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Detection and Adhesion Gene Profiling of Biofilm-Producing *Staphylococcus aureus* from Chicken Meat and Raw Milk Samples

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Abstract

The present study, aimed to investigate the presence of *S. aureus* (*Staphylococcus aureus*) in food samples (chicken meat and raw milk), as well as the biofilm-forming ability of isolates, and to characterise different adhesion genes. Out of 20 chicken meat samples, five (25%) tested positive for *S. aureus*. Out of 20 raw milk samples, 6 (30%) tested positive for S. aureus. Microtitre plate assay revealed that all isolates were weak to moderate biofilm producers. The isolates were characterized and confirmed to be *S.aureus* by PCR using 16S rRNA. The adhesion genes were detected using PCR with specific primers. Among the isolates, nuclease gene *nuc* (60%), coagulase gene *coa* (80%), *agr* (80%), and *sdrD* (80%) were detected in *S.aureus* isolated from chicken meat. In *S.aureus* isolated from milk samples *nuc* (66%), *coa* (100%), *agr* (50%) and *sdrD* (50%) genes were detected. All isolates were negative for *fnpB*, bap, and *icaD* genes. This study identified biofilm-forming *S. aureus* in food samples, which serves as a contaminant that contributes to food spoilage. Furthermore, they pose risks to consumer health and safety.

Keywords: PCR, adhesion genes, *S. aureus*

Introduction

Foodborne infection and spoilage is a serious problem around the world. According to the World Bank report (2018) on the economic burden of the foodborne diseases, the total productivity loss associated with food borne disease in low and middle-income countries was estimated to cost US$ 95.2 billion per year, and the annual cost of treating foodborne illnesses is estimated at US$ 15 billion.

In India, foodborne diseases are the 5th leading cause of disease burden and diarrhea is the third leading cause of childhood mortality (Lakshminarayanan and Jayalakshmy, 2015). More than 100 million cases of food-related illnesses are anticipated to occur each year in India by 2030.

A biofilm is a structured colony of microorganisms that sticks to a surface and form their own matrix, which is mostly made up of proteins, polysaccharides, and deoxyribo nucleic acid (DNA). This matrix offers various advantages to the bacterial community, including immune cell protection and improved adherence, which is assisted by bacterial adhesins. Biofilms are clusters of bacterial cells that adhere to the surface of any material that exhibits resistance to antimicrobial agents (Carrascosa *et al*., 2021; Selem *et al*., 2022).

Biofilm formation occurs in three main stages which includes bacterial adhesion, quorum sensing, and biofilm biomass formation (Thakur *et al*., 2016; Alshammari *et al*., 2023).

The capacity of many bacterial organisms to form biofilms through surface adhesion has major implications for the food sector, where biofilms pose a continuous risk of contamination. Biofilm formation is influenced

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by various factors such as the extracellular polymeric matrix of cells, lipopolysaccharides, antibiotic resistance, oxidative stress, and other traits, as well as environmental variables (Van Houdt and Michiels, 2010; Carrascosa *et al.,* 2021).

Biofilms on food contact surfaces may lead to transmission of diseases, food spoilage, shortened time between cleaning events, contamination of product by nonstarter bacteria, metal corrosion in pipelines and tanks and reduced heat transfer efficacy of the heat equipment (Cappitelli *et al.,* 2014).

Staphylococcus aureus is a gram-positive bacterium that causes a wide range of diseases in humans and animals. *S. aureus* is widely regarded as one of the most important foodborne pathogens globally. *S. aureus* pathogenicity can cause infection and biofilm formation on the surface area is related to the presence of a variety of virulence factors and adhesion proteins (Achek *et al.,* 2020). The contaminated foods includes egg products, vegetables, raw milk and dairy products, meat and meat products and seafood (Liang *et al.,* 2023b).

Staphylococcus aureus can produce various toxins that lead to food poisoning with symptoms of diarrhea, nausea and abdominal cramps. *S. aureus* can produce biofilms in various environments, such as food-processing surfaces, for avoiding host defenses and antimicrobial agents. This can create an ideal environment for S. aureus proliferation, leading to colonisation and biofilm formation on the surface (Ryu *et al.*, 2021).

Staphylococcal biofilms are facilitated by microbial surface components that recognise adhesive matrix molecules (MSCRAMMs) by biofilm-associated proteins (*Bap*), serine-aspartate repeat family proteins (*SdrC, SdrD*, and *SdrE*), fibrinogen-binding protein (*Fib*), clumping factors (*ClfA, ClfB*) (Peng *et al.*, 2022). Biofilm formation in *S. aureus* is regulated by two genetic loci: the staphylococcal accessory regulator (*sarA*) and accessory gene regulator (*agr*) quorum-sensing systems (Achek *et al.*, 2020).

Fibronectin-binding *S. aureus* possesses a diverse repertoire of MSCRAMMs, including fibronectin-binding proteins A and B (*fnbpA/ fnbpB*) (Chen *et al.*, 2020; Speziale and Pietrocola, 2020). Serine-aspartate repeat family proteins contain motifs required for cell wall anchoring. *SdrD* is an important factor for the survival of *S. aureus* and mediates its adhesion to the surface for biofilm formation (Peng *et al.,* 2022). *S. aureus* biofilm formation is critically regulated by the agr gene, which is responsible for the population-sensing system of *Staphylococci*(Liang *et al.*, 2023).

The present study was designed to investigate the prevalence of *S.aureus* in chicken and raw milk samples and to characterize the 16S rRNA gene associated with biofilm formation.

Materials and Methods

Chemicals and media: Mannitol Salt Agar, Nutrient Broth, Brain Heart Infusion (BHI) broth, PCR Master Mix were used for isolation and detection of *S. aureus.*

Collection of food samples: Twenty chicken meat samples and raw milk were collected aseptically in separate containers in and around Chennai and Thiruvallur districts of Tamil Nadu. The samples were transported in ice to laboratory for further investigation.

Primary isolation of *S.aureus:* About 25 g of sample was mixed with 225 ml of nutrient broth for enrichment at 37°C for 18–24 hrs. After enrichment, 100 µL of the inoculum was plated on mannitol salt agar (MSA). The plates were incubated at 37°C for 18-24 hrs. Yellow/ white colour colonies on MSA were subjected to gram staining and biochemical test. After

biochemical confirmation, each *S.aureus* isolate was inoculated and incubated in test tubes containing BHI broth medium at 37°C for 24 hrs and subsequently stored at -20°C in glycerol for further analysis.

Quantification of biofilm: The biofilm production of *S.aureus* was characterized by microtitre plate method (Obe *et al.,* 2021). The S. aureus isolate was grown in Brain Heart Infusion broth (BHI), and the turbidity of the bacterial culture was adjusted to 0.08 optical density (OD) at 600 nm. In each well, the inoculumn were mixed with BHI broth and was incubated at 37°C for 48 hrs to promote biofilm formation. After, incubation each wells were washed three times with sterile distilled water to remove planktonic cells. Then each well was added with 200 µL of 0.1% crystal violet solution to stain the cells attached to the wells. After staining, the wells are washed three times with sterile distilled water. Then, resolve the stained cells by addition of 200 µL of a mixture consisting of 75% ethanol and 25% acetone. The ability of each strain to form biofilms was evaluated by measuring optical density at 597 nm using a microplate reader (Biotek).

Molecular identification and amplification of adhesion genes: The isolated colonies were subjected by PCR using 16S rRNA primers specific to *S. aureus*. Adhesion-associated genes for biofilm formation in *S. aureus* were identified using primers targeting various genes. Gene primers used in this study are listed in Table 1. PCR was carried out using a thermal cycler (QIAGEN). Genomic DNA was extracted by using the QIAamp DNA Mini kit (QIAGEN) according to the manufacturer's instructions. The PCR reaction was optimized with 25µL reaction with amplicon master mix (12.5 µL), forward primer (0.5 µL), reverse primer (0.5 µL), DNA template (1.0 µL), nuclease free water (10.5 µL). Polymerase chain reaction was performed with the following conditions 95^0C for 3 min, 30 cycles of 95^0C for 30 sec, 55^0C for 30 sec, 72^0C for 1.5 min and final extension at 72^0C for 7 min. The annealing temperature of different genes are given in Table 1. The products were analyzed using 1.8% agarose gel electrophoresis with a molecular weight marker of 100 bp and documented with a UV light transilluminator.

Results and Discussion

Isolation and identification: In this study, 20 chicken meat and 20 raw milk samples were collected for isolation of *S. aureus* using Mannitol Salt Agar. Six samples each from chicken meat and raw milk showed yellow colonies on Mannitol Salt Agar plates. Biochemical and sugar fermentation tests were carried out for the six isolates from chicken meat and raw milk for further confirmation. Out of the six isolates five from chicken and all the six from raw milk were confirmed as *S.aureus*.

A prevalent study of *S. aureus* was conducted in chicken meat samples in Jalandhar, Punjab and it showed 46.61% positive results (Herve and Kumar, 2017). Sharma *et al.* (2017) reported that 73 *S. aureus* isolates were obtained from raw milk samples collected from 13 districts of Rajasthan. Several studies have been conducted on the isolation and characterization of foodborne microorganisms, including *S. aureus* (Kumar 2010; Bhardwaj *et al.,* 2021; Neelam *et al.,* 2022). Deepak *et al.* (2023) reported that 34% of milk samples collected from Chennai were positive for *S. aureus.*

Quantification of biofilm forming ability of *S. aureus* isolates: The biofilm-forming abilities of *S. aureus* isolates from chicken meat and raw milk samples are presented in Tables 2 and 3 respectively. The isolates confirmed as S.aureus by biochemical tests were screened for biofilm formation.

In the present study, *S. aureus* isolates from chicken meat and raw milk showed

weak to moderate biofilm forming ability. Chen *et al.* (2020) concluded that 72% of the *S. aureus* isolates from various food produced biofilms. Among these isolates, 54.64% were weak biofilm producers, whereas 14.43% and 3.09% produced moderate and strong biofilms, respectively. Among the *S. aureus* isolates obtained from the food contact surfaces, 20%, 77%, and 3% were strong, intermediate, and non-biofilm producers respectively (Ballah *et al.,* 2022).

Molecular identification and amplification of adhesion genes: The results of PCR amplification of adhesion genes are presented in Fig 1 – 4 and Table 4 – 5. Molecular characterisation of *S. aureus* isolated from chicken meat, and raw milk using 16S rRNA, revealed that all the samples were positive for *S. aureus.*

S. aureus isolated from chicken meat samples revealed genetic markers which target biofilm-associated genes including the nuclease gene *nuc* (60%), coagulase gene *coa* (80%), accessory regulator gene *agr* (80%), and *sdrD* (80%). In raw milk, the results of the adhesion gene amplification revealed that the percentage of the nuclease gene (*nuc*), coagulase gene (*coa*) accessory gene regulator (*agr*) and serine-aspartate repeat protein D (*sdrD*) were 66%, 100%, 50% and 50% respectively. All isolates were negative for *fnpB,* bap, and *icaD*. All isolates from chicken meat and raw milk were negative for *fnpB*, bap, and *icaD*.

In this study, the thermo nuclease gene was detected in most *S. aureus* isolates. The thermo nuclease gene has also been implicated in *S. aureus* biofilm formation (Tang *et al.*, 2013; Yu *et al.*, 2021). The quorum sensing mechanism of *S. aureus* is regulated by the *agr* gene which contributes to the secretion of virulence factors (Ganesh *et al.*, 2022). Achek *et al.* (2020) reported that *sdrD* gene was found in 87.5% of slime-producing *S. aureus* strains originating from food. The gene, *sdrD* was found more frequently in slime producers than in non-slime producers which was consistent with the results obtained in the present study.

In this study, 80 % and 100 % of *S. aureus* isolates from chicken meat and raw milk, respectively, contained the *coa* gene. Sharma *et al.* (2017) also reported that 41% of *S. aureus* isolates showed the presence of *coa* gene.

Conclusion

This study provides insights into *S. aureus* contamination in food samples in and around Chennai. The biofilm film forming ability of the *S. aureus* strains isolated from food samples was moderate. The prevalence of adhesion-encoding genes also varied greatly within strains. However, the majority of isolates contained the thermo nuclease, coagulase, *agr*, and *sdrD* genes, indicating that increasing the biofilm-forming ability leads to an increased risk of foodborne infection. Further research with large numbers of food samples is required to explore the connection between these genes and the biofilm-forming ability of *S. aureus*.

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Table I: Adhesion associated gene specific primers for *S. aureus*

Gene	Nucleotide sequences (5′→3′)	Amplicon size (bp)	Annealing temperature	Reference
16S rRNA	GTGCACATCTTGACGGTACC CGAAGGGGAAGGCTCTATC	565	54°C	(Tang *et al.,* 2006)
Thermonuclease (nuc)	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	447/279	55°C	(Brakstad *et al.,* 1992)
Coagulase (coa)	ACCACAAGGTACTGAATCAACG TGCTTTCGATTGTTCGATGC	750	55°C	(Neelam *et al.,* 2022)
Serine-aspartate repeat protein D (sdrD)	GGAAATAAAGTTGAAGTTTC ACTTTGTCATCAACTGTAAT	500	45°C	(Azara *et al.,* 2017)
Intra cellular adhesion (icaD)	AGGCAATATCCAACGGTAA GTCACGACCTTTCTTATATT	526	59°C	(Ma *et al.*, 2012)
Biofilm associated protein (Bap)	CCCTATATCGAAGGTGTAGAATTGCAC GCTGTTGAAGTTAATACTGTACCTGC	971	53°C	(Cucarella et al., 2004)
Fibronectin-binding protein (fnbpB)	TCTGCGTTATGAGGATTT ACAGTAGAGGAAAGTGGG	452	54°C	(Tang *et al*., 2013)
Accessory gene regulator (agr)	GTGCCATGGGAAATCACTCCTTCC TGGTACCTCAACTTCATCCATTATG	976	55°C	(Liang *et al.,* 2023)

Table 2: Biofilm production assay of *S. aureus* isolates from chicken meat samples

Sample	Biofilm formation
RC10ST	Weak
RC11ST2	Weak
RC12ST2	Moderate
RC13ST3	Moderate
RC14ST1	Moderate

Table 3: Biofilm production assay of *S.aureus* isolates from raw milk samples

Sample	Biofilm formation
RM10ST1	Weak
RM11ST1	Weak
RM12ST2	Weak
RM13ST1	Moderate
RM13ST2	Moderate
RM2ST2	Weak

Table 4: Amplification of adhesion genes of *S. aureus* isolates from chicken meat

Sample	Genes associated with biofilm formation							
	16S rRNA	***nuc***	***coa***	***fnbpB***	***agr***	***bap***	***icaD***	***sdrD***
RC10ST	+	-	-	-	-	-	-	-
RC11ST2	+	-	+-	-	-	-	-	-
RC12ST2	+	+	+	-	+	-	-	+
RC13ST3	+	+	+	-	+	-	-	+
RC14ST1	+	+	+	-	+	-	-	-

Table 5: Amplification of adhesion genes of *S. aureus* isolates from raw milk

Sample	Genes associated with biofilm formation							
	16S rRNA	***nuc***	***coa***	***fnbpB***	***agr***	***bap***	***icaD***	***sdrD***
RM10ST1	+	-	+	-	-	-	-	-
RM11ST1	+	+	+	-	-	-	-	-
RM12ST2	+	+	+	-	+	-	-	-
RM13ST1	+	+	+	-	+	-	-	+
RM13ST2	+	+	+	-	+	-	-	+
RM2ST2	+	-	+	-	-	-	-	+

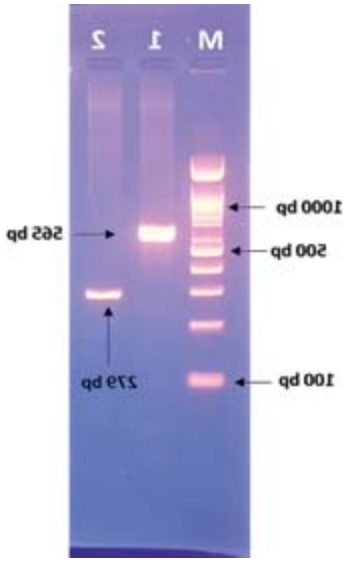

Fig. 1: 1.8% agarose gel showing PCR products of *16S rRNA* and *nuc* gene in *S. aureus* isolate. M- Marker (100 bp), Lane 1 (*16S rRNA*) and lane-2 (*nuc* gene)

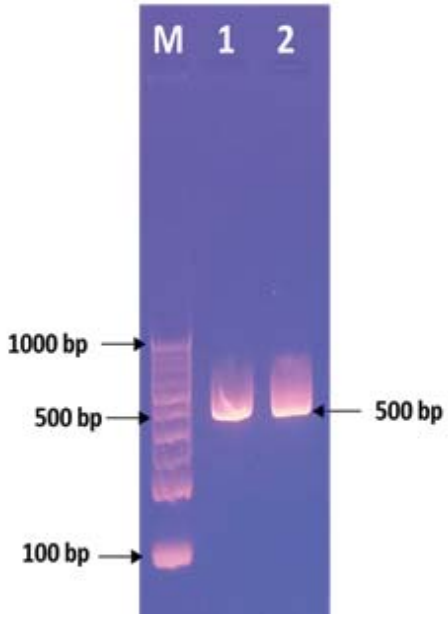

Fig. 2: 1.8% agarose gel showing PCR products of *agr* gene in *S. aureus* isolates. M- Marker (100 bp), Lanes 1-2 *S. aureus* isolates

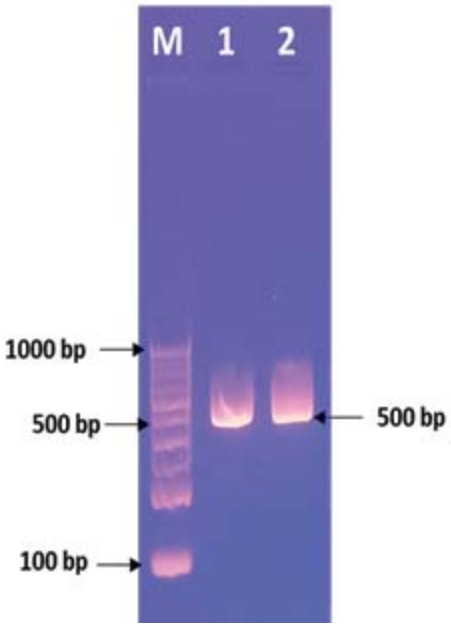

Fig.3: 1.8 % agarose gel showing PCR products of *sdrD* gene in *S. aureus* isolates. M- Marker (100 bp), Lane 1-2 *S. aureus* isolates

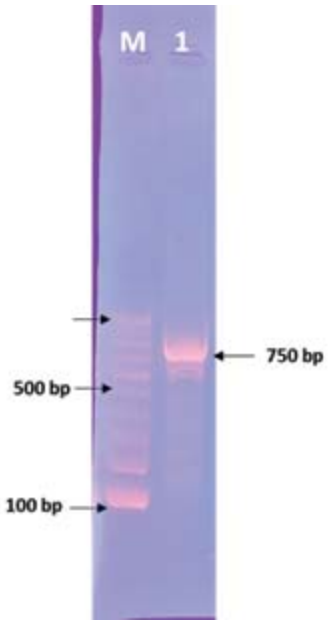

Fig.4: 1.8% agarose gel showing PCR product of *coa* gene in *S. aureus* isolates. M- Marker (100 bp), Lane 1- *S. aureus* isolate