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Phenotypic and Genotypic Characterization of *Staphylococcus aureus* Isolated from Bovine Mastitis for Biofilm Formation

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a major contagious pathogen responsible for both the clinical and subclinical mastitis in dairy cattle all over the world. The ability of *S. aureus* to form biofilm is considered to be a major virulence factor influencing its pathogenesis. In addition, it often creates intricacy in treatment of bovine mastitis using conventional antibiotics and produce recalcitrant drug resistant infections. This indeed demands urgent remedial measures as outbreaks of livestock associated methicillin resistant *S. aureus* (LA-MRSA) and community associated methicillin resistant *S. aureus* (CA-MRSA) are on the rise and currently being implicated as a rapidly emerging cause of numerous life threatening and therapy refractory human infections as well. In this study, a total of 22 *S. aureus* isolates from bovine clinical mastitis were evaluated for their ability to form biofilm by using phenotypic and genotypic methods. Using Congo red agar method, 9.09 per cent and 22.72 per cent of *S. aureus* were considered as strong and intermediate biofilm producers respectively. The presence of *icaA* and *eno* genes associated with biofilm formation was confirmed by using polymerase chain reaction (PCR). This study indicates a high prevalence of the *ica* (63.63 per cent) and *eno* (100 per cent) genes among *S. aureus* isolates from bovine mastitis.

HIGHLIGHTS

- The mere presence of biofilm associated genes is not always associated with *in vitro* biofilm formation.
- A combination of phenotypic and genotypic tests is recommended for the investigation of biofilm formation

Keywords: Mastitis, biofilm, Congo red agar, *icaA*, *eno* genes

India is an agricultural country, where the dairy industry is closely interwoven with the socioeconomic fabric of the rural farming community. Mastitis, often exacerbated by antimicrobial resistance is the prime challenge faced by dairy farmers all over the world. *Staphylococcus aureus* being a ubiquitous bacterium, acts as a significant contagious pathogen responsible for persistent and recurrent intramammary infections with minimal response to the antimicrobial therapy.

The success of this pathogen could be attributed to the multitude of its virulence factors, extensive resistance mechanisms that enable them to withstand the currently available antibiotic repository, and perhaps due to the dissemination of various multidrug resistant (MDR) strains. However, the biofilm formation is yet another multi-layered defense mechanism that augments the success of staphylococci. The possibility of biofilms as an important virulence determinant and a multi-layered defense mechanism that could result in a loss of sensitivity to virtually all class of antibiotics, has recently garnered the attention of many researchers.

Biofilms are organised sessile or motile aggregates of microorganisms encased in a self-produced polymeric

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matrix composed of exopolysaccharides, DNA and proteins (Hoiby *et al.*, 2010; Melchoir *et al.*, 2006). It is the result of expression of a complex network of signal molecules orchestrated under the coordinated expression of certain genes and bacterial quorum sensing (QS) system (Aricola *et al.*, 2012).

Biofilm formation in bacteria is a two- step process which involves the bacterial adhesion followed by tissue invasion and multiplication (Vasudevan *et al.*, 2003). An exopolysaccharide, intercellular adhesion/ polymeric N-acetyl-glucosamine (PIA/PNAG) that are regulated by proteins coded by the intercellular adhesion (ica) locus consisting of icaADBC genes regulates the bacterial adhesion (Fitzpatrick *et al.,* 2005; Hennig *et al.,* 2007). Among the *ica* genes*, icaA* and *icaD* have been reported to a play a significant role in biofilm formation in *S. aureus* and *S. epidermidis*. The i*caA* gene encodes N-acetylglucosaminyl transferase, the enzyme involved in the synthesis of the polysaccharide intercellular adhesin (PIA) and capsular polysaccharide/ adhesin (PS/A) that mediates the bacterial adhesion. (Arciola *et al.*, 2001). On the otherhand, the *eno* gene encodes the laminin binding protein that facilitates adhesion and ultimately helps in tissue invasion.

The decreased antimicrobial susceptibility of bacteria in biofilms is caused by multiple factors such as the nutritional limitation, genetic basis, composition and interactions among organisms of biofilms, occurrence of persister cells and biofilm-specific protective stress responses in bacteria (Hall and Mah, 2017). The presence of biofilm matrix reduces the antimicrobial penetration and paves way to the tolerance and resistance of the organism towards the particular antibiotic. This protects the pathogen and lead to recurrent and chronic intramammary infections (Melchoir *et al.*, 2007) ultimately aggravating the antimicrobial resistance crisis which is emerging as a silent pandemic.

Hence, early and rapid detection of the pathogen and the dynamics of biofilm formation is an absolute necessity for addressing the challenges of antimicrobial resistance. Therefore, the present study was envisaged to investigate the biofilm forming potential of *S. aureus* isolated from bovine mastitis using congo red agar method and also to determine the presence of *icaA* and *eno* genes associated with biofilm formation. This could help in developing crucial strategies and therapeutic concepts to effectively combat and mitigate the spread of these omnipresent pathogen which has tremendous impact on livestock production, animal welfare, global food safety and public health.

MATERIALS AND METHODS

Sample collection

The present study was conducted on 83 cross bred lactating dairy cows with clinical mastitis in and around Thrissur district, Kerala. The midstream lacteal secretions of each animal were collected aseptically in sterile screw capped vials.

Isolation and identification of *Staphylococcus aureus*

Isolation of bacteria was attempted by direct streaking of a loopful of milk sample on to brain heart infusion agar (BHIA- M211, Himedia), followed by incubation of the plates at 37ºC for 12 to 18 h. The isolates were identified based on morphological characteristics on Gram's staining. The presumptively identified *S. aureus* isolates were further verified by direct streaking and subsequent incubation at 37°C for 24 h on mannitol salt agar (MSA-M118, Himedia) and hichrome staph selective agar (SSA-M1931, Himedia) which was prepared according to the manufacturer's instruction. Biochemical characterisation of the isolates were done based on positive catalase and coagulase tests and negative oxidase test as per Barrow and Feltham (1993) and Quinn *et al.* (2013).

Isolation of genomic DNA

The DNA was extracted from the presumptively identified *S. aureus* isolates using snap chill method (heat lysis method) as described by Junior *et al.* (2016) with slight modifications. For this, approximately three millilitres of overnight grown *S. aureus* culture in mannitol salt broth (MSB-M383, Himedia) was centrifuged at 10,000 rpm for 10 min at 4ºC (Hispeed Centrifuge, KEMI). The pellet formed was resuspended in one milliliter of sterile nuclease (DNAase & RNAase) free milli-Q-water (ML064, Himedia), followed by centrifugation at 10,000 rpm for 10 min at 4ºC and the supernatant was discarded. The pellet obtained was further resuspended in

100µl tris EDTA buffer with pH 8, mixed in a vortex and kept in boiling water bath for 15 min. Then snap chilled on crushed ice for 30 min and centrifuged at 10,000 rpm for 10 min, at 4ºC. After that the supernatant containing DNA was collected in sterile nuclease free centrifuge tubes and further checked for concentration and purity using a Nanodrop Spectrophotometer (Nanodrop™ 1000 Spectrophotometer). The DNA samples with 260/280 ratio greater than 1.8 were selected and used as template for the polymerase chain reaction (PCR).

Genotypic characterisation of *Staphylococcus aureus* and its biofilm formation

Molecular confirmation of the presumptively identified *S. aureus* isolates were done by PCR targeting the *16SrRNA* for identification of *Staphylococcus* spp. and *23SrRNA* for identification of the *S. aureus*. The genotypic analysis of bacterial biofilm formation was carried out by PCR targeting the *icaA* (intercellular adhesion gene A) and *eno* (encoding lamininbinding protein) genes respectively.

The PCR was performed in a total volume of 25 µl reaction mixture by combining the reagents as shown in table 1 using the programmable S1000 Thermal cycler, (BioRad, USA). The details of the primers used and the PCR protocol are shown in table 2 and 3 respectively. PCR products were analysed on 1.2 per cent agarose gel stained with ethidium bromide, visualised under the ultraviolet transilluminator ((Genei™, Bengaluru) and photographed using GelDoc apparatus (Doc™ Gel EZ imager, BIO-RAD, USA).

Table 1: Components of PCR reaction mix (**Source:** Original)

Components	Volume (µl)
Master Mix (2X PCR Smart mix, Takara, Japan)	12.5
Forward Primer (100nM/ml, Sigma Aldrich)	1
Reverse Primer (100nM/ml, Sigma Aldrich)	1
Nuclease Free Water	5.5
Template DNA	5
Total	**25**

Table 2: Primers used for genotypic characterisation of *S. aureus* and its biofilm formation (**Source:** Original)

Sl. No.	Organism	Gene	Primer sequence	Amplicon size	References
1	*Staphylococcus* spp.	*16SrRNA*	F: 5'- AAC TCT GTT ATT AGG GAA GAA CA- 3' R: 5'- CCA CCT TCC TCC GGT TTG TCA CC-3'	756 bp	Ciftici *et al.* 2009
2	*S. aureus*	*23SrRNA*	F: 5'- GGA CGA CAT TAG ACG AAT CA- 3' R: 5'- CGG GCA CCT ATT TTC TAT CT-3'	1318 bp	El-Razik *et al.* 2010
3	Intercellular cell adhesion gene	*icaA*	F: 5'- ACA CTT GCT GGC GCA GTC AA-3' R: 5'-TCT GGA ACC AAC ATC CAA CA-3'	188 bp	Notcovich *et al.* 2018
4	Gene encoding laminin binding protein	*eno*	F: 5'- ACG TGC AGC AGC TGA CT- 3' R: 5'- CAA CAG CAT TCT TCA GTA CCT TC-3'	301 bp	Kot *et al.* 2018

F: Forward primer, R: Reverse Primer

Table 3: PCR protocol for amplification of various genes (Source : Original)

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	No. of cycles
16SrRNA	94°, 5 min	94°, 45 sec	56.9°, 45 sec	72°, 90 sec	72°, 10 min	30
23SrRNA	94°, 5 min	94°, 45 sec	55.8°, 45 sec	72°, 2 min	72°, 10 min	30
icaA	95°, 5 min	94°, 1 min	57°, 30 sec	72°, 45 sec	72°, 7 min	35
eno	94°, 5 min	94°, 1 min	56°, 1 min	72°, 1 min	72°, 10 min	35

Phenotypic characterization of biofilm formation

The biofilm formation of each *Staphylococcus aureus* isolate were evaluated qualitatively by culturing the organism on congo red agar (CRA) as described by Mathur *et al.* (2006). The CRA was composed of brain heart infusion broth (BHI - 37g/l) (M210, Himedia), sucrose (50g/l) (GRM 601, Himedia), agar (10g/l) (GRM 026, Himedia) and congo red dye (0.8g/l) (GRM 927, Himedia). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121° for 15 min. Then, it was added to autoclaved BHI agar with sucrose at 55°. Plates were inoculated with test organism and incubated aerobically at 37° for 24 to 48 h. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centres of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

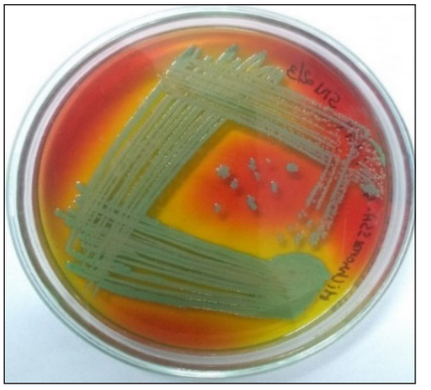

Fig 1: Greenish blue coloured colonies of *Staphylococcus aureus* on Hichrome Staph selective agar (*Source:* Original)

RESULTS AND DISCUSSION

In the present study, isolation of bacteria from bovine mastitic milk was achieved by standard microbiological

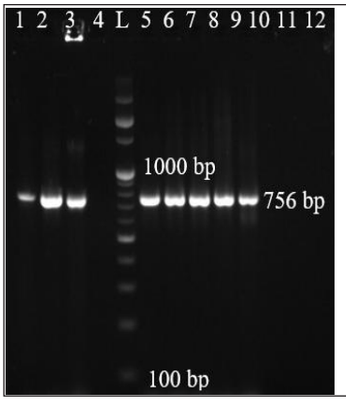

Fig. 2: Agarose gel electrophoresis of *16S rRNA* specific PCR of *Staphylococci* spp. (Source : Original)

Lane L	: DNA marker 100 bp
Lane 5	: Positive control
Lane 4	: Negative control
Lane 1, 2, 3, 6, 7, 8, 9	: Positive samples (756 bp)
Lane 10,11,12	: Negative samples

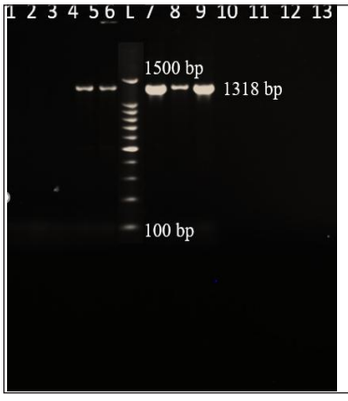

Fig. 3: Agarose gel electrophoresis of *23S rRNA* specific PCR of *Staphylococcus aureus* (Source : Original)

Lane L	: DNA marker 100 bp
Lane 9	: Positive control
Lane 10	: Negative control
Lane 1, 2, 3, 11,12,13	: Positive samples (1318 bp)
Lane 5, 6, 7, 8	: Negative samples

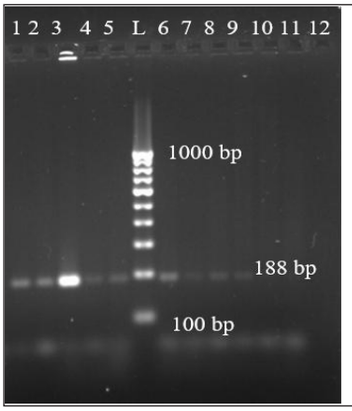

Fig. 4: Agarose gel electrophoresis of *icaA* gene specific PCR of *Staphylococcus aureus* (Source : Original)

Lane L	: DNA marker 100 bp
Lane 7	: Positive control
Lane 6	: Negative control
Lane 10,11,12	: Negative samples
Lane 1, 2, 3, 4, 5, 8, 9	: Positive samples (188 bp)

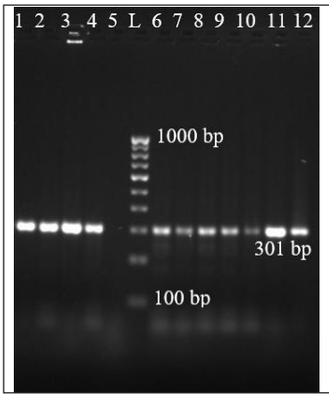

Fig. 5: Agarose gel electrophoresis of *eno* gene specific PCR of *Staphylococcus aureus* (Source : Original)

Lane L	: DNA marker 100 bp
Lane 6	: Positive control
Lane 5	: Negative control
Lane 1 to 4, 7 to 10, 11,12	: Positive samples (301 bp)

culture techniques. Upon initial inoculation of 83 mastitic milk samples on to BHIA, 26 samples (31.33 per cent) did not produce any colonies whereas, 57 samples yielded bacterial growth (68.67 per cent), among which *S. aureus* was isolated from 22 samples (26.5 per cent). The bacterial isolates were identified based on their morphology, colony characteristics on selective media and biochemical characteristics (Barrow and Feltham, 1993; Quinn *et al.*, 2013).

The CRA method was often used as an indirect indicator of biofilm formation. (Darwish and Asfour, 2013). Out of the 22 *S. aureus* isolates, 9.09 per cent, 22.72 per cent and 68.19 per cent were strong, intermediate and negative biofilm producers, respectively. Similar findings were reported by Bose *et al.* (2009) who reported a total of 32.96 per cent of *S. aureus* isolates to be biofilm producers by CRA method. In contrast, Darwish and Asfour (2013), reported that 32.5 per cent and 35 per cent of the *S. aureus* isolates from bovine mastitis to be strong and intermediate biofilm producers where they used a plant based culture medium - Tryptose soya broth when compared to the animal based brain heart infusion broth that was used in our study. Hence, this could not be extrapolated to our study as the phenotypic expression of biofilm in bacteria may vary based on the difference in chemical composition of the culture media.

Though there are numerous low cost, easily adaptable, phenotype based tools for the qualitative and quantitative assessment of biofilm formation (Notowich *et al.*, 2018) several previous studies of *S. aureus* have reported *in vivo* biofilm formation eventhough they were phenotypically non biofilm producers. This might be either due to the heterogeneous nature of the phenotypic expression of biofilms (Darwish and Asfour, 2013) or may be due to the fact that the data obtained from most phenotypic typing tools are not guided by the host and mammary tissue factors involved in biofilm formation. This had limited the practical utility of various phenotypic assays in the diagnosis and containment of biofilm formation.

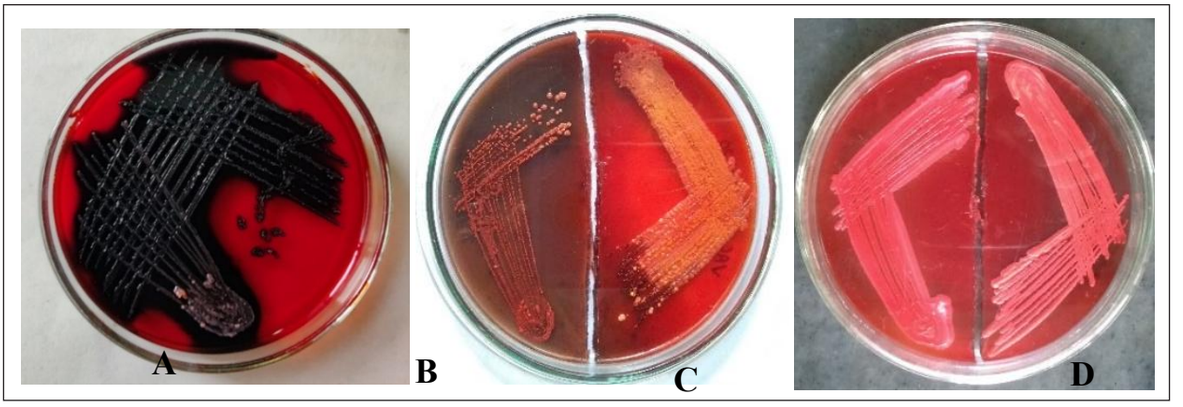

Fig. 6. Colonies of *Staphylococccus aureus* on Congo red agar. **(A)** Black coloured dry crystalline colonies indicative of strong biofilm producers; **(B & C)** Rough, dry red coloured colonies indicative of weak biofilm producers; **(D)** Indeterminate result

Molecular approaches, such as those focused on PCR, were therefore regarded as a method of choice, for the rapid and reliable diagnosis as well as for epidemiological investigations of outbreaks involving biofilm forming strains. It also eliminates the need for the cumbersome cultural procedures and combats for the heterogeneous nature of the phenotypic expression of biofilm formation. Therefore, in the current study apart from the *in vitro* congo red agar method, PCR was employed to assess the biofilm formation of *S. aureus* isolates.

Numerous PCR-based systems targeting different genes such as those enoding the intercellular adhesion genes - i*caA* and *icaD*, *bap gene* encoding biofilm associated protein, *eno* gene encoding laminin binding protein, *aap* encoding an accumulation associated protein, *embP* encoding fibronectin adhesion, *fbe* encoding a fibrinogen adhesion, *atlE* gene encoding an adhesion and autolysin etc. have been documented in literature (Darwish and Asfour, 2013; Srednik *et al.*, 2017) for the detection of biofilm formation. In the current study, molecular characterisation of biofilm formation was done by PCR targeting the *icaA* and *eno* genes. The results indicated that *eno* gene had the highest prevalence (100 per cent) followed by the *icaA* gene which was detected in 63.63 percent of the isolates. The presence of *eno* genes was analogous to the study conducted by Darwish and Asfour (2013) who reported 75 per cent and 92.5 per cent prevalence of *eno* genes among the *S. aureus* and coagulase negative staphylococci isolated from bovine mastitis.

A comparison of the phenotypic and genotypic methods for assessment of biofilm formation revealed that the mere presence of *icaA* and/or *eno* genes does not always result in phenotypic expression of biofilms. The inability of *S. aureus* isolates that were positive for *icaA* and /or *eno* genes to produce biofilm *in vitro* can be due to point mutations in the locus and/or any other unidentified factors that negatively regulates and influence the biofilm formation (Darwish and Asfour, 2013). Another probable reason behind this disparity might be the phenomenon of phase variation in response to the *in vitro* conditions (Baselga *et al.*, 1993).

Thus, it can be concluded from the present study that the *S. aureus* isolated from bovine clinical mastitis exhibited a greater ability to form biofilms, with different degrees of production using the CRA method. The higher prevalence of the *icaA* and *eno* genes responsible for biofilm formation must be considered as an alarming situation since biofilms are increasingly being recognized as a bacterial defense mechanism, that could result in loss of sensitivity to virtually all class of antibiotics, which are considered to be the holy grail of mastitis therapy. Therefore, novel ways of effective treatment, prophylaxis & control of such infections in dairy farms is of paramount importance. The study also highlights the need for modification of i*n vitro* antimicrobial susceptibility tests, which selects antibiotics that are effective only in inhibiting the planktonic microbial population, ignoring the bacteria in biofilms that resist and survive treatment and provides materials for further growth leading to the recurrence or chronicity. Further research involving genomic, transcriptomic

and proteomic approaches is required to unravel the complexities of bacterial biofilm formation and related antimicrobial resistance that could reverse a century of medical revolution.

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