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, cephalxin, 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 warmth 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-affected udders, while 52 were from sub clinically-affected 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-Akins-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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Concentration of antibiotics that inhibited growth on BA.

The MIC and MBC were determined using micro dilutions of antibiotics. The MIC was the lowest concentration of antibiotics that inhibited visible growth after 24 h of incubation. The MBC was the lowest concentration of antibiotics which 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.

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.

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)

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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/mL)</th>
<th>Range of concentrations studied (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>≤0.25 [23]</td>
<td>0.125-64</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>&lt;2 [21]</td>
<td>0.125-64</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>&lt;2 [24]</td>
<td>0.25-128</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>≤4 [25]</td>
<td>0.25-128</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&lt;16 [26]</td>
<td>0.5-256</td>
</tr>
</tbody>
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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.

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.
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].

Acknowledgments

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Conflict of Interest

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

References


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.


