Prevalence Study of Biofilm Marker ica D Gene among Coagulase Positive Staphylococcus of Mastitic Origin

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ABSTRACT: Bovine Mastitis is an inflammation of the mammary gland, usually due to microbial infection, which causes economic loss to dairy producer every year. Staphylococcus is one of the most common pathogens responsible for contagious mastitis in bovines. The aim of this study was to identify coagulase positive Staphylococci isolated from bovine subclinical mastitis. In vitro biofilm forming ability of the isolates, an important virulence factor of mastitogen and prevalence of biofilm marker icaD gene among the isolates. In the present study, 70 isolates were biochemically identified as S. aureus. Biochemically characterized strains of S. aureus were screened for biofilm formation. Phenotypic detection of biofilm formation of S. aureus were done by two methods, namely, Congo Red Agar (CRA) method and Tube Method (TM). The frequency of biofilm formation in S. aureus strains was 28.57 per cent on CRA plates while 71.42 per cent were biofilm non-producers. The frequency of biofilm formation in S. aureus strains was 38.57 per cent in total as shown by tube method while 61.42 per cent were biofilm non-producers. Employing PCR, molecular detection technique of icaD gene was carried out. In this study, 72.85 per cent S. aureus strains were found to be positive for icaD gene which produced an amplicon of 381bp.

KEYWORDS: biofilm, coagulase, CRA, ica D, mastitis, PIA

INTRODUCTION

The dairy sector in India has grown substantially over the years, making India the world’s largest producer of milk. In India, 85% of total milk produced is handled by the noncommercial sector [1]. Staphylococcus aureus is the bacterium responsible for various diseases in animals and humans [2]. Staphylococcus aureus (S. aureus) is a significant cause of mastitis in ruminants such as cows, sheep, and goats worldwide [3]. Staphylococcus aureus is a highly prevalent cause of mastitis in dairy herds worldwide [4].

Subclinical mastitis (SCM) with no sign of inflammation is more severe than clinical forms and is responsible for a huge loss to the dairy industry [5]. Preventive strategies could be better implemented by understanding the prevalence, genetic patterns, and the presence of enterotoxin and biofilm-producing genes along with the antibiotic susceptibility of this organism [2].

Biofilm-producing S. aureus strains, especially the enterotoxigenic and antibiotic-resistant ones, are the major cause of several persistent bacterial diseases in the livestock sector, including mastitis [6]. The ability to form biofilm is a trait associated with bacterial virulence and many chronic bacterial infections [7]. During biofilm formation, initially, the bacteria adhere to each other by polysaccharide intercellular adhesion (PIA) and then propagate. The PIA is under the control of the icaABCD operon, and the strains possessing this gene cluster have been reported as strong biofilm producers. Several genes are involved in the production and maintenance of biofilms by staphylococci, of which the most widely studied are the icaA and icaD (intercellular adhesion A and B) genes responsible for the production of polysaccharide intercellular adhesion (PIA) that includes N-acetyl glucosamine as a primary component of the exopolysaccharide matrix surrounding the bacterial cells within the biofilm [8].

The formation of biofilm also provides a shielding effect to the bacterium from antibiotic attacks [2]. The production of biofilms is an important factor and makes these bacteria resistant to antimicrobial therapy [9].

Therefore, this study aimed to assess the prevalence and identify Coagulase Positive Staphylococci isolated from bovine subclinical mastitis. In vitro biofilm forming ability of the isolates and prevalence of biofilm marker icaD gene among the isolates.
MATERIAL AND METHODS

1. Identification of coagulase positive Staphylococcus (CPS)

The isolates were subjected to Gram’s method to study morphology and staining reaction. Cultures of Gram positive cocci typical to that of Staphylococcus were selected for further characterization. Identification of CPS was carried out as per the procedure of Cowan and Steel (1970) [10] and Cruickshank et al. (1975) [11]. The isolates were further processed for Biochemical tests for the identification and characterization of CPS. The Gram-positive cocci cultures were tested for oxidation fermentation (OF) test as per the procedure of Baker and Breach (1980) [12]. The OF positive cultures i.e. Staphylococci were further subjected for coagulase and Sugar fermentation and biochemical tests in order to identify *S. aureus*. All the tests were performed as per Sperber and Tatini (1975) [13].

Phenotypic characterization of CPS for biofilm formation: *In vitro* qualitative biofilm formation was assessed by following methods:

A. Congo Red Agar (CRA) method:

Qualitative detection of biofilm production in CPS strains was performed by cultivation on CRA as described by Vasudevan et al. (2003) [14]. The medium composed of brain heart infusion (BHI) broth (37gms/L), sucrose (36gms/L), agar powder (30gms/L) and Congo red dye (0.8gms/L). CRA plates were kept at room temperature overnight for sterility test. Inoculated CRA plates were incubated at 37°C for 24 hrs followed by subsequent storage at room temperature. Colonies on CRA were observed at 48 and 72 hrs. Along with CPS test strains, known biofilm positive strains served as control.

Strains producing black colonies with a rough, dry and crystalline consistency were considered biofilm producer. Strains producing red colonies with rough, dry and crystalline consistency or smooth colonies were classified as biofilm non-producer.

B. Tube Method (TM):

A qualitative assessment of biofilm formation was determined as described by Christensen et al., (1982) [15] with some modifications. Briefly, 5mL trypticase soya broth (TSB) with 0.25 per cent glucose was inoculated with loopful of broth culture and incubated by 24 hrs at 37°C. The tubes were decanted and washed for three times with sterile phosphate buffer saline (PBS, pH 7.4) and dried. After drying, tubes were stained with 0.1 per cent safranin. Excess stain was removed and tubes were washed with sterile distilled water. Tubes were dried in an inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible stained film lined the wall and bottom of the tube. Ring formation at the liquid interface was not considered an indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as strong, moderate and weak.

**Genotypic identification of icaD gene in S. aureus by Polymerase Chain Reaction**

The primers for the detection of intercellular adhesion gene, icaD used in this study were synthesized from Genetix, Biomed Asia Pvt. Ltd. New Delhi. The details of the primer sequence are shown in Table 1.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Product Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaD</td>
<td>5’-AAA CGT AAG AGA GGT GG-3’</td>
<td>381 bp</td>
<td>Vasudevan et al. (2003)[14]</td>
</tr>
<tr>
<td></td>
<td>5’-GGC AAT ATG ATC AAG ATA C-3’</td>
<td></td>
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</table>

**Preparation of the Genomic DNA:** The *S. aureus* isolates were grown in 2mL of BHI broth overnight at 37°C. Cultures were harvested by centrifugation (8000g for 10 min) and suspended in 400 µL of TE solution (10mM Tris HCl; 1mM EDTA, pH8.0). Bacteria were lysed by addition of 10 µL of proteinase K (20mg/mL) and 100 µL of 10% SDS, followed by incubation of 1 hr. The cell wall debris, denatured proteins, polysaccharides and polymeric matrix were eliminated by precipitation with addition of 80 µL of 5M NaCl and 64 µL of CTAB solution (10% CTAB in 0.7 M NaCl) and incubation at 65°C for 10 min. DNA was purified by two extractions with phenol:chloroform (1:1) and chloroform:isoamylalcohol (24:1). DNA was precipitated by adding 100 µL of isopropanol and kept at -20°C for 30 min or overnight and later centrifuged at 8000g for 15 min. The pellet was washed...
in ethanol (80%) and air dried to remove alcohol and resuspended in 50 µL TE buffer. Two µL of suspended pellet was used as the template.

**Single-plex PCR targeting the intercellular adhesion gene icaD of S. aureus.**

The PCR was standardized for the detection of icaD gene. The methodology described by Vasudevan *et al.* (2003) [14] was suitably modified. The standardized PCR protocol was used for screening of all the *S. aureus* strains for icaD gene.

PCR was set for 25 µL reaction volume for the detection of icaD gene in *S. aureus* strain. Reaction mixture for PCR was optimized as follows- 2.5 µL of 10x PCR buffer with 15mM MgCl2, 0.75 µL of dNTP mix (10mM with a final concentration of 0.2 mM), 1 µL of forward and reverse primer of each set (final concentration 0.1 µL each), 1 µL of Taq DNA polymerase, 2 µL of template DNA and sterilized milliQ water to make up the reaction volume.

PCR tube (0.2mL) containing the reaction mixture was flash spun on a micro centrifuge (Remi, C 24) to get reactants at the bottom. The reaction was performed in Px2 Thermal cycler (Thermal electronic corporation, USA) with a pre-heated lid. The cycling conditions included an initial denaturation at 94°C for 3 min followed by 40 cycles each of 45 seconds denaturation at 92°C, 45 seconds annealing at 49°C and 1 min extension at 72°C. After the reaction, PCR products were kept at -20°C until further analysis by agarose gel electrophoresis.

**Comparative evaluation of methods employed for detection of biofilm formation in *S. aureus***

Sensitivity and specificity of various tests namely Congo Red Agar (CRA) method and Tube Method (TM) for detection of biofilm formation by strains of *S. aureus* from bovine SCM was worked out as per the method described by Thapliyal and Mishra (1996) [16]. PCR for the detection of icaD genes was considered as golden standard test for comparison.

**RESULTS**

Worldwide, mastitis is the most common infectious disease affecting dairy cows and the most economically important disease of the dairy industry [17]. The first step in pathogenesis is adhesion of microorganisms to the extra-cellular matrix promoting colonization to host tissue. Another step-in colonization is the formation of biofilm [18].

In the present study, 70 isolates were biochemically identified as *S. aureus* were screened for biofilm formation.

**Phenotypic characterization of *S. aureus* for biofilm formation:**

**Congo Red Agar (CRA) method:**

Phenotypic detection of biofilm formation by *S. aureus* isolates was evaluated by culturing on CRA plates. In the present study, strains producing black rough colonies on CRA were considered as biofilm producing strains as per Vasudevan *et al.* (2003) [14]. Whereas, black smooth, red rough, red smooth and white smooth colonies were classified as non-biofilm producing strains.

The colony morphology of *S. aureus* strains observed in present investigation are shown in Figure 1 and results of biofilm production by CRA method is presented in Table 2.

**Table 2: In vitro biofilm production in *S. aureus***

<table>
<thead>
<tr>
<th>Staphylococcus aureus</th>
<th>Biofilm positive (CR+ve)</th>
<th>Biofilm negative (CR-ve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 (28.57%)</td>
<td>50 (71.42%)</td>
<td></td>
</tr>
</tbody>
</table>

**Tube Method (TM):**

Phenotypic detection of biofilm formation was assessed by Tube method as described by Christensen *et al.*, (1982) [15] with some modifications. By Tube Method, a visible stained film lined the inner wall and the bottom of the tube was considered as positive for biofilm formation. Ring formation at the liquid interface was not taken as an indicative of biofilm formation. The amount of biofilm formation was scored as strong, moderate and weak. (Table 3; Figure 2)

The frequency of biofilm formation in *S. aureus* strains was 28.57 per cent on CRA plates while 71.42 per cent were biofilm non-producers. Likewise, Vasudevan *et al.* (2003) [14], screened 35 isolates out of which 24 (68.57%) *S. aureus* were biofilm producing. Yazdani *et al.* (2006) [19] also recorded 52% biofilm positive *S. aureus* isolates by CRA method. Dhanawade *et al.* (2009) [20] examined a total of 102 *S. aureus* isolates of bovine subclinical mastitis cases of which biofilm producing strains...
detected by Congo red agar (CRA) method were 48.03 per cent. Salina et al., 2020 [21], studied biofilm production in *Staphylococcus aureus* from subclinical and clinical bovine mastitis and reported that total of 25 (25%) *S. aureus* isolates produced biofilms on CRA.

**Tube Method (TM):**

Table 3: Biofilm producing *S. aureus* strains as detected by Tube Method

<table>
<thead>
<tr>
<th>Biofilm non-producer</th>
<th>Biofilm producer</th>
</tr>
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<tbody>
<tr>
<td>43 (61.42%)</td>
<td>Strong</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
</tr>
<tr>
<td>10 (14.2%)</td>
<td>6 (8.5%)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
</tr>
<tr>
<td>11 (15.71%)</td>
<td></td>
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</tbody>
</table>

The frequency of biofilm formation in *S. aureus* strains was 38.57 per cent in total as shown by tube method while 61.42 per cent were biofilm non-producers. The present results of biofilm formation by Tube Method are in agreement to those of Dhanwade et al., (2009) [20] who observed 36.27 per cent biofilm production by Tube Method.

**Genotypic characterization of *S. aureus* strains:**

The present study was undertaken to detect the biofilm marker icaD gene among Coagulase positive *Staphylococcus* of mastitic origin. The PCR was standardized for detection of icaD gene in *S. aureus*. The primer set for icaD gene allowed positive amplification of 381 bp product represented by a single band in the corresponding region of the 100 bp DNA ladder. (Fig.3). The result of biofilm formation of *S. aureus* strains of mastitic origin by PCR is shown in Table 4.

Table 4: *S. aureus* strains detected a icaD positive by Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Positive for icaD gene</th>
<th>51 (72.8%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative for icaD gene</td>
<td>19 (27.1%)</td>
</tr>
<tr>
<td>Total samples</td>
<td>70</td>
</tr>
</tbody>
</table>

Overall by PCR, 51 (72.8%) *S. aureus* strains were observed positive for icaD gene and 19 (27.1%) strains were negative. Vasudevan et al. (2003)[14] reported 100 per cent prevalence of icaAD genes. Likewise Yazdani et al. (2006) [19] also reported similar results. Ciftci et al. (2009) [22] reported 64.40 per cent strains positive for icaD gene. In order to isolate and identify *S. aureus*, 1555 milk samples were collected from 401 cows, located in different regions of the Republic of Serbia. Using the conventional microbiological methods 100 isolates were characterized as coagulase-positive staphylococci. The presence of icaD gene alone was confirmed in 40.9% of cases totaling icaD positive isolates to 65.9% [23]. Comparison of methods for the detection of biofilm formation by *Staphylococcus aureus* isolated from bovine subclinical mastitis was studied and reported ninety strains (95%) were positive by PCR for both icaA and icaD genes [24].

*Staphylococcus aureus* isolated from bovine subclinical mastitis, the results revealed that 85% of the isolates tested produced slime on the Congo red agar and 95.7% of the isolates carried the icaD genes. The study recorded that 215 *S. aureus* strains collected from human and dairy cow's infections and the most prevalent gene was icaD found in 88.4% of the isolates [25].

The distribution of the frequencies of icaD gene in *S. aureus* isolates was 83%.(Salina et al 2020) [21]. The prevalence and characteristics of staphylococcal biofilm formation in raw milk samples was studied and reported the icaD was found in both CoNS strains (21.4%) and *S. aureus* (100%) [26]Over all biofilm formation by *S. aureus* by different methods is presented in Table 5.

Table 5: Overall Biofilm formation in mastitic *S. aureus* as seen by different methods

<table>
<thead>
<tr>
<th>Test</th>
<th>Total samples</th>
<th>No. of positive strains</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congo Red Agar (CRA) method</td>
<td>70</td>
<td>20</td>
<td>28.57</td>
</tr>
<tr>
<td>Tube Method (TM)</td>
<td>70</td>
<td>27</td>
<td>38.57</td>
</tr>
<tr>
<td>Polymerase Chain Reaction (PCR)</td>
<td>70</td>
<td>51</td>
<td>72.85</td>
</tr>
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</table>
CONCLUSION
The present study was undertaken for the evaluation of biofilm formation among Coagulase positive *Staphylococcus* of mastitic origin and also to study the prevalence of *icaD* gene in *S. aureus* recovered from bovine subclinical mastitis. Bacterial cultures of mastitic origin were taken from the Department of Veterinary Microbiology and Animal Biotechnology, Nagpur Veterinary College, Nagpur. The cultures were identified as CPS and characterized as *S. aureus*. This study was performed to generate useful information for field veterinarians and policy makers to form strategies for controlling *S. aureus*-mediated infections and ultimately improving the quality of milk.

Phenotypic detection of biofilm formation of *S. aureus* was done by two methods, namely, Congo Red Agar (CRA) method and Tube Method (TM). In present study, by CRA method 28.57 per cent and by TM 38.57 per cent of *S. aureus* of mastitic origin were found to be biofilm producer. Employing PCR molecular detection of *icaD* gene was carried. In this study, 72.85 per cent *S. aureus* strains were found to be positive for *icaD* gene which produced an amplicon of 381bp. It seems that the biofilm in *Staphylococcus aureus* has a high phenotypic expression, and the high ability of this bacterium to form a biofilm can be particularly important in the emergence of infections and the creation of multiple antibiotic strains.

ACKNOWLEDGMENT
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Conflict of Interest: The authors declare that there are no conflicts of interest.

REFERENCE
**Figure 1:** Colony morphology of *S. aureus* originated from bovine subclinical mastitis on CRA plate

**Figure 2:** Screening of biofilm producing *S. aureus* of bovine subclinical mastitis by TM method (S : Strong ; M : Moderate ; W : Weak )
Lane 1, 2, 3: Positive sample of icaD gene
Lane 4: DNA ladder
Lane 6, 7, 8: Negative sample of icaD gene

**Figure 3**: Agarose gel electrophoresis of PCR amplified product of icaD gene with amplification of 381 bp product with 100 bp DNA ladder.