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Antimicrobial Resistance of Biofilm-Forming *Streptococcus agalactiae* Isolated from Bovine Mastitis

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Abstract

Streptococcus agalactiae is a contagious mastitis pathogen commonly found in dairies in northern Thailand. During infection, *S. agalactiae* may form biofilms which is known to be associated with increased antimicrobial resistance of bacteria. We aimed to investigate changes in antimicrobial resistance of biofilm producing *S. agalactiae* associated with bovine mastitis. We measured biofilm formation, Minimum Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC), and Minimum Biofilm Eradication Concentration (MBEC) of 56 archived isolates from bovine milk in Chiang Mai, Thailand. Quantitative biofilm evaluation found no (0%) strong, 21 (37%) moderate, and 30 (54%) weak biofilm producers, as well as 5 (9%) non-biofilm producers. Qualitative biofilm assay found only 11 isolates (20%) to be biofilm producers; these were further investigated for resistance to ampicillin, cloxacillin, cephalexin, gentamicin and tetracycline. All 11 isolates showed higher MBECs compared to MICs and MBCs. Some *S. agalactiae* strains from cows with clinical or subclinical mastitis can produce biofilms *in vitro*, and these appear more resistant to common antibiotics. Such resistance can be an obstacle in the eradication of *S. agalactiae* from infected herds. Determination of biofilm formation by *S. agalactiae* cultured from milk may be useful for creating an effective treatment plan and prognosis of bovine mastitis.

Keywords: Biofilm; *Streptococcus agalactiae;* Antibiotic; Mastitis

Introduction

Streptococcus agalactiae is a pathogen causing subclinical and mild-to-moderate clinical mastitis in dairy cows, which causes significant economic losses to dairy farmers [1]. This microorganism poorly survives in the environment, but can persist indefinitely within the mammary gland. It is therefore the only mastitis pathogen that can be eliminated from a herd using blanket therapy with penicillin or its derivatives [2]. However, eradication from a herd may not be achieved if *S. agalactiae* becomes resistant to the antibiotic used, either by genetic mutation or by production of a coating to shield cells from the antibiotic.

A biofilm is a structured and self-produced exopolysaccharide with multiple layers of cells adhering to a surface [3], which contributes to the resistance to antibiotics and innate host defense mechanisms [4,5]. Biofilm production is an important virulence factor for several human infectious pathogens, such as *Escherichia coli* causing biliary tract infection, *Pseudomonas aeruginosa* and *Burkholderia cepacia* causing cystic fibrosis pneumonia, and other bacteria causing nosocomial infections [3]. Bacteria causing bovine mastitis that can produce biofilms include *Staphylococcus aureus* [6-9], *Staphylococcus epidermidis* [8,10], and *Streptococcus uberis* [11].

S. agalactiae is a very common pathogen in dairy farms in Chiang Mai --a major dairy-producing province of the northern Thailand region -- and moderately resistant to most of the commonly used antibiotics for mastitis [12]. Previously, we investigated the biofilm-producing ability of *Staphylococci* and *Streptococci* strains isolated from bovine mastitis in Chiang Mai, and found that most *S. agalactiae* isolates produce biofilms *in vitro* [13]. The objective of the present study was to investigate changes in antimicrobial resistance of biofilm producing *S. agalactiae* associated with bovine mastitis. We evaluated biofilm formation and compared antimicrobial resistance between planktonic and biofilm-forming phases of *S. agalactiae* isolated from bovine cases of subclinical and clinical mastitis.

Materials and Methods

Bacterial isolates

We selected fifty-six *S. agalactiae* frozen isolates which had been derived from sampled milk from cows with both subclinical and clinical mastitis in Chiang Mai, Thailand. Subclinical mastitis cases were determined using California Mastitis Test performed either by farmers or veterinarians. Clinical mastitis cases were determined when clinical signs including abnormal milk, increased firmness and warmness of udders were examined by veterinarians. The 56 isolates were among a collection of 377 bacterial genera from cows sampled from July 2012 to February 2013 [14]. The selected isolates came from 35 cows on 8 farms. Only 4 isolates were from clinically-infected udders, while 52 were from sub clinically-infected udders. The isolates were re-grown in Brain Heart Infusion (BHI) broth and 5% bovine blood agar (BA). Species identification was performed by standard tests, including Gram's stain, catalase reaction, Christie-Atkins-Munch-Petersen (CAMP) test, hydrolysis of esculin and hippurate, fermentation of inulin, raffinose, salicin and Mannitol [15].

Quantitative evaluation of biofilm formation

One colony of each isolate that grew on BA was cultured in Todd-Hewitt broth with 1% yeast extract (THY broth) and incubated at 37°C for 18-24 h. The inoculated THY broths were then adjusted for

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turbidity to be approximately 0.5 McFarland. Biofilm formation was induced using the Tissue-Culture-Plate (TCP) biofilm assay, adapted from methods described previously [16]. Briefly, 5 µL of cell suspension from each isolate was transferred into each well of a "U-bottom" polystyrene tissue culture plate containing 195 µL of THY with 0.25% (w/v) glucose. 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, and then stained with 0.1% (w/v) crystal violet. After washing with PBS and drying, 200 µL of 95% (v/v) ethanol was added to dissolve the biofilm formed in each well. The absorbance or optical density (OD) at 570 nm was then measured using a microplate reader. Wells with uninoculated culture media were used as blanks. The biofilm assays were performed in triplicate for each isolate and repeated three times. The cut-off OD value (ODc) was defined as the averaged OD of the blanks plus 3 times their standard deviation. The ability to produce biofilm of each *S. agalactiae* isolate was classified according to the following criteria [17]:

OD ≤ ODc=Not a biofilm producer

ODc<OD ≤ 2x ODc=Weak biofilm producer

2x ODc<OD ≤ 4x ODc=Moderate biofilm producer

4x ODc<OD=Strong biofilm producer

Qualitative evaluation of biofilm formation

For qualitative evaluation of biofilm formation, the modified TCP assay was again used. However, in contrast to the quantitative method above, the biofilm formed in each well of the tissue culture plates was stained with 0.25% (w/v) crystal violet for 1 min, and washed with PBS until a clear PBS rinse was observed. Wells were dried 1 h before the absorbance was measured at OD570 nm using a microplate reader. Wells with uninoculated culture media were used as blanks. Isolates with blank-corrected means >0.1 were considered biofilm producers [9]. The biofilm assay was performed in triplicate for each isolate and repeated three times. The positive and negative controls used were *S. aureus* DMST 4745 (ATCC 29123) and *S. epidermidis* DMST 15505 (ATCC 12228; Department of Medical Science, Ministry of Public Health, Thailand), respectively.

Minimum inhibitory and bactericidal concentrations

The MIC and MBC were determined using micro dilutions of ampicillin, cephalexin, cloxacillin, gentamicin and tetracycline by the standard methods of the National Committee on Clinical Laboratory Standards [18]. Different ranges of micro dilutions of each antibiotic were selected according to MICs previously described (Table 1). The MICs were defined as the lowest concentration of antibiotics which inhibited visible growth after 24 h of incubation. The MBCs were determined by inoculating 10 µL from all broth with no visible growth onto BA and incubated at 37°C for 24 h. The MBC was the lowest concentration of antibiotics that inhibited growth on BA.

Antibiotics	MIC (µg/mL)	Range of concentrations studied (µg/mL)
Ampicillin	≤ 0.25 [23]	0.125-64
Cloxacillin	<2 [21]	0.125-64
Tetracycline	<2 [24]	0.25-128
Cephalexin	≤ 4 [25]	0.25-128
Gentamicin	<16 [26]	0.5-256

Table 1: Previously reported minimum inhibitory concentration (MIC) of *Streptococcus agalactiae* and ranges of antibiotic solutions performed in the study.

Minimum biofilm eradication concentrations

To determine MBEC, *S. agalactiae* isolates were induced for biofilm formation using the same TCP assay described above, followed by methods adapted from Chamdit and Siripermpool [19]. Briefly, 200 µL of serial dilutions of antibiotics were added into each well, with antibiotic-free wells and biofilm-free wells included as positive and negative controls, respectively. The plates were incubated at 37°C for 24 h. After removing the antibiotic solutions, the remaining bacteria were cultured on BA and incubated at 37°C for another 24 h. MBECs were determined from the lowest concentration of antibiotics that inhibited growth on BA.

Results

Biofilm formation

All 56 studied *S. agalactiae* frozen isolates were viable when re-grown on BA. Most *S. agalactiae* isolates were weak biofilm producers (30/56, 54%) followed by moderate biofilm producers (21, 37%) (Figure 1). Only 5 (9%) *S. agalactiae* isolates were non-biofilm producers. No strong biofilm producers were identified. Upon qualitative assay, 11 (20%) *S. agalactiae* isolates were biofilm producers (Figure 2). These biofilm-producing isolates were from only 3 of the 8 farms represented among the samples.

MIC, MBC and MBEC

The observed MICs of the 11 biofilm-producing *S. agalactiae* ranged from 4-64 µg/mL for ampicillin, 16-32 µg/mL for cloxacillin, 8-16 µg/mL for cephalexin, 32-128 µg/mL for gentamicin, and 32-512 µg/mL for tetracycline (Table 2). Most isolates demonstrated greater or equal MBCs compared to MICs. Similarly, MBECs of tested isolates tended to be at higher concentrations compared to MICs and MBCs.

Discussion

The present study reaffirmed the ability of *S. agalactiae* to produce biofilms on abiotic surfaces previously reported [13,20]. It was interesting that all biofilm-producing isolates were from only 3 of the 8 farms. This suggested localized spread of biofilm-producing *S. agalactiae* within affected farms. We found a much lower fraction of biofilm-producing *S. agalactiae* (20%) by qualitative assay, compared to 76.5% of human isolates found by Kaur et al. [20], and 73.3% of bovine isolates reported by Boonyayatra and Jupia [13]. This difference may have been due to different amounts of initial inoculum of bacteria and/or different strains of *S. agalactiae*.

Biofilm formation can be detected and quantified directly or indirectly by many methods [17]. The qualitative and quantitative biofilm assays we performed measured surface-attached bacterial cells by crystal violet staining. The different rates of biofilm production we found between the two assays may be because the qualitative assay of Christensen et al. [16] measures cells attached to the bottoms of the wells, whereas the quantitative assay of Stepanović et al. [17] allows cells attached to the bottoms and the walls of the wells to be measured. We determined MICs, MBCs and MBECs only with 11 biofilm producers as categorized by the qualitative assay because the method has been more widely used compared to the quantitative assay.

Similar to other microorganisms, biofilm-producing *S. agalactiae* showed increased antibiotic resistance (MBECs) measured on sessile cells in well surfaces, compared to those of the planktonic phase of cells measured by MIC and MBC assays [4,21]. The drug-resistance mechanisms for biofilm formation have been hypothesized to (1)

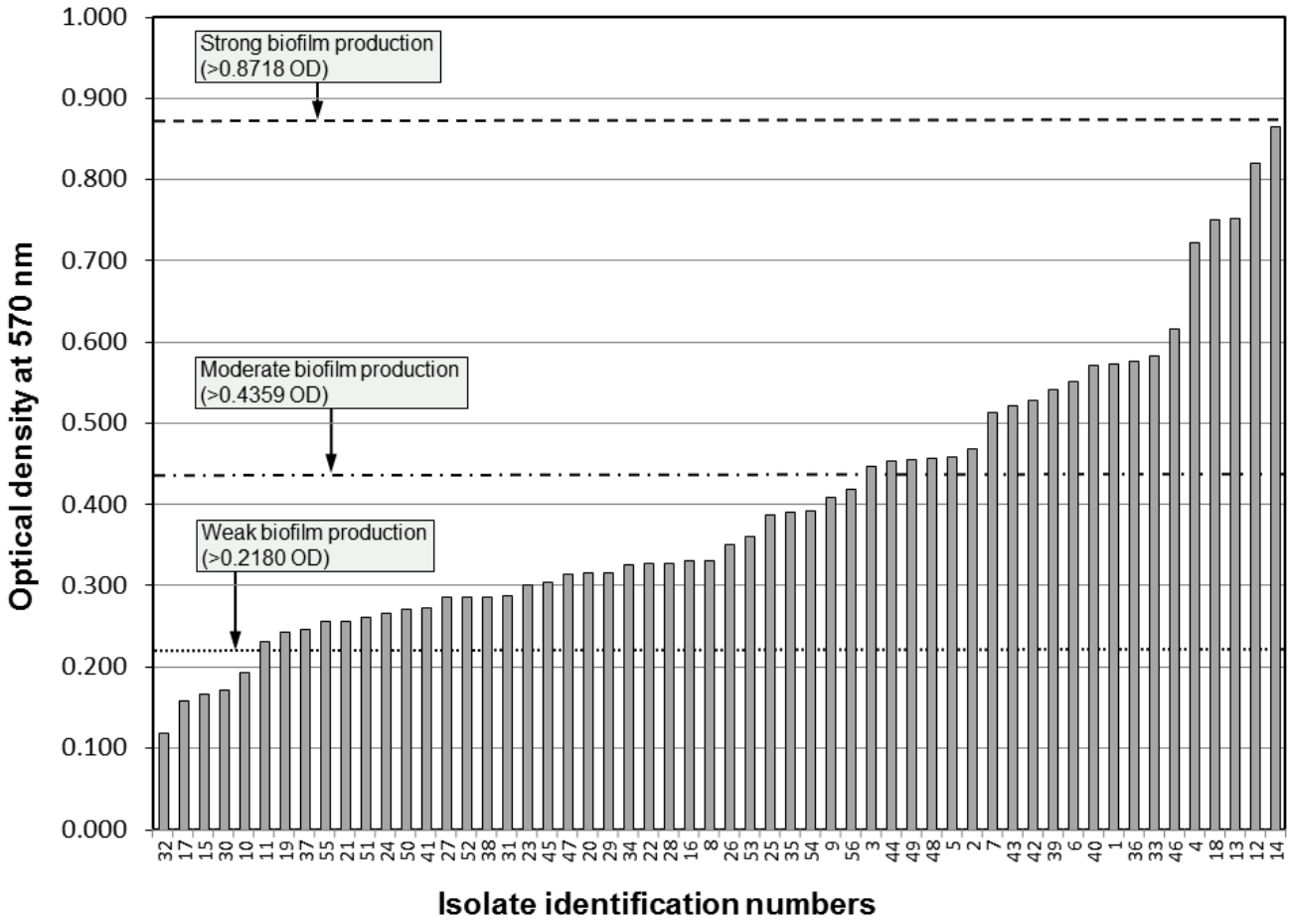

Figure 1: Classification of *Streptococcus agalactiae* isolates based on the quantitative biofilm assay. Isolates with averaged OD570 ≤ 0.2180, 0.2180<OD570 ≤ 0.4359, and 0.4359<OD570 ≤ 0.8718 were classified as non, weak and moderate biofilm producers respectively.

Antibiotics	Assay	Numbers of isolates inhibited at concentrations (μg/mL)														
		0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024
Ampicillin	MIC						2		6	2	1					
	MBC						2		2	3	1	3				
	MBEC									1	1	4	3	2		
Cloxacillin	MIC								2	9						
	MBC								1	6		1	2			
	MBEC												1	2	3	4
Cephalexin	MIC							2	9							
	MBC							1	7	3						
	MBEC												4	2	2	2
Gentamicin	MIC									6	4	1				
	MBC									4	5	1		4		
	MBEC												1	6	4	
Tetracycline	MIC									8	1		1	1		
	MBC								1	1	3	2	1	2		1
	MBEC										1		2	3	4	2

Table 2: Number of isolates among 11 biofilm-producing *Streptococcus agalactiae* by minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), and minimum biofilm eradication concentrations (MBEC) for five antibiotics.

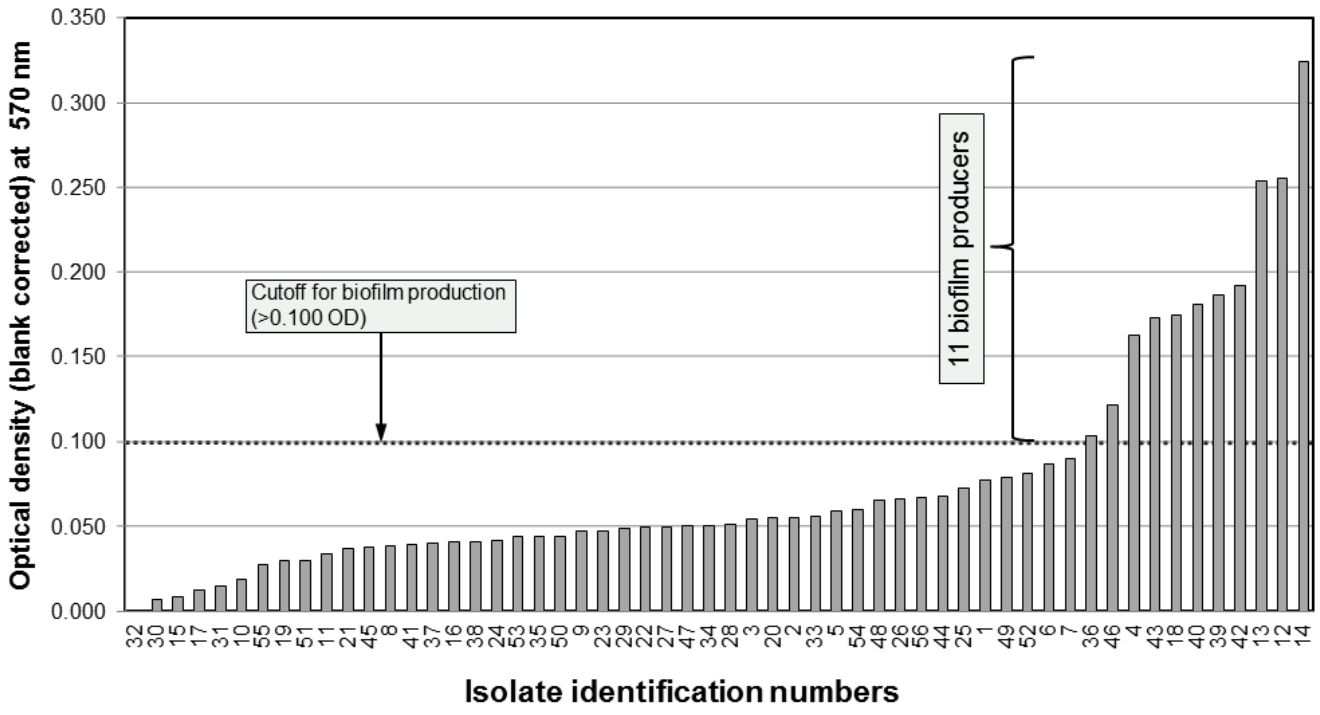

Figure 2: Classification of *Streptococcus agalactiae* isolates based on the qualitative biofilm assay. Isolates with blank corrected mean of OD570 nm >0.1 were classified as biofilm producers.

reduce the penetration of antibiotics through the biofilm, (2) reduce the growth rate of biofilm cells in rendering them less affected by antibiotics, and (3) generate "persister" biofilm cells genetically mutated to be highly antibiotic-resistant [4,22]. Findings of the current study could not confirm any of these hypotheses. However, regarding to the short induction time (18 hours) of biofilm formation performed in the laboratory, the increased antibiotic resistance of biofilm-producing *S. agalactiae* observed in this study might be mainly due to the reduction of accessibility of antibiotics through the biofilm structure [23,24]. The increased antibiotic resistance of biofilm-producing *S. agalactiae* suggests that treatment can fail if infected cows are treated with drug doses based on routine antibiotic sensitivity tests. However, as biofilm evaluation was based only on *in vitro* methods, *in vivo* studies should be pursued.

In conclusion, this study indicates that some strains of *S. agalactiae* were biofilm producers which may spread within dairy farms. Biofilm-producing strains of *S. agalactiae* were associated with increased antimicrobial resistance as determined *in vitro*. We recommend that determination of biofilm formation by *S. agalactiae* cultured from milk may be useful for creating an effective treatment plan and prognosis of bovine mastitis [25,26].

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Conflict of Interest

The authors declare that they have no conflict of interests to be concerned.

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