SHORT COMMUNICATION

Detection of Biofilm Forming *Streptococcus* species from Bovine Mastitis

D. K Parasana[1]*, B. B Javia[1], D. B Barad[1], D. T Fefar[2], S. N Ghodasara[1]

Abstract

Mastitis is a common disease condition of farm animals, which causes production loss in animals and heavy economic loss to the dairy farmers. *Streptococcus* genus is one of the important etiological agents for mastitis. Biofilm formation by organisms leads to chronic infection due to more antibiotic resistance. The purpose of our study was to determine the ability to produce biofilm among bacteria of the genus *Streptococcus* isolated from bovine mastitis. In present study, micro-titer plate assay was used to detect the biofilm formation in *Streptococcus* isolates. Out of 98 *Streptococcus* isolates, 30 (30.61%) were non-biofilm former, 43 (43.87%) were weak biofilm former, 15 (15.30%) were moderate biofilm former and 10 (10.20%) were strong biofilm former.

Key words: Biofilm, Bovine, Mastitis, *Streptococcus.*

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Introduction

Bovine mastitis is an inflammatory response of the udder tissue caused by microorganism infections or physical trauma. Bacterial intra-mammary infection (IMI) is considered to be the main cause of bovine mastitis (Galie *et al*., 2018). It has well-known negative impacts on the welfare of the animals as well as the profitability of dairy farms (Ruegg, 2017). Mastitis causes destruction of the milk secreting cells and scar or connective tissue replaces the milk secreting tissue, resulting in a permanent loss of production ability (Sharma *et al*., 2012). Due to decreased milk production in infected animals and costs associated with the application of proper therapy, both clinical and subclinical mastitis results into significant financial losses in the dairy industry (Rato *et al*., 2013). Healthy quarters are most frequently contaminated due to poor hygiene, primarily by infectious pathogens or an infection brought on by environmental germs from dairy cow's natural surroundings (Kaczorek *et al*., 2017).

Bacteria form biofilms in response to environmental stresses such as UV radiation, desiccation, limited nutrients, extreme pH, high temperature, high salt concentrations, high pressure, and antimicrobial agents (Galie *et al*., 2018). Microcolonies covered in extracellular polysaccharide molecules make up the biofilms. The development of biofilms was related to the virulence and pathogenicity of bacteria (Saginur *et al.,* 2006). By creating such a structure, bacteria increase their chances of surviving in adverse environments, improve their defenses against the human immune system, and become less vulnerable to the effects of antibiotics and disinfectants (Felipe *et al.,* 2017). The first studies investigating biofilm forming abilities of bovine mastitis pathogens emerged in the early 1990s. In 1993, "slime production" (exopolysaccharide matrix) was observed in bovine coagulase-negative Staphylococci (CNS) strains (Baselga *et al*., 1993).

Biofilms are known to be very resistant due to a number of processes, including minimal antibiotic penetration, enzyme neutralisation, heterogeneous nature, the presence of persistent cells, and sluggish cell growth rates (Jamal *et al*., 2018). *Streptococcus* spp. isolated from mastitis could be able to form biofilm and also possess multiple virulence genes that influences the course of disease and treatment (Kaczorek *et al*., 2017). Hence this study was undertaken to detect biofilm forming *Streptococcus* species from bovine mastitis.

[1]Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh, 362001, Gujarat, India

[2]Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh, 362001, Gujarat, India

Corresponding Author: D. K Parasana, Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Junagadh, 362001, Gujarat, India, E-mail: dkpatel784@gmail.com

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Materials and Methods

Bacterial Isolates

In the present study, 98 *Streptococcus* spp., including *Str. agalactiae* (n=42), *Str. dysgalactiae* (n=10), *Str. uberis* (n=16) and other *Streptococcus* (n=30) previously isolated from bovine mastitis milk samples were used for detection of biofilm formation.

Microtiter Plate Assay for Biofilm

The experiment was performed using polystyrene flat bottom microtiter plates as described by Tendolkar *et al*. (2004). All *Streptococcus* spp. isolates were grown in tryptic soy broth (TSB, HiMedia) at 37 °C for 16 h. The bacterial cells were centrifuged at 6000 g for 10 min. Supernatants were removed and the cell pellets were re-suspended in 5 mL of fresh medium (TSB). The optical density (OD) of the bacterial suspensions was measured using the Multiskan Microplate spectrophotometer (Thermo Scientific, India) and normalized to an absorbance of 1.00 at 595 nm. The suspensions were then diluted 1:40 in fresh TSB, and 200 µL of the diluted cell suspension was added to each well of the microtiter and plates were incubated at 37 °C for 24 h. The plates were then washed three times with sterile phosphate buffered saline (PBS). The inverted plates were dried at ambient temperature for 1 h. Then, the plates were stained with 200 µL of 0.2% aqueous crystal violet solution for 15 min. The plates were then washed three times with sterile PBS to remove excess dye. Crystal violet bound to the biofilms was extracted with 200 µL of an 80:20 (v/v) mixture of ethyl alcohol and acetone and the absorbance was measured at 595 nm using an ELISA plate reader (Thermo Scientific, India). Non-inoculated TSB wells stained with crystal violet were used as negative controls. The assay was performed in triplicate and mean OD (ODs) of the sample was considered for final analysis. Interpretation of biofilm formation was done according to the criteria described by Stepanovic *et al*. (2007). The mean optical density (OD) of the negative control +3 standard deviations of negative control was considered as the cut-off (ODc). The biofilm producers were categorized as:

- Non biofilm former : ODs ≤ ODc
- Weak biofilm former : ODc < ODs ≤ 2 × ODc
- Moderate biofilm former : 2 × ODc < ODs ≤ 4 × ODc
- Strong biofilm former : ODs > 4 × ODc
- Where ODc = cut-off OD and ODs = Mean OD of sample

Results and Discussion

Based on the results of microtiter plate assay all *Streptococcus* isolates were divided into 4 categories (Table 1 and Figure 1.)

Table 1: Distribution of biofilm formation in *Streptococcus* isolates

	Non biofilm former	Weak biofilm former	Moderate biofilm former	Strong biofilm former
***Streptococcus* spp. (n=98)**	30(30.61%)	43(43.87%)	15(15.30%)	10(10.20%)
***Str. agalactiae* (n=42)**	19(45.23%)	15(35.71%)	6 (14.28%)	2(4.76%)
***Str. dysgalactiae* (n=10)**	0(0%)	4(40%)	5(50%)	1(10%)
***Str. uberis* (n=16)**	4(25%)	9(56.25%)	0(0%)	3(18.75%)
Other than major *Streptococcus* spp. (n=30)	7(23.33%)	15(50.00%)	4(13.33%)	4(13.33%)

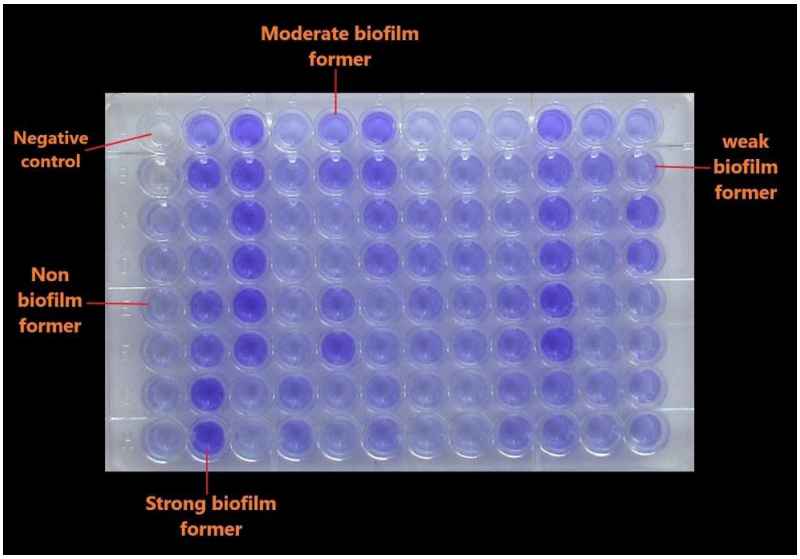

Fig. 1. Microtiter plate assay for biofilm

In our study around 69.38% isolates of *Streptococcus* spp. demonstrated an ability to produce biofilm. In major *Streptococcus* spp. overall 54.76% *Str. agalactiae*, 100% *Str. dysgalactiae* and 75% *Streptococcus uberis* showed biofilm producing ability. Overall 10.28% isolates of *Streptococcus* spp. showed strong biofilm producing ability. Among the major *Streptococcus* spp. studied here, *Streptococcus uberis* showed highest (18.75%) strong biofilm producing ability, while *Streptococcus agalactiae* showed least (4.76%) strong biofilm producing ability.

Similar findings were observed by many scientists. Ebrahimi *et al.* (2013) reported that out of 31 isolates of *Streptococcus agalactiae*, 6 (19.3%) were strong biofilm producer, 14 (45.1%) were moderate biofilm producer and 8 (25.8%) were weak biofilm producer. Sohail *et al.* (2019) screened twelve *Streptococcus agalactiae* isolates for biofilm formation using Luria-Bertani (LB) medium with 1 % glucose. They found 25 % of the *Str. agalactiae* isolates were strong biofilm producers, 16.67 % were moderate biofilm producers and 58.33 % as non-biofilm producers. Abundantly *in vitro* studies inspect the biofilm forming abilities of mastitis pathogens isolated from milk samples. However, disadvantages are the risk of contamination from the environment and *in vitro* studies only detect bacteria present in or released into the milk, however bacteria embedded in the tissue, encapsulated bacteria, low shedding bacteria and potential biofilms might not be detectable in milk samples. Hence to find the actual role of biofilm in bovine mastitis, the approach needs to change from *in vitro* to *in vivo* investigations of biofilms in infected udders (Pedersen *et al*., 2021).

Higher incidence of biofilm producing *Streptococcus* spp. in the studied geographical area presents a serious challenge for veterinarians and dairy farmers. Biofilm production by bacteria can have a considerable impact upon treatment efficiency. From a clinical point of view there is significant relationship between biofilm production and antibiotic resistance. Bacteria that forms biofilms are having very low sensitivity to antibiotics. During mastitis biofilm formation by bacteria facilitate their persistence into udder which leads to chronic infection.

Conclusion

Streptococcus spp. isolated from this region showed very high ability to form biofilm which may affect the success rate of mastitis treatment. High prevalent *Str. agalactiae* showed less biofilm forming ability, while less prevalent *Streptococcus dysgalactiae* showed high biofilm forming ability which indicates its clinical importance in forming strategies for treatment, prevention and control of bovine mastitis.

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