastitis and Their Antibacterial Susceptibility in Smallholder Dairy Farms, Chiang Mai Province, Thailand

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Abstract: The objectives of this study were to identify the bacteria causing subclinical mastitis at the beginning of the rainy season to define the factors associated with subclinical mastitis caused by various pathogens and to identify antibiotic susceptibility and resistance. About 42 farms from the Mae-On Dairy Cooperative participating in the Herd Health Management Program (HHPM), Faculty of Veterinary Medicine, Chiang Mai University were included in the study. The study was conducted in June, 2008. From the protocol of HHPM, all farms had to collect a milk sample from each milking cow for measurement of Somatic Cell Counts (SCC) once a month. At the cut-off point of SCC = 200,000 cells mL^{-1} the cows were deemed to haveintramammary infection. Cows in 3 groups were checked for subclinical mastitis in quarter levels using the California mastitis test within 2 weeks after SCC measurement. A cow with CMT score ≥+1 for at least one quarter was identified as a subclinical mastitis cow and was included into the study. Milk samples from subclinical mastitis quarters were collected with aseptic techniques. The fisher exact χ^2-tests were used to evaluate the association of pathogens with antibiotic resistant and the associated factors. The significant levels were defined at $p<0.05$. In total, 133 quarters from 68 cows and 22 dairy farms were included in the study. *C. bovis* (28%) and Coagulase Negative Staphylococci (CNS) (28%) were the main bacteria isolated in this study. *Stap. aureus* (8%) and *St. agalactiae* (2%) as contagious pathogens were at low levels. An occurrence of subclinical mastitis from *S. aureus* was significantly associated with subclinical mastitis status in which most *S. aureus* subclinical mastitis showed chronic status of subclinical mastitis. Most subclinical cases occurred during late lactation (54%). Occurrences of subclinical mastitis from *Stap. aureus* and *C. bovis* were associated with period of lactation ($p<0.05$). About 99 bacterial identifications were used for antibiotic susceptibility test. Bacteria that were significantly associated with resistant patterns were *St. uberis*, *S. aureus* and *S. dysgalactaie*. Subclinical mastitis with *St. uberis* was resistant to most antibiotics ($p<0.05$); subclinical mastitis with *Stap. aureus* and CNS wassusceptible for most antibiotics.

Key words: Subclinical mastitis, pathogens, antibiotic resistant, somatic cell counts, associated factor, chronic status

INTRODUCTION

Mastitis is a costly disease in the dairy industry through decreased production, discard of milk, drug and veterinary expense, extra labor and increased rate of cow replacement (Bartlett *et al*., 1990). A subclinical form is the most prevalent type of mastitis; it can be a reservoir of infection that can spread micro-organisms to other cows and can develop into a clinical case. Bacterial intramammary infection is the most common cause of mastitis. The various bacterial species causing mastitis are different depending on geographic area, management and environment. Many epidemiological studies on mastitis-causing micro-organisms in western countries have reported that the prevalence of the classical contagious bacteria such as *S. aureus*and *St. agalactiae* has decreased but that CNS and *C. bovis* considered to be minor mastitis pathogens have become more common (Makovec and Ruegg, 2003; Osteras *et al*., 2006; Pittkala *et al*., 2004).

Pathogens causing subclinical mastitis are quite different in tropical countries. *S. aureus* has shown more predominance than environmental pathogens (Mekonnen *et al*., 2005; Shitandi and Kihumbu, 2004). Some studies in Thailandhave reported that minor pathogens such as CNS and *Streptococcus* sp. have become dominant pathogens (Ajariyakhajorn *et al*., 2003; Boonyayatra *et al*., 2007; Boonyayatra and Chaisri, 2004). Intramammary Infection (IMI) involvesmany risk factors. The season has been reported as influencing occurrence of IMI in western countries. Thailand has a different climate and different seasonal patterns but information

relating its seasonal risk factor is scant. Antimicrobial agents play an important role in the treatment and control of mastitis. Therapy decisions are usually based on previous susceptibility information for the herd. Susceptibility patterns of antibacterial agents depend on the type of causal micro-organisms. The different resistancepatterns among various mastitis pathogens have been widely reported (Gentilini *et al*., 2002; Mekonnen *et al*., 2005; Pittkala *et al*., 2004). However, information on the susceptible or resistant patterns of mastitis pathogens in the small dairy farm in this area is limited. Therefore, the objectives of this study were to identify the bacteria causing subclinical mastitis at the beginning of the rainy season to define the factors associated with subclinical mastitis caused by various pathogens and to identify the antibiotic susceptibility and resistance.

MATERIALS AND METHODS

Farm selection: About 42 farms from the Mae-On Dairy Cooperative participating in the Herd Health Management Program, Faculty of Veterinary Medicine, Chiang Mai University were included in the study. All farms were small-holder dairy farms with <20 milking cows per farm and using budget-type milking machines with an average of 8 cows/machine set.

Most cows were fed post-harvest corn stem and straw ad lib and concentrates according to their milk production and the vast majority of the cows were crossbred Holstein-Friesian. Veterinarians and para-veterinarians were available on call as health and reproductive consultants (the para-veterinarians were animal health and reproduction volunteers trained by the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand from either the private sector or the Satellite Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University.

Cow and samples collection: The study was conducted in June, 2008. From the protocol of HHPM, all farms had to collect milk samples from each milking cow for measurement of Somatic Cell Counts (SCC) once a month. At the cut-off point of SCC = 200,000 cells mL^{-1} the cows were deemed to have intramammary infection. Based on the results of SCC at May and June, cows were defined into acute stage (ACUTE) for cows that had SCC<200,000 cells mL^{-1} in May and >200,000 cells mL^{-1} in June, chronic stage (CHRONIC) for cows that had SCC>200,000 cells mL^{-1} in both May and June and other stage (OTHER) for cows that had SCC< 200,000 cells mL^{-1} in June but had SCC>200,000 cells for the previous months. Cows in the three groups were checked for subclinical mastitis in quarter levels using California mastitis test within 2 weeks after SCC measurement. The reaction was interpreted as follows: score 0 = no reaction; trace = slight slime which disappears with continued swirling; +1 = distinct slime but without gel formation; +2 = immediate formation of gel which moves as a mass during swirling; +3 = gel develops a convex surface and adheres to the bottom of the paddle.

A cow with a CMT score ≥+1 inat least one quarter was identified as a subclinical mastitis cow and was included in the study. Milk samples from subclinical mastitis quarters were collected with aseptic techniques in accordance with National Mastitis Council guidelines (NMC, 1999). Briefly, an udder and teat were cleaned with disinfectant. The teat ends were scrubbed with cotton balls moistened with 70% ethanol solution until they were no longer visibly dirty. The first stream of milk was discarded before collecting the samples into a sterile test tube. The samples were kept in cool temperature and were transported to the laboratory immediately for bacterial identification. The data on owner, cow identification, quarter, sampling and date of sampling were recorded.

Bacterial identification: Microbiological examination was performed according to the standards described in the National Mastitis Council's guideline (NMC, 1999). About 10 μL of an individual quarter milk sample was cultured on 5% bovine blood agar plates and MacConkey agar plates. Plates were incubated at 37°C for 24-48 h. Bacterial colonies were identified based on gross morphology, number of colonies and hemolytic pattern.

Appropriate tests were performed on the colonies isolated to identify the pathogens including gram staining and a catalase test to distinguish between streptococci and staphylococci. The hemolytic patterns and coagulase reaction with rabbit plasma were used to distinguish between *S. aureus* and CNS and the esculin hydrolysis and CAMP reaction were used to differentiate *Streptococcus agalactiae* and environmental streptococci. *C. bovis* was identified by using culture characteristic on blood agar, motility and catalase reaction test. Gram-negative bacteria were identified to *Enterococci* sp. using culture morphology on MacConkey agar (Merk, Germany), lactose fermentation, motility and reaction in triple sugar iron.

Other colony types were grouped as other micro-organisms. Degree of confidence in diagnosing an infection was classified asnot significant, questionably significant, probably significant or 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. However, isolates of either *St. agalactiae* or *S. aureus* 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 in NMC guideline. Firstly, all isolates were checked for purity by subculturing on proper media. About 3-5 colonies of pure isolated pathogens were picked up and suspended in trypticase soy broth and incubated at 37°C for 2-8 h 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 sterile cotton swab. Disks containing 10 μg of ampicillin, 30 μg of cloxacillin, 30 μg of cephazolin, 10 μg of gentamicin, 10 μg of tetracyclineand 10 μg of sulfa-trimethroprim were placed onto the agar surface and gently pressed to ensure contact. Plates were incubated at 37°C for 24 h. Subsequently, the diameter of the zone of inhibition around the disk was measured. The isolated micro-organisms were categorized to susceptibility and resistance according to methods and criteria described by National Committee for Clinical Laboratory Standards (NCCLS, 2002).

Statistical analyses: Contaminated milk samples were excluded from statistical analysis when quarters with any bacteria were defined as quarters with intramammary infection. Factors associated with subclinical mastitis in the rainy season included status of subclinical mastitis, levels of SCC: (HIGH, SCC in June<400,000 cells cc^{-1} and LOW, SCC in June>400,000 cells cc^{-1}), levels of 4 months averaged SCC ($HIGH_{av}$, SCC in June<400,000 cells cc^{-1} and LOW_{av}, SCC in June>400,000 cells cc^{-1}) and 4 lactation periods: days in milk at <100 (EARLY), 100-200 (MID), >200 (LATE). Frequencies of quarters with subclinical mastitis cows were described as percentages. From the results of susceptibility testing, percentages of resistance for all bacteria were described and analyzed separately.

Antibiotic resistant subclinical mastitis case (RESIST) was defined when the case had <4 antibiotic susceptibility tests out of 6. Effects of associated factors, bacterial resistance and antibiotic susceptibility on cloxacillin, gentamicin, tetracycline and sulfa-trimethroprimgentam as the most used antibiotic for mastitis in this area were analyzed separately for all pathogens including *S. aureus*, *Step. uberis*, *Step. dysgalactiae*, CNS and *C. bovis*. The Fisher exact chi-square tests were used to evaluate the association of pathogens with antibiotic susceptibility, RESIST and the associated factors. The significant levels were defined at $p<0.05$.

RESULTS AND DISCUSSION

In total, 133 quarters from 68 cows and 22 dairy farms were included in the study. Frequencies of bacteria isolation from subclinical mastitis cases during the rainy season were shown in Fig. 1. *C. bovis* (28%) and CNS (28%) were the most common bacteria isolated in this study. *S. aureus* (8%) and *St. agalactiae* (2%) as contagious pathogens were atlow levels. Environmental streptococci including *St. uberis* (5%) and *St. dysgalactiae* (6%) were found at approximately 11%.

Associations of subclinical mastitis status are shown in Table 1. An occurrence of subclinical mastitis from *S. aureus* was significantly associated with subclinical mastitis status in which most *S. aureus* subclinical mastitis showed chronic status. Levels of both recent SCC and averaged SCC were associated with occurrences of *S. aureus*and *St. dysgalactaie*, respectively (Table 2). Cows with recently high SCC (>400,000 cells mL^{-1}) were associated with *Stap. aureus* but cows with low levels (<400,000 cells mL^{-1}) of 4 months averaged SCC were associated with subclinical mastitis from *St. dysgalactaie*. Most subclinical cases occurred during late lactation (54%). Occurrences of subclinical mastitis from *S. aureus* and *C. bovis* were associated with period of lactation ($p<0.05$). Subclinical mastitis from *S. aureus* mostly occurred during mid lactation (20.83%) but from *C. bovis* mostly occurred in late lactation Table 3.

From the overall antibiotic susceptibility testing (n = 99), cephalosporin wasmost susceptible for subclinical mastitis pathogens (95%) excluding 3 cases from the other pathogens group and a case from coliform bacteria. Percentages of antibiotic susceptibility for

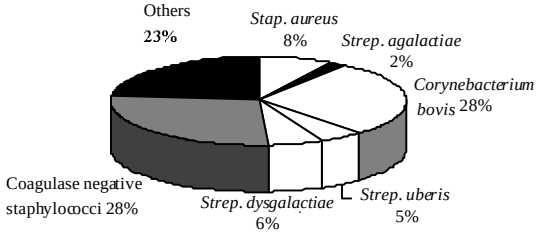

Fig. 1: Distribution of bacterial isolation from subclinical mastitis cases (n = 133)

Table 1: Associations of subclinical mastitis status as CHRONIC, ACUTE and OTHER on occurrences of subclinical mastitis from specified pathogens (n = 111)*

	CHRONIC (N = 46)		ACUTE (N = 25)		OTHER (N = 40)		
Pathogens	n	(%)	n	(%)	n	(%)	p value
S. aureus	7	15.22	1	4.00	0	0	0.02
*St. uberis**	2	4.35	0	4.17	2	5	0.68
St. dysgalactiae	3	6.52	1	5.00	2	5	1.00
CNS	10	21.74	5	20.00	16	40	0.13
C. bovis	12	26.09	10	40.00	12	30	0.47

*Missing data = 22 caused by unspecified classes

Table 2: Associations of recent Somatic Cell Count (SCC) and 4 month averaged SCC on occurrences of subclinical mastitis from specified pathogens (n = 133)

	LOW		HIGH		
Pathogens	n	(%)	n	(%)	p value
Recent somatic cell count*					
S. aureus	3	3.85	8	14.55	0.05
St. uberis	4	5.13	3	5.45	1.00
St. dysgalactiae	6	7.69	2	3.64	0.47
CNS	26	33.33	11	20.00	0.12
C. bovis	21	26.92	16	29.09	1.00
Averaged somatic cell count**					
S. aureus	6	7.14	5	10.20	0.53
St. uberis	4	4.76	3	6.12	0.71
St. dysgalactiae	8	9.52	0	0.00	0.03
CNS	27	32.14	10	20.41	0.16
C. bovis	20	23.81	17	34.69	0.23

*Total number of low (<400,000 cells mL^{-1}) and high (>400,000 cells mL^{-1}) were 78 and 55, respectively. **Total number of low (<400,000 cells mL^{-1}) and high (>400,000 cells mL^{-1}) were 84 and 49, respectively

Table 3: Effects of period of lactation as days in milk <100 (early), 100-200 (mid) and >200 (late) on occurrences of subclinical mastitis from specified pathogens

	Early (N = 33)		Mid (N = 24)		Late (N = 72)		
Species	n	(%)	n	(%)	n	(%)	p-value
S. aureus	3	9.09	5	20.83	3	4.00	0.02
*St. uberis**	3	9.09	1	4.17	3	4.00	0.50
St. dysgalactiae	4	12.12	0	0.00	4	5.33	0.18
CNS	11	33.33	6	25.00	20	26.67	0.75
C. bovis	4	12.12	5	20.83	27	36.00	0.02

*missing value = 1

Table 4: Percentages of antibiotic susceptibility on pathogens causing subclinical mastitis (n = 99)

	S. aureus		*St. uberis*		*St. dysgalactiae*		CNS		*C. bovis*	
Antibiotics	S	R	S	R	S	R	S	R	S	R
Cloxacillin	23.4**	0.0**	2.1*	11.5*	10.6	3.9	20.3	19.2	17.0	28.9
Gentamicin	17.5**	2.4**	1.8**	14.3**	7.0	7.1	33.3**	2.4**	28.1	16.7
Oxytetracycline	20.0**	3.7**	2.2*	11.1*	2.2*	11.1*	37.8**	5.6**	17.8	27.8
Sulfa-trimetroprim	24.4**	0.0**	2.2*	11.1*	2.2*	11.1*	28.9**	13.0**	33.3	14.8
At least 4 susceptible out of 6 antibiotics	23.4**	0.0**	0.0**	13.5**	6.4	7.7	31.9**	9.6**	23.4	23.1

ampicillin, cloxacillin, tetracycline, gentamycin and sulfa-trimetroprim were 55.1, 47.5, 45.5, 57.6 and 45.5%, respectively. Therefore, the efficiency of cephalosporin in this study was 100% susceptible for *St. uberis*, *S. aureus*, CNS, *St. dysgalactiae* and *C. bovis*. For ampicillin, there was no significant association between antibiotic susceptibility on occurrences of subclinical mastitis from specified pathogens. Associations between antibiotic susceptibility for cloxacillin, gentamicin, tetracycline and sulfa-trimetrophim on pathogens causing subclinical mastitis were shown in Table 4. Most antibiotics tested here except cloxacillin were susceptible to *S. aureus* causing subclinical mastitis. Except for cephalosporin, most CNS were susceptible to gentamicin ($p<0.01$) and tetracycline ($p<0.01$) and *C. bovis* was susceptible to sulfatrimetroprim. Bacteria that were significantly associated with the resistant pattern (4 susceptible out of 6 antibiotics) were *St. uberis*, *Staph. aureus* and *St. dysgalactaie*. Subclinical mastitis with *St. uberis* was resistant to most antibiotics ($p<0.05$); subclinical mastitis with *S. aureus* and CNS were susceptible for most antibiotics. Environmental *C. bovis* (28%) and CNS (28%) were the most commonly isolated bacteriain this study. This is in agreement with previous studies in USA and Europewhere CNS, *S.* sp. which is not *S. aureus* were the predominant bacteria causing mastitis

(Makovec and Ruegg, 2003; Pittkala *et al*., 2004). In a recent study on dairy cows in Brandenburg, Germany, CNS and *C. bovis* were the pathogens most frequently isolated in the study on prevalence of mastitis pathogens and their resistance against antimicrobial agents. There is disagreement with many reports on studies in Thailand (Ajariyakhajorn *et al*., 2003; Boonyayatra *et al*., 2007; Boonyayatra and Chaisri, 2004). However in Northeastern Thailand, Ethiopia and Kenya, *S. aureus* was the most common cause of subclinical mastitis (Mekonnen *et al*., 2005; Shitandi and Kihumbu, 2004). CNS and *C. bovis* were common isolated bacteria in the current study. These results were similar to the previous report from Finland (Pittkala *et al*., 2004).

Subclinical mastitis cases from *S. aureus* were mostly chronic caseshaving an increase of SCC >200,000 cells mL^{-1} for 2 months. In the study, we also found the differences of SCC on mastitis pathogen causing subclinical mastitis (Table 2). Cows with recently high SCC (>400,000 cells mL^{-1}) were associated with *S. aureus* but cows with low levels (<400,000 cells mL^{-1}) of 4 months averaged SCC were associated with subclinical mastitis from *St. dysgalactaie*. In the view, there is a limited number of studies on pathogen-specific subclinical mastitis and SCC. Few studies have investigated pathogen-specific clinical mastitis and its effects on SCC curves.

However, De Haas *et al*. (2004) estimated associations between pathogen-specific cases of clinical mastitis and SCC patterns based on deviations from a typical curve for SCC during lactation. They found that clinical *E. coli* mastitis was associated with short peaks in SCC whereas *S. aureus* was associated with long-lasting increases in SCC. No particular SCC patterns were found for *St. dysgalactiae* and *St. uberis*. Chaffer *et al*. (1999) found that CNS were associated with increased SCC and caused chronic infections in heifers similar to those caused by *S. aureus*. These results indicate that breeding for lower SCC is more likely to reduce the number of clinical mastitis due to bacteria such as *S. aureus* and CNS as against bacteria such as *E. coli*.

In this study, *S. aureus* was likely to be susceptible to most antibiotics testing *in vitro*. Selection of antibiotics for treatment based on *in vitro* susceptibility to antibiotics is no guarantee for treatment success *in vivo*. According to one study *in vitro* testing can be used as a predictor for cure for *S. aureus* infections of <2 weeks duration but not for chronic intramammary infection (the intramammary infection of >4 weeks (Owens *et al*., 1997). *S. aureus* is susceptible to a variety of antibiotics *in vitro*. However, farmers often complain that *in vivo* cure rates are disappointing. Several factors including the ability of *S. aureus* to survive inside neutrophils (Mullarky *et al*., 2001; Yancey *et al*., 1991) to form small-colony variants or L-forms (Brouillette *et al*., 2004; Owens and Nickerson, 1989) to induce fibrosis and formation of microabscesses (Erskine *et al*., 2003; Sordillo *et al*., 1989; Ziv and Storper, 1985) and to invade into mammary epithelial cells (Dego *et al*., 2002; Lammers *et al*., 2000) are potential contributors to the poor response of chronic *S. aureus* to antimicrobial treatment. Excepting for cephalosporin, antibiotic susceptibility tests showed that CNS was more susceptible for gentamicin and tetracycline. For environmental streptococci especially *St. uberis*, most antimicrobials used in this study had high levels of resistance (Table 4).

These results are higher than those in previous reports (Busato *et al*., 2000; Erskine *et al*., 2002; Mekonnen *et al*., 2005). The high percentage of resistance to penicillin is probably related to its being the most common antibiotic available on themarket and consequently the first choice. Furthermore, widely used sulfa-trimethroprim, tetracycline and gentamicin to treat gastro-intestinal and other diseases in cattle have probably developed the resistance of these antimicrobial agents.

CONCLUSION

The observation of subclinical mastitis during the rainy season has shown that most pathogens isolated were coagulase negative staphylococci and *C. bovis*. Occurrences of subclinical mastitis from *S. aureus* were associated with increased SCC >200,000 cells mL^{-1} for 2 months as chronic intramammary infection and also with increased recent SCC >400,000 cells mL^{-1}. *St. dysgalactiae* was associated with increase of 4 month averaged SCC >400,000 cells mL^{-1}. Most mastitis pathogens were susceptible to cephalosporin. *S. aureus* and CNS were susceptible to most antibiotic testing *in vitro* in this study but Strepuberis was resistant to antibiotic susceptibility testing. Coagulase negative staphylococci were mostly susceptible to gentamicin and tetracycline.

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