International Journal of
Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

International Journal of Dairy Science 9 (3): 89-95, 2014
ISSN 1811-9743 / DOI: 10.3923/ijds.2014.89.95

Association of Intramammary Infection Caused by Biofilm-producing Pathogens with Chronic Mastitis in Dairy Cows

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ABSTRACT

Biofilm is a self-structure of exopolysaccharide with multiple layers of cells. Biofilm formation is associated with a reduced susceptibility to antibiotics, resulting in chronic infections by pathogens. This study aimed to determine the association between intramammary infection with biofilm-producing bacteria and chronic clinical mastitis in dairy cows. Eighty milk samples from acute and chronic clinical mastitis cases were collected from 52 farms in Chiang Mai and Lamphun provinces in northern Thailand. Forty-eight bacterial isolates were identified from the milk samples. The most prevalent isolated bacteria were streptococci, followed by staphylococci and *Escherichia coli*. Among these bacterial isolates, only 14 (29.17%) could produce a biofilm, 10 of these isolates were from chronic cases and 4 were from acute cases. There was no statistically significant association between intramammary infection with biofilm-producing bacteria and chronic clinical mastitis. However, biofilm production *in vivo* and its association with the severity of mastitis should be investigated in future studies.

Key words: Biofilm, mastitis, dairy cow

INTRODUCTION

Mastitis is considered the most costly disease in the dairy industry. Bovine mastitis is generally treated with antibiotics and is the most common reason for antibiotic use in dairy herds (Halasa *et al*., 2007). However, the cure rates for bovine mastitis with antibiotic treatment are unsatisfactory, ranging from 0-52% in lactating cows (Owens *et al*., 1997). Treatment of chronic intramammary infection (IMI) or a prolonged high Somatic Cell Count (SCC) in milk is usually not successful (Deluyker *et al*., 1999; Pyorala and Pyorala, 1998; Shephard *et al*., 2000). Understanding the pathogenesis of chronic bovine mastitis is crucial for the development of an effective treatment regimen.

Biofilm is a structured and self-produced exopolysaccharide with multiple layers of cells adhering to a surface (Costerton *et al*., 1999). Biofilm formation contributes to the resistance to antibiotics and host defense mechanisms (Melchior *et al*., 2006; Raza *et al*., 2013). Pathogenic bacteria can produce a biofilm which is recognized as an important virulence factor for several human infectious diseases, such as biliary tract infection with *Escherichia coli*, cystic fibrosis pneumonia caused by *Pseudomonas aeruginosa* and *Burkholderia cepacia* and many nosocomial infections (Costerton *et al*., 1999). Several studies have investigated mastitis-associated bacteria that can produce a biofilm, including *Staphylococcus aureus* (Melchior *et al*., 2009;

Oliveira *et al*., 2006; Fox *et al*., 2005; Vasudevan *et al*., 2003), *S. epidermidis* (Oliveira *et al*., 2006; Simojoki *et al*., 2012) and *Streptococcus uberis* (Varhimo *et al*., 2010). The objectives of the present study were to investigate biofilm formation with bacteria isolated form clinical mastitis and whether the ability to produce a biofilm is associated with chronic bovine mastitis.

MATERIALS AND METHODS

Animals and milk samples: We enrolled in the study lactating cows suspected of mastitis that presented to the Satellite Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand, between July 1, 2012 and February 28, 2013. The cows were from farms located in the Chiang Mai and Lamphun provinces in northern Thailand. In total, 80 cows with mastitis from 52 farms were included in the study. These cows showed signs of clinical mastitis, such as an abnormal milk appearance with or without udder swelling or systemic signs. A veterinarian recorded each cow's history including the duration of clinical signs, previous treatments, lactation number and Days In Milk (DIM). The severity of clinical signs was categorized as either acute or chronic mastitis. Cows showing clinical signs for <5 days without previous antibiotic treatments were identified as acute mastitis cows, whereas cows showing clinical signs ≥5 days with previous antibiotic treatments were identified as chronic mastitis cows. Milk samples were aseptically collected from infected udders and submitted for bacterial culture within 24 h of collection.

Culture condition and bacterial identification: Milk samples were cultured using standard methods (Hogan *et al*., 1999). The bacterial isolates were primarily categorized as staphylococci, streptococci, gram-negative bacteria, or other bacterial species using Gram's staining and catalase reactions. Staphylococci were speciated using the API Staph 32 test (bioMerieux Thailand Ltd., Bangkok, Thailand). The species of streptococci were identified based on the results of biochemical tests, including a CAMP test, esculin and hippurate hydrolysis and fermentation of inulin, raffinose, salicin and mannitol. Gram-negative bacteria were speciated based on the results of an oxidase reaction, Triple Sugar Iron (TSI), motility, citrate and urea tests. After speciation, bacterial isolates were kept in Brain Heart Infusion broth (BHI) with 10% glycerol (v/v) at -80°C until use.

Biofilm assay: The tissue culture plate biofilm assay was adapted from the method previously described by Christensen *et al*. (1985). Isolates of staphylococci, streptococci and gram-negative bacteria were incubated at 37°C for 18 h in Tryptone Soy Broth (TSB), Todd-Hewitt broth with 1% (w/v) yeast extract (THY) and Luria-Bertani (LB) broth, respectively. Five microliter of cell suspension from each isolate was transferred into each well of a "U" bottom polystyrene tissue culture plate containing 195 μL of TSB with 0.25% (w/v) glucose, THY with 0.25% (w/v) glucose and AB-minimal medium with 0.45% (w/v) glucose for staphylococci, streptococci and gram-negative bacteria, respectively. The plates were then incubated at 37°C for 18 h. The wells were washed 3 times with phosphate buffered saline (PBS, pH 7.0), dried at room temperature for 2 h, stained with 0.5% crystal violet for 1 min and washed with PBS until a clear PBS rinse was observed. Wells were dried 1 h before the absorbance at OD 570 nm was measured using a microplate reader. Wells with uninoculated culture media were used as blanks. *S. aureus* DMST 4745 (ATCC 29123) and *S. epidermidis* DMST 15505 (ATCC 12228; Department of Medical Science, Ministry of Public Health, Thailand) were used as positive and negative controls. Isolates with blank corrected means >0.1 were considered as biofilm producers (Vasudevan *et al*., 2003). The biofilm assay was performed in triplicate for each isolate.

Statistical analysis: The data regarding lactation number was categorized and coded into the following 4 levels: 1 = lactation number 1, 2 = lactation number 2, 3 = lactation number 3 and 4 = lactation number >3. The DIM data was classified and coded into 4 levels; 0 = DIM <101, 1 = DIM <201, 2 = DIM <301 and 3 = DIM >300. Logistic regression model was performed to analyze the associations among factors including lactation number, DIM and IMI with biofilm-producing bacteria (Yes = 1, No = 0) with chronic clinical mastitis (Yes = 1, No = 0) using R (R Core Team, 2013). The p-values less than 0.05 were considered statistically significant.

RESULTS

Bacterial isolation associated with clinical mastitis: Eighty milk samples were submitted for bacterial culture. Thirty-two of these samples were excluded from the study because they showed no growth (21) or yeast infections (11). Consequently, bacterial isolates from only 48 milk samples were investigated for biofilm production. These milk samples included 11 samples from acute mastitis cows and 37 samples from chronic mastitis cows. The most prevalent gram-positive bacteria isolated from the samples were streptococci (26/48) followed by staphylococci (14/48) and *Corynebacterium* spp. (2/48). We found only 6 isolates of gram-negative bacteria which were identified as *E. coli* (Table 1).

Biofilm production: Only 14 bacterial isolates (29.17%) could produce a biofilm in *in vitro*, including 2 staphylococci isolates and 12 streptococci isolates. *E. coli* and *Corynebacterium* spp. isolated in this study could not produce biofilm. The 14 isolates producing biofilm were isolated from milk samples obtained from 4 acute (4/11; 36.36%) and 10 chronic mastitis cows (10/37; 27.03%), as shown in Table 2.

Association between IMI with biofilm-producing bacteria and chronic clinical mastitis: There was no significant association between IMI with biofilm-producing bacteria and chronic clinical mastitis. Neither lactation number nor DIM included in the logistic regression analysis was statistically significant.

Table 1: Identification of bacteria isolated from milk samples of clinical mastitis cows

Identification	No. of isolates	Acute mastitis	Chronic mastitis
Staphylococci			
S. xylosus	4	0	4
S. simulans	1	0	1
S. homonis	2	0	2
S. sciuri	3	0	3
S. epidermidis	2	1	1
S. chromogenes	1	0	1
Other staphylococci	1	0	1
Total	14	1	13
Streptococci			
S. agalactiae	5	1	4
S. uberis	14	5	9
S. dysgalactiae	2	1	1
Other streptococci	4	1	3
Total	26	8	18
Corynebacterium spp.	2	0	2
Escherichia coli	6	2	4

Table 2: Identification of biofilm and non-biofilm producing bacteria isolated from acute and chronic clinical mastitis cases

	Biofilm producers		Non-biofilm producers	
Identification	Acute mastitis	Chronic mastitis	Acute mastitis	Chronic mastitis
Staphylococci	1	1	0	12
Streptococci	3	9	5	9
Escherichia coli	0	0	2	4
Corynebacterium spp.	0	0	0	2
Total	4	10	7	27

DISCUSSION

The most prevalent bacteria isolated in the present study were streptococci, particularly *S. uberis* and *S. agalactiae*. This result is similar to that of a previous study conducted in the same region of Thailand (Boonyayatra *et al*., 2007). Approximately 45% of IMI cases with *S. uberis* can cause clinical mastitis in lactating cows (Todhunter *et al*., 1995). Chronic infection of *S. uberis* can increase SCC for weeks (Wang *et al*., 1999). *S. agalactiae* is a known contagious mastitis pathogen and is usually the cause of subclinical or mild clinical mastitis (Boonyayatra, 2012; Keefe, 1997). However, IMI with *S. agalactiae* can be infinite with a very low self-cure rate and therefore needs to be treated with antibiotics (Keefe, 1997). As a result, IMI with streptococci is probably the major reason for antibiotic use in dairy herds in northern Thailand.

Staphylococci identified in the current study were Coagulase-Negative Staphylococci (CNS). The CNS can be isolated from the teat end and udder skin of dairy cows and can opportunistically infect mammary glands during either milking or non-milking periods. About half of all CNS infections are chronic (Taponen *et al*., 2007). Staphylococcal species frequently isolated from milk included *S. chromogenes* (Todhunter *et al*., 1993; Taponen *et al*., 2007), *S. simulans* (Aarestrup *et al*., 1999; Taponen *et al*., 2006) and *S. epidermidis* (Myllys, 1995; Thorberg *et al*., 2006, 2009), whereas species infrequently isolated included *S. xylosus* and *S. haemolyticus* (Thorberg *et al*., 2009). Six species were identified in the current study: *S. xylosus* was the most common species followed by *S. sciuri*, *S. homonis* and *S. epidermidis* (Table 1). Such differences in results could be due to differences between the studies' speciation methods. A commercial test kit, API Staph ID 32 test, was used to identify staphylococci. Even though it was recommended by the National Mastitis Council (Hogan *et al*., 1999), a previous study revealed only a limited accuracy when the API was used with staphylococci isolated from milk samples (Park *et al*., 2011).

Biofilm producers associated with clinical mastitis in northern Thailand in the current study were less prevalent than what was previously reported (29.17% vs. 50.33%) (Boonyayatra and Jupia, 2013). The decreased prevalence in the current study could have occurred because milk samples were collected only from clinical mastitis cows, whereas the previous study investigated biofilm formation with bacteria isolated from milk but without specification. Moreover, *S. epidermidis*, the most common CNS species that can produce a biofilm (Simojoki *et al*., 2012; Arciola *et al*., 2001; Los *et al*., 2010), was isolated from only 2 samples in this study. However, a high prevalence of biofilm producers among streptococci isolated from milk was observed not only in the present study but also in previous studies (Boonyayatra and Jupia, 2013; Varhimo *et al*., 2010).

We found no significant association between IMI with biofilm-producing bacteria and chronic clinical mastitis in the current study. This finding agrees with that of a previous study by

Simojoki *et al.* (2012). They investigated the association of slime production by CNS with chronic IMI but could not find a significant association. However, that study used different criteria for chronic infection. In addition, because the current study was conducted with mastitis cases that presented for treatment by veterinarians, a bias towards a higher prevalence of chronic cases than acute cases may have resulted from unsuccessful treatments performed by the farmer. Finally, a limited number of isolates could have affected the present results.

CONCLUSION

In conclusion, this study indicates that the bacteria most commonly associated with bovine clinical mastitis in northern Thailand are streptococci and staphylococci and about one-third of the isolated bacteria were biofilm producers. However, the ability to produce a biofilm *in vitro* was not associated with chronic bovine mastitis. Therefore, biofilm production *in vivo* and its association to the severity of mastitis should be further investigated.

ACKNOWLEDGMENTS

This study was granted by Chiang Mai University. We are grateful to the veterinarians at the Satellite Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University, for their help in collecting samples and the farmers for their contributions.

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