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Phenotypic and genotypic determination of biofilm forming *Staphylococcus aureus* causing bovine mastitis

Jasleen Kaur, Pawanjit Singh Cheema, Naresh Kakkar, Jeenu Manoj and Rajesh Chhabra

Abstract
Mastitis is causing huge economic losses to the dairy industry. Among different mastitis causing pathogens, *Staphylococcus aureus* is the leading bacterial cause involved in mastitis with more than 30 virulence factors. Biofilm production is one of the important virulence factor leading to treatment failure. Therefore, the present study was planned to investigate the percent prevalence of biofilm producing *Staphylococcus aureus* on the basis of genotypic and phenotypic basis. A total of 102 *Staphylococcus aureus* isolated from mastitic milk samples were cultured on congored agar with 5% glucose for phenotypic expression of biofilm and for molecular detection PCR assay targeting *icaA* and *icaD* gene was employed. Characteristic black and brown colnies typical of congored agar were seen in 88.23% isolates. Positive amplicon of 1315 bp and 381 bp was generated in 91.17% and 92.15% isolates corresponding to amplification of *icaA* and *icaD* gene, respectively.

Keywords: Mastitis, *Staphylococcus aureus*, *icaA*, *icaD*, congo red agar

Introduction
Mastitis is a multifactorial disease imparting huge economic loss to the dairy sector worldwide (Constable *et al*., 2017) [10]. In India, losses have been estimated to the tune of Rs. 7165.51 crores per annum (Bansal and Gupta, 2009) [4]. There are over 250 known causative agents of mastitis, including bacteria, fungi, algae and viruses (Bhuvana and Shome, 2013) [5]. Among different causative agents, *Staphylococcus aureus* (*S. aureus*) is the most common cause leading to both clinical and subclinical mastitis in bovines (Charaya *et al*., 2014; Sharma *et al*., 2018, Chhabra *et al*., 2020) [6, 21, 8].

S. aureus produce more than 30 virulence factors, including fibrinogen-, fibronectin-, collagen-binding proteins, exotoxins, hemolysins (alpha, beta, delta, and gamma), leukocidins, toxic shock syndrome toxin, epidermolysins, antibiotic resistance genes (*mec*A, *bla*Z) and, biofilm formation. These factors allow it to colonize, invade and multiply in bovine mammary epithelial cells which in turn make antimicrobial agents poorly effective (Marechal *et al*., 2011; Spoor *et al*., 2013) [22]. Biofilm is a coherent cluster of bacterial cells embedded in a biopolymer matrix, which, compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense (Pedersen *et al*., 2021) [19]. Sessile bacteria present in biofilm have low replication rates, which is contradictory on account of action of antibiotics, which act on organisms in active metabolic state (Christner *et al*., 2010) [9]. Bacteria on biofilm structures are protected from environmental conditions, antimicrobial agents, and host immune responses, and they also exhibit up to 1000-fold-increased antibiotic resistance to a wide range of antimicrobial agents, thus leading to persistence of infection (Chen *et al*., 2018) [7]. Treatment of biofilm-related infections has become an important part of antimicrobial chemotherapy because biofilms are not affected by therapeutic concentrations of antibiotics (Dosler & Karaaslan, 2014; Fabres-Klein *et al*., 2015) [13, 14]. Multiple genes are responsible for biofilm formation in *S. aureus*. The genes present in *ica* locus (*icaA* and *icaD*) play a significant role in biofilm formation (Aslantaş Ö, & Demir C., 2016) [2]. Gene, *icaA* encodes for *N*-acetylglucosaminyl transferase which is responsible for the *N*-acetylglucosamine oligomers from UDP-*N*-acetylglucosamine (Arciola *et al*., 2001) [1], whereas *icaD* helps in the expression of *N*-acetylglucosaminyl transferase, leading to the phenotypic expression of the polysaccharide (Gerke *et al*., 1998) [15]. The biofilm formed by *S. aureus* is an important virulence factor in the establishment of infections, as it helps the bacterium to survive hostile environments within the host (Otto, 2013) [18]. Therefore, the present study was conducted for determination of biofilm formation property in *S. aureus* isolated from bovine milk samples.

Materials and methods

One hundred and two *S. aureus* colonies isolated from milk samples of bovines suffering from mastitis were characterized both phenotypically and genotypically for biofilm formation.

Phenotypic identification of biofilm forming *S. aureus*

S. aureus colonies were inoculated on Congo Red Agar (CRA) for 24 hrs at 37°C for determination of biofilm forming ability. Black and brown coloured colonies on CRA were considered as biofilm forming, whereas red colour colonies were considered as non-biofilm forming.

Genotypic identification of *S. aureus* for biofilm production

Primers used for PCR assay

Published oligonucleotide primers for *icaA* (F: CCT AAC TAA CGA AAG GTA G and R: AAG ATA TAG CGA TAA GTG C) and *icaD* (F: AAA CGT AAG AGA GGT GG and R: GGC AAT ATG ATC AAG ATA C) genes as per Vasudevan *et al,* 2003 were used for determination of presence of biofilm formation property. Oligonucleotide primers were purchased from Integrated DNA Technology (IDT) in purified and lyophilized form. Stock solution (100 µM) of primers was prepared according to the instructions supplied by the manufacturer. For preparation of working solution (10 µM), 10 µl of stock solution was added to 90 µl of NFW.

Positive and negative control for PCR assay

DNA extracted from MRSA ATCC strain no. 700699 and MSSA ATCC strain no. 25923 were used as positive controls for PCR assay for detection of *icaA* and *icaD* gene for biofilm formation. NFW was taken as negative control.

DNA extraction

Genomic DNA of *S. aureus* isolates was extracted using QIA amp Blood Kit (Qiagen, Germany) following the manufacturer's protocol.

Amplification of DNA by PCR assay

DNA extracted from pure colonies of *S. aureus* was used for amplification by PCR assay. A total of 25 µl reaction mixture was prepared in 200 µl thin walled PCR amplification tubes. The reaction contained 12.5 µl GoTaq Green Master Mix (2X) (Promega), 1 µl of each forward and reverse primer of 10 µM concentration and 3 µl template DNA of sample to be tested. Volume of NFW was calculated and added to complete the reaction volume to 25 µl. PCR tube was vortexed and spinned for 10 sec. PCR amplification was performed in thermo cycler (Bio-Rad, USA).

Thermo cyler conditions

The similar thermo cycler conditions used by Vasudevan *et al,* 2003 were used for PCR assay. The initial denaturation was done at 94°C-3min, 30 cycles of denaturation at 94°C-45 sec, annealing at 49°C-45 sec and extension at 72°C-60 sec followed by final extension at 72°C-7 min for both genes.

Agarose gel electrophoresis for PCR products

PCR products were run on a 1% w/v agarose gel containing ethidium bromide (0.5 µg/ml in 1x TAE buffer (Tris-acetate-EDTA) at 75 V for 1 hr, after loading 10 µl of PCR product per well. A 200 bp and 100 bp plus ladder (Molecular marker) were included for estimation of size of amplicon for *icaA* and *icaD* genes respectively. After the completion of electrophoresis, the gels were viewed on a UV transilluminator and photographed for record.

Results

Phenotypic characterization of 102 *S. aureus* isolates was done to determine biofilm formation property by growing on CRA (fig. 1), and 90 (88.23%) showed the typical growth. On using PCR assay for determination of *icaA* and *icaD* gene, 93 (91.17%) and 94 (92.15%) isolates were found to possess respective genes. Correlating phentotypic and genotypic expression, 75 (73.50%), 12 (11.76%), 02 (1.96%) and 08 (7.84%) isolates manifested biofilm forming feature by characteristic growth on CRA as well as amplification of both *ica* A and *ica* D genes, presence of *icaA* and *icaD* genes, CRA growth and presence of *icaD* gene and by growth on CRA alone, respectively. Five (4.90%) isolates were found to be negative by all the three parameters.

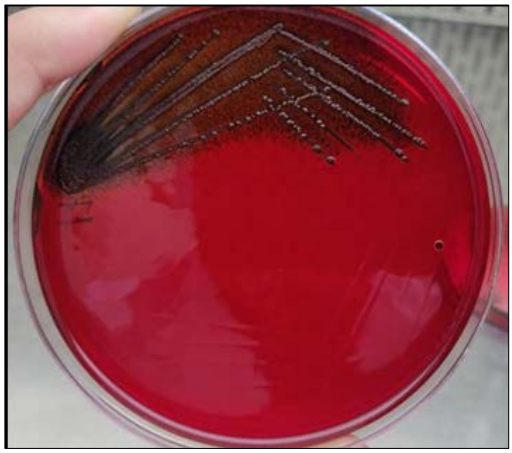

Fig 1: Growth of biofilm producing *S. aureus* on CRA showing black colonies

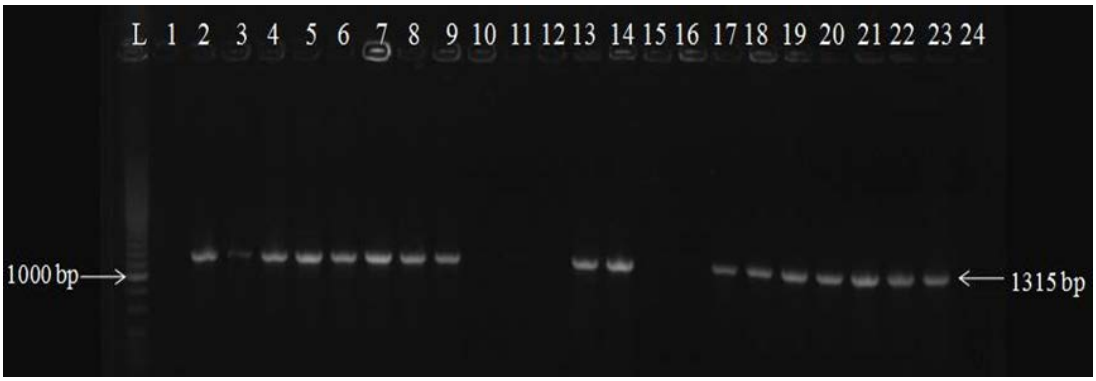

Fig 2: Agarose gel electrophoresis results for molecular identification of biofilm forming *S. aureus* showing 1315 bp amplicons of *icaA* gene. L: 200 bp ladder, 2-9, 13, 14, 17-22: Positive test samples, 1, 10-12, 15, 16: Negative test samples 23: *S. aureus* ATCC 25923, 24: Negative control

Fig 3: Agarose (1%) gel electrophoresis result of PCR for molecular identification of biofilm forming *S. aureus* showing 381 bp amplicons of *icaD* gene. L: 200 bp ladder, 1-17, 19, 21, 22: Positive test samples; 18, 20: Negative test samples; 23: *S. aureus* ATCC 25923; 24: Negative control

Discussion

Among the *S. aureus* isolates screened for biofilm activity in present study, 73.50% isolates showed growth on CRA, as well as found to possess gene *icaA* and *icaD* determining the biofilm forming activity whereas, 7.84% isolates showed growth on CRA alone without having both the genes controlling biofilm forming activity tested in the present study. This can be attributed to the fact that *icaA* and *icaD* are the genes responsible for biofilm formation but not the sole reason as other genes can also result in biofilm formation. Phenotypic differentiation of biofilm forming to non-biofilm forming bacteria can be easily done by culturing on CRA. Biofilm forming (slime producing) colonies form blackish colonies whereas non-biofilm forming colonies form red coloured colonies. This method is based on the principle that bacteria release some secondary metabolites on fermentation of sugars, which combine with Congo red dye and give black color colonies. Similar to the present study, Aslantas and Demir, 2016 [2], have also reported same percentage of biofilm producing *S. aureus* whereas Szweda *et al.*, 2012 [23]; *de* Castro Melo, 2013 [12] and Marques *et al.*, 2017 [17] reported higher percentage of isolates having biofilm forming ability by *icaA* and *ica D* gene detection. In contrary to present study.

Darwish and Asfour, 2013 [13]; Avila-Nova *et al.*, 2018 reported comparatively lower percentage of isolates to possess biofilm producing genes. By CRA method, *de* Castro Melo, 2013 [12], showed nearly equal percentage of biofilm harbouring *S. aureus*, whereas Szweda *et al.*, 2012 [23] showed higher percentage and Farbes-Klein *et al.*, 2015 and Aslantas and Demir, 2016 [2] showed lower percentage comparatively. Biofilm forming bacteria gurantees bacterial adherence and maintenance in mammary tissue making their clearance difficult even by using antibacterial agents. This virulence factor of *S. aureus* along can be detrimental to dairy industry and its spread because of ignorance of farmers and veterinarians in field to test milk prior to treatment is an invitation to upcoming threats of pandemic. Sugar concentration in agar and incubation period of culture has also been reported to play crucial role in results of biofilm production. It was difficult to find a borderline in the color of the growing colonies that should classify as a reference test for slime production. This unreliability has also been presented by many authors in past (Rohde *et al.*, 2001; Vancraeynest *et al.*, 2004; Szweda *et al.*, 2012) [20, 24, 23].

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