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Research Article

GENOTYPIC AND PHENOTYPIC ANALYSIS OF BIOFILM FORMATION IN *STAPHYLOCOCCUS EPIDERMIDIS* ISOLATES FROM HUMAN AND ANIMAL SKIN AND SOFT TISSUE INFECTIONS

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ABSTRACT: The present study aims at determining the prevalence and biofilm characterization of *Staphylococcus epidermidis* (*S. epidermidis*) isolates from both humans and animals with skin and soft tissue infections. Out of 250 samples investigated, 70 revealed the presence of *S. epidermidis* based on cultural and morphological characteristics. Of those, 37 were confirmed as *S. epidermidis* based on species-specific PCR amplification. The phenotypic determination of biofilm production among *S. epidermidis* isolates revealed that approximately 80% of *S. epidermidis* isolates were biofilm producers (CRA, 78.37%; TA and TCP, 81.08%) with varying intensities (43.24% strong, 35.13% moderate, 21.63% weak/non; 54.05% strong, 27.02% moderate, 18.52% weak/non and 35.14% strong, 45.94% moderate, 18.92%). The genetic analysis of biofilm production revealed an overall prevalence of 27.02%, 21.62%, 21.62%, 18.91%, and 13.51% of *ica*D, *clf*A, *cna*, *fnb*A, and *bap* genes, respectively, while none of the isolates showed the presence of the *icaA* gene.

Keywords: *Staphylococcus epidermidis,* Skin infection, Biofilm, *icaAB* gene.

INTRODUCTION

The development of resistance among microorganisms and the spread of such organisms through nosocomial infections are creating huge problems in contemporary healthcare [1, 2, 3]. The use of antimicrobials for many other purposes than the treatment of diseases, their misuse, and insufficient investment in the field of development of new or potent antimicrobials are increasing the problems far more [4, 5, 6]. The synthesis of biofilm by the bacteria to surround themselves is another problem in the effective use of antibacterial drugs. Bacterial biofilms are multilayered structures formed by clusters of bacteria attached and/or to a surface and encased in a self-generated matrix that may consist of substances like polysaccharides, proteins, and DNA. Inside biofilms, the bacterial cells are more resistant to environmental stress and host defense mechanisms. Both Gram-negative and Gram-positive bacteria are reported to cause biofilm-associated infections [7]. *Escherichia coli* O157: H7 in biofilms on salads is reported in food-borne illness [8]. *Vibrio cholera* uses biofilms to survive in water bodies and colonizes the intestine whenever it enters its mammalian host [9]. Also, the oral microbiota includes several biofilm-producing microbial communities [10]. Similarly, *Staphylococcus epidermidis* (*S. epidermidis*), a coagulase-negative staphylococcus (CoNS), has an intense capability of biofilm formation over the skin and surfaces of medical devices, which provides an evolutionary advantage and gives phenotypic benefits, gradually increasing the survival rate [11]. The extracellular polysaccharide adhesion required for biofilm formation is reported to be a key virulence determinant in *S. epidermidis* infections [12, 13, 14]. Some clinical studies have reported that isolates of

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S. epidermidis are positive for polysaccharide intercellular adhesion (PIA). PIA is synthesized by ica operon gene products, and the ica operon is made up of four genes, *viz. ica*A, *ica*D, *ica*B, and *ica*C [15, 16]. The *ica*A and *ica*D collaborate to produce the N-acetyl glucosamine transferase, which is used in the synthesis of PIA oligomers. The *ica*C is a membrane protein that transports oligomers synthesized by the *ica*A and *ica*D genes across the cell membrane, and the *ica*B genes synthesize some proteins associated with bacterial cells. Shanks *et al.* [17] describe the fibronectin-binding proteins (fnbA gene) in Staphylococcus species as important virulence factors that contribute to bacterial adhesion. The fibronectin-binding proteins A and B (FnbA and FnbB) both can adhere to fibronectin to cause disease, which is a ubiquitous host protein present in soluble form in the blood [18]. The clumping factor (clf) is also responsible for the pathogenesis and is used for identifying virulent strains. There are two types of clumping factors, clf A and B, which bind to fibrinogen. The clfA binds to the complement regulator I protein and is responsible for the clumping of blood plasma. The clfA gets attached to the plasma protein-coated biomaterials and helps the bacteria colonize and form biofilm. The *cna* are cell wall-anchored protein genes responsible for the adhesion of bacteria to collagen-rich tissues [19]. It is responsible for generating adhesion factor and immune evasion factor and plays an important role in pathogenicity [20]. The *bap* gene is associated with the production of biofilm formation proteins and plays an important role in biofilm formation in both gram-positive and gram-negative organisms [21]. The bap C protein is responsible for the structural functionality of bacteria to form biofilm and maintain elongated protein conformation on cell surfaces [16, 22]. In recent times, *S. epidermidis* has emerged as a pathogen commonly reported from healthcare-associated infections like catheter-associated bacteremia, cardiovascular infections, prosthetic joints, heart valves, and polymer or metal implants [23], and damaged skin and soft tissue infections like burns, cuts, and wounds among humans [24]. Although the bacteria is reported as the major cause of CONS infections in animals, studies on animal infections are prone to causing infection on the skin, and thus the present study was undertaken with the aim of phenotypic and genetic identification and determination of biofilm production capacity among *S. epidermidis* isolates from both human and animal skin and soft tissue infections.

MATERIALS AND METHODS

Sample collection and isolation of *S. epidermidis*

A total of 250 swab samples from different clinical conditions such as post-operative wounds, skin, and soft tissue wounds, abscesses, etc. were collected from humans (n = 200) and animals (n = 50) of different age groups from June 2017 to September 2018 from local clinics and dispensaries in Mathura, Uttar Pradesh, India (Table 1). For the isolation of *S. epidermidis*, collected pus swabs were immediately brought to the laboratory on ice and inoculated into the sterile nutrient broth (NB) (HiMedia, India) with 6.5% NaCl (HiMedia, India) under sterile conditions and incubated at 37ºC under aerobic conditions. After 16-18 hours of incubation, the culture growth obtained was streaked over a nutrient agar (NA) (HiMedia, India) media plate and incubated overnight at 37ºC under aerobic conditions to obtain single discrete colonies.

Identification of *Staphylococcus epidermidis*

Phenotypic identification

Based on the cultural characteristics like white, raised, cohesive colonies about 1-2 mm in diameter, the *S. epidermidis* suspected colonies on NA media plates were selected and subjected to Gram's staining for observing the bacterium morphology, and the pure cultures showing gram-positive cocci arranged in grape-like bunches were selected to subculture over mannitol salt agar (MSA) (HiMedia, India) media to differentiate from mannitol fermenting *Staphylococcus* sps. The identification of *S. epidermidis* by performing the biochemical analysis was done using the KB004 Hi Staph identification kit (HiMedia, India) utilizing 12 conventional biochemical tests (Voges prokaeurs, alkaline phosphatase, ONPG, urease, arginine utilization, mannitol, trehalose, sucrose, arabinose, lactose, and maltose) based on the change in pH and substrate utilization. After inoculation of the culture isolates, the test results were observed as Voges prokaeurs (+), alkaline phosphatase (+), ONPG (-), urease (+), arginine utilization (+), mannitol (-), trehalose (-), sucrose (+), arabinose (-), lactose (+), and maltose (+) for identification of *S. epidermidis*.

Genotypic identification

Genetic confirmation of presumptive *S. epidermidis* isolates was done by PCR amplification of a genus-specific *gap* gene using primers and amplification conditions as described by Ghebremedhin [25], followed by species identification by PCR amplification

of a *16S rDNA* sequence specific for *S. epidermidis* [26] using a TC-5000 thermal cycler (GeneiTMMerck Specialities Pvt. Ltd., Maharashtra, India).

Determination and characterization of biofilm formation in *Staphylococcus epidermidis* isolates

Phenotypic determination of biofilm formation

The biofilm formation in *S. epidermidis* isolates was determined using the Congo red agar (CRA) plate, tube adherence (TA), and tissue culture plate (TCP) methods. The CRA plate method was performed as described by Kaiser *et al.* [27]. Single colonies of *S. epidermidis* isolates were inoculated on CRA media and incubated aerobically at 37ºC for 24 hours. The bacterial isolates developing black colonies with dry crystal consistency indicate strong biofilm production, while moderate biofilm producers were represented by less dark colonies and weak biofilm-producing bacteria with red-colored colonies. In the TA method [28], a loopful of the overnight-grown culture of *S. epidermidis* on NA was taken and inoculated in 10 ml of trypticase soy broth (TSB) (HiMedia, India) containing 1% glucose (HiMedia, India). The inoculated broths were incubated at 37 ºC for 24 hours. The cultures were decanted, and the tubes were washed with phosphate-buffered saline (PBS; pH 7.3), dried, and stained with 0.1% crystal violet (HiMedia, India). Excess stain was washed with deionized water, and tubes were dried in an inverted position and observed for biofilm formation. A visible film lining the wall and bottom of the tube was considered positive for biofilm production. The formation of rings only at the liquid interface was not considered indicative of biofilm formation, while the tube was examined for the number of rings at the bottom of the tubes and scored as 1-weak, 2-moderate, and 3-strong [28]. In the TCP method, *S. epidermidis* was inoculated on trypticase soy agar (TSA) media (HiMedia, India) and incubated for 18-24 hours at 37ºC under aerobic conditions. From TSA media, a single colony was inoculated into brain heart infusion (BHI) broth (HiMedia, India) and incubated for 16-18 hours at 37°C. PBS was used to adjust the cultures to a 0.5 McFarland scale (HiMedia, India) concentration, and 100 µl of each culture suspension was transferred into the microliter plate well and incubated for 24 hours at 37 °C. A negative control of a blank well containing only broth and two positive controls of known cultures of *E. coli* with weak and strong biofilm-producing capability was also performed in parallel with the test isolates. After 24 hours of incubation, the plankton and nutrient suspensions were aspirated and washed three times with 300 µl of PBS. After washing, fixation was done using 200 µl of methanol (Sigma-Aldrich, St. Louis, USA) for 15 minutes. Then, the wells were decanted and stained with 200 µl of 1% crystal violet for 20 minutes. The wells were again decanted and washed with distilled water. Finally, 160 µl of 33% glacial acetic acid (Sigma-Aldrich, St. Louis, USA) was added to the wells to extract the stain of adherent cells. The optical density (OD) of each well is measured at 490 nm using an automated ELISA plate reader (iMarkTMMicroplate Absorbance Reader, Bio-RAD, California, USA). The analysis of the OD was done as per Dekha [28] to assess the biofilm-forming capability of test isolates. Isolates with a cutoff OD of 0 to 2, 2 to 4, and more than 4 were considered weak, moderate, and strong biofilm producers, respectively.

Genotypic determination of biofilm formation

Genetic identification of biofilm-forming ability was performed by PCR amplification of six adhesion genes, *viz*., *fnb*A, *cna*, *ica*A, *ica*D, *clf*A, and *bap* genes. The PCR amplification of adhesion genes was carried out in a 25 µl reaction volume containing 12.5 µl of 2x EmeraldAmpMax PCR master mix (TakaRa Bio Inc., Japan), 25 pmol of each primer, 2 µl of template DNA, and nuclease-free water (TakaRa Bio Inc., Japan) to make up the reaction volume. Initial denaturation at 94ºC for 5 minutes is followed by 32 cycles of denaturation at 94ºC for 30 sec., annealing at 49ºC for *ica*A and *ica*D genes, 60ºC for *clf*A and *bap* genes, 52ºC for *fnb*A and 54ºC for 30 sec. for *cna* gene, extenstion at 72ºC for 30 sec. and final extension at 72ºC for 10 minutes [29, 30]. PCR products were analyzed by 1.5% agarose gel electrophoresis. The details of the primer sequence used for the amplification of genes associated with biofilm formation are mentioned in Table 2.

RESULTS AND DISCUSSION

S. epidermidis isolates identified based on cultural, morphological, and biochemical tests were confirmed by the PCR amplification of a genus-specific *gap* gene followed by a species-specific *16S rDNA* gene sequence. Out of 250 pus samples, 70 samples have shown cultural and morphological characteristics similar to *Staphylococcus* spp. These 70 presumed *Staphylococcus* isolates comprise 45 isolates of human and 25 isolates of animal origin. Out of 70 presumptive Staphylococcus isolates, only 50 isolates showed

Table 1. Details of the pus samples swabs collected for the present investigation.

Sl. No.	Samples source	Location of clinics/dispensaries and veterinary hospital	Sample Description		No. of Samples
			Clinical condition	Site (No. of samples)	
1	Humans (Male and females of all the age groups presented with superficial pyogenic infections)	Jarrah Clinics, Janmbhumi road, Mathura	Skin and soft tissue wounds	Thigh (11), armpit (6), leg (9), burn on hand (10), burn on leg (18), sole of leg (22), Burn on finger (4), head (24), neck (12), cheeks (6), back side (8), knee (10), nail removed (20), behind ear (5), nose (9), chest (5).	200
			Post-operative Skin and soft tissue wounds	Hand facture open wound (05), leg fracture open wound (07), cut nose stitches (02), cuts on hand skin (02), deep cuts on muscle of leg (02), cut on forehead stitches (02), abdomen stitches (01)	
2	Animal (Cattle, buffaloes, and dogs of either sex and of all age group presented with superficial pyogenic infections)	Veterinary Clinic Complex, College of Veterinary Science and A.H., DUVASU, Mathura	Skin and soft tissue wounds	Pus from thigh (4), paws (5), leg (5), scrotum pus (2), tail necrosis pus (1), toe (6), tail amputation (5), head (5), neck (4), lower abdomen (3), teat (8)	50
			Post- operative Skin and soft tissue wounds	Abdomen stitches (01), inguinal region (01)	
			Total		250

Table 2. Details of the PCR primers used for detection of biofilm formation associated genes.

Target Gene	Primer Sequence 5' to 3'	Product size (bp)	Reference
*ica*A	F- CCTAACTAACGAAAGGTAG R-AAGATATAGCGATAAGTGC	1351 bp	[30]
*ica*D	F- AAACGTAAGAGAGGTGG R-GGCAATATGATCAAGATAC	381 bp	[30]
*clf*A	F- CCGGATCCGTAGCTGCAGATGCACC R-GCTCTAGATCACTCATCAGGTTGTTCAGG	1000 bp	[29]
Bap	F- CCCTATATCGAAGGTGTAGAATTG R- GCTGTTGAAGTTAATACTGTACCTGC	971 bp	[30]
*fnb*A	F- GATACAAACCCAGGTGGTGG R- TGTGCTTGACCATGCTCTTC	191 bp	[29]
cna	F- AAAGCGTTGCCTAGTGGAGAC R- AGTGCCTTCCCAAACCTTTT	192 bp	[29]

Table 3. Prevalence of biofilm production in *S. epidermidis* isolates.

Origin	Number of Samples	*S. epidermidis* positive	Prevalence of Biofilm								
			Congo Red Agar method			Tube adherence Method			Tissue Culture Plate Method		
			S	M	W	S	M	W	S	M	W
Human	200	20	7	9	4	11	6	3	5	11	4
	Percentage		35	45	20	55	30	15	25	55	20
Animal	50	17	9	4	4	9	4	4	8	6	3
	Percentage		53	24	24	53	24	24	47	35	18
Total percentage (%)			43.24	35.13	21.63	54.05	27.02	18.92	35.14	45.94	18.92
Total positive/negative (%)			78.37		21.63	81.08		18.92	81.08		18.92

* S- Strong, M- Moderate, and W- Weak biofilm producers/Biofilm non-producer.

Table 4. PCR amplification results of biofilm associated genes.

Isolate*	*icaA* gene	*ica*D gene	*fnb*A gene	*cna* gene	*clf*A gene	*bap* gene	PCR positive for biofilm
H1.	-	-	+	-	-	+	+
H2.	-	-	-	-	+	-	+
H3.	-	-	-	-	-	-	-
H4.	-	+	-	+	-	-	+
H5.	-	-	+	-	-	-	+
H6.	-	-	+	-	-	-	+
H7.	-	-	-	-	-	-	-
H8.	-	-	-	-	-	-	-
H9.	-	-	-	-	-	-	-
H10.	-	-	+	+	-	-	+
H11.	-	-	-	+	+	+	+
H12.	-	+	-	+	-	-	+
H13.	-	+	-	+	+	-	+
H14.	-	-	-	+	-	-	+
H15.	-	-	+	-	-	-	+
H16.	-	+	-	-	-	-	+
H17.	-	+	-	-	+	+	+
H18.	-	-	-	-	+	-	+
H19.	-	-	-	-	-	-	-
H20.	-	+	-	+	+	+	+
Total	0	6	5	7	6	4	15
A1.	-	+	+	-	-	-	+
A2.	-	-	-	-	-	-	-
A3.	-	-	-	-	-	-	-
A4.	-	-	-	-	-	-	-
A5.	-	-	-	-	-	-	-
A6.	-	-	-	-	-	-	-
A7.	-	+	-	-	-	-	+
A8.	-	-	-	-	-	-	-
A9.	-	-	-	-	-	-	-
A10.	-	-	-	-	-	-	-
A11.	-	-	-	-	-	-	-
A12.	-	-	-	-	-	-	-
A13.	-	+	-	+	+	+	+
A14.	-	-	-	+	-	-	+
A15.	-	-	+	-	-	-	+
A16.	-	+	-	-	+	-	+
A17.	-	-	-	-	-	-	-
Total	0	4	2	2	2	1	6
Overall total	0	10	7	9	8	5	21

* H=Human origin isolates; A=Animal origin isolates.

Table 5. Comparative results of biofilm formation assessment by phenotypic and genetic methods.

Isolate ID	Phenotypic Method (Tissue culture plate method)	Genetic Method (PCR amplified gene)
H1	Moderate	*fnb*A, *bap*
H2	Moderate	*clf*A
H4	Strong	*ica*D, *cna*
H5	Strong	*fnb*A
H6	Moderate	*fnb*A
H10	Weak	*fnb*A, *cna*
H11	Moderate	*cna*, *clf*A, *bap*
H12	Moderate	*ica*D, *cna*
H13	Moderate	*ica*D, *cna*, *clf*A
H14	Moderate	*cna*
H15	Weak	*fnb*A
H16	Weak	*ica*D
H17	Strong	*ica*D, *clf*A, *bap*
H18	Moderate	*clf*A
H20	Weak	*ica*D, *cna*, *clf*A, *bap*
A1	Strong	*ica*D, *fnb*A
A7	Weak	*ica*D
A13	Moderate	*ica*D, *cna*, *clf*A, *bap*
A14	Strong	*cna*
A15	Moderate	*fnb*A
A16	Strong	*ica*D, *clf*A

positive amplification of the genus-specific 933 bp of the *gap* gene, confirming them as *Staphylococcus* (Fig. 1). Among these 50 *gap* gene - positive *Staphylococcus* isolates, 31 isolates were of human origin, while 19 isolates were of animal origin, corresponding to a *Staphylococcus* sps prevalence rate of 20.0% among the total pus samples under investigation, while the prevalence was 15.5% among human samples and 38.0% among animal samples. In PCR amplification of *S. epidermidis* specific sequence, 37 isolates revealed the desired amplification of 124 bp among 20 isolates of human and 17 isolates of animal sample origin (Fig. 2), corresponding to an overall 14.8% prevalence of *S. epidermidis* with 10.0% prevalence among human samples and 34.0% prevalence among animal samples. All the *S. epidermidis* isolates subjected to assessment of biofilm production by phenotypic and genetic methods reveal variable biofilm production in different methods used (Table 3). Among the 20 human-origin *S. epidermidis* isolates tested, 7 (35%) appeared to be strong biofilm producers, 9 (45%) moderate biofilm producers, and 4 (20%) weak biofilm producers in the CRA method

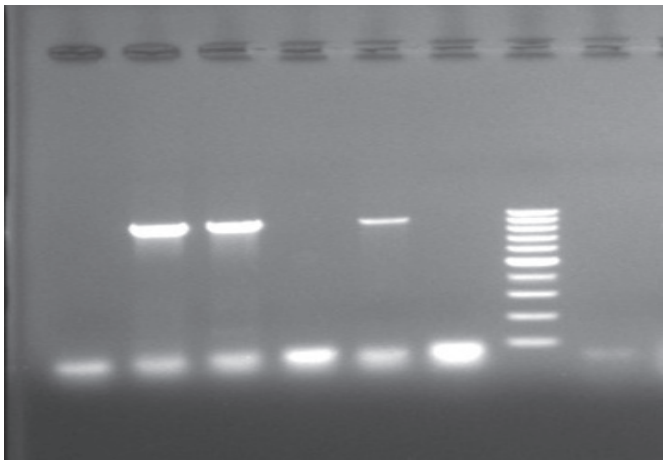

Fig. 1. AGE (1.5%) of *gap* gene PCR amplification (933 bp) of representative sample with 100 bp DNA marker.

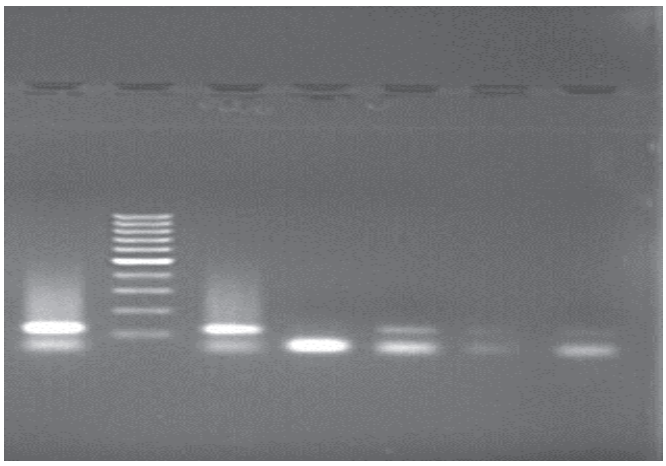

Fig. 2. AGE (1.5%) of *16S rDNA* gene PCR amplification (124 bp) of representative sample with 100 bp DNA marker.

(Fig. 3). In the tube adherence method, 11 isolates (55%) were found to be strong biofilm producers; 6 (30%) isolates were moderate biofilm producers; and 3 isolates (15%) were weak biofilm producers, while in the tissue culture plate method, 5 (25%), 11 (55%), and 4 (20%) isolates revealed strong biofilm, moderate biofilm, and weak biofilm production. Similarly, in the case of animal origin, of 17 *S. epidermidis* isolates, 53% (9/17) were found to be strong biofilm producers, 24% (4/17) moderate biofilm producers, and 24% (4/17) weak biofilm producers in both the CRA and tube adherence methods, whereas in the tissue culture plate method, 47% (8/17) showed strong biofilm production, 35% (6/17) moderate biofilm production, and 18% (3/17) weak biofilm production.

In *S. epidermidis*, biofilm formation is governed by the presence of many different genes. In the present

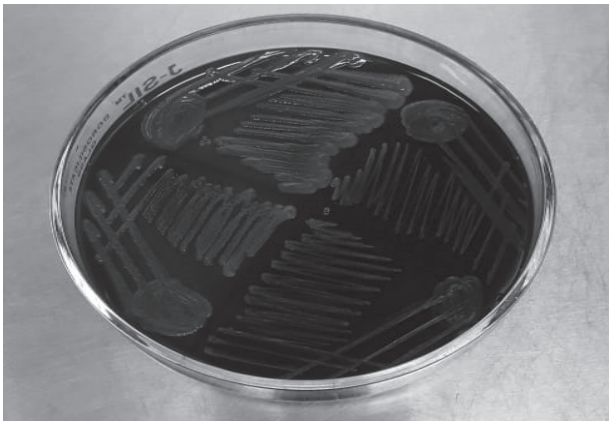

Fig. 3. Representative *S. epidermidis* isolates growth on Congo red agar at 37 ºC after 18 hr incubation. (Black colour colonies represents biofilm producer while red colour colonies represents non-biofim producer).

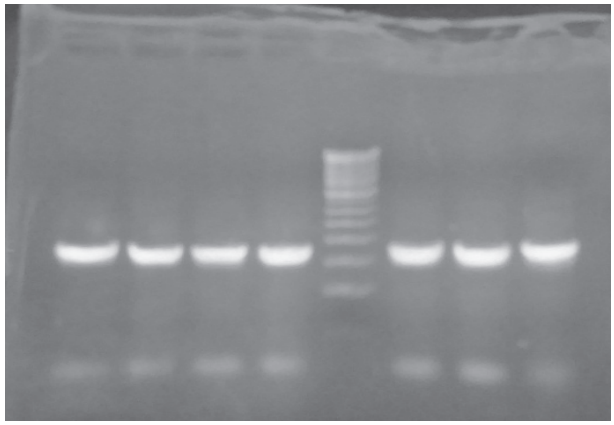

Fig. 4. AGE (1.5%) of PCR amplification of *ica*D gene (381 bp) of representative samples with 100 bp DNA marker.

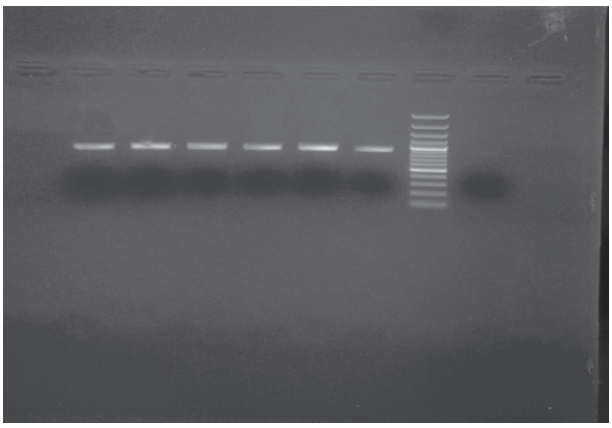

Fig. 5. AGE (1.5%) of PCR amplification of *clf*A gene (381 bp) of representative samples with 100 bp plus DNA marker.

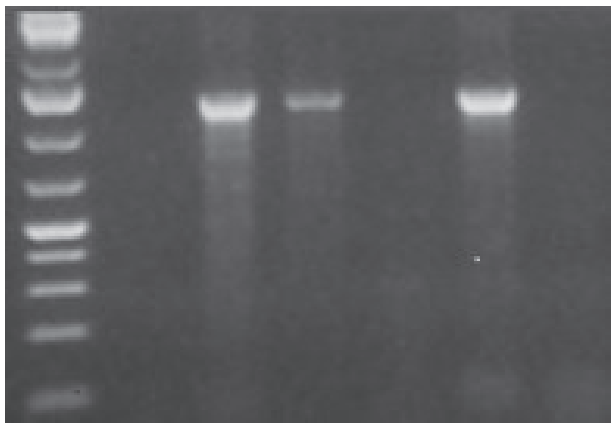

Fig. 6. AGE (1.5%) of PCR amplification of *bap* gene (971 bp) of representative samples with 100 bp DNA marker.

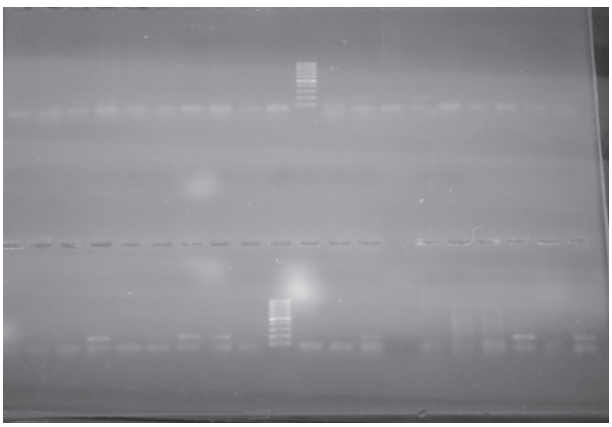

Fig. 7. AGE (1.5%) of PCR amplification of *fnb*A gene (191 bp) of representative samples with 100 bp DNA marker.

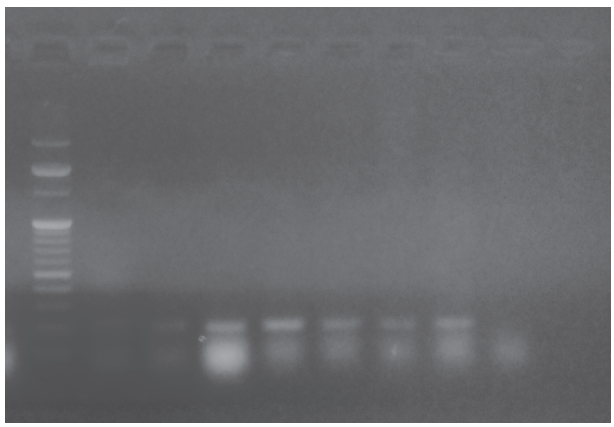

Fig. 8. AGE (1.5%) of PCR amplification of *cna* gene (192 bp) of representative samples with 100 plus bp DNA marker.

study, PCR amplification of six genes associated with biofilm formation was attempted. The amplification of *ica*A (1351 bp), *ica*D (381 bp), *bap* (971 bp), *cna* (192 bp), *fnb*A (191 bp), and *clf*A (1000 bp) genes from genomic DNA was considered positive for the presence of biofilm formation capability (Fig. 4 to Fig. 8). In PCR amplification of biofilm formation-associated genes, 5 out of 20 human-origin isolates and 11 out of 17 animal-origin isolates did not show amplification for any of the genes under investigation, while the remaining 15 human-origin and 6 animal-origin isolates were positive for one or the other gene(s) associated with biofilm formation (Table 4). Among the PCR-positive 15 human-origin isolates, 7 isolates revealed the presence of only 1 gene, 4 isolates revealed the presence of 2 genes, 3 isolates revealed the presence of 3 genes, and one isolate showed the presence of 4 genes. Similarly, in the case of PCR-positive animal origin isolates (N = 6), 3 revealed the presence of only one gene, 2 isolates revealed the presence of two genes, and one isolate showed the presence of four genes. The *cna* gene was the most commonly found among the human-origin isolates, followed by *ica*D and the *clf*A gene. In the case of animal-origin isolates, the *ica*D gene was the most amplified, followed by the *cna*, *clf*, and *fnb*A genes. None of the isolates under study showed amplification for the *ica*A gene, while the *ica*D gene was amplified in 6 human and 4 animal isolates. The *cna* gene was amplified in 9 isolates, comprising 7 human isolates and 2 animal isolates, while the *clf*A gene was amplified in 8 isolates, comprising 6 human isolates and 2 animal isolates. The *bap* genes were found in 5 isolates, including 4 human and 1 animal isolate, and the *fnb*A genes were amplified in 7 isolates, including 5 human and 2 animal isolates. Overall, the presence of the *ica*D gene was highest, followed by the *clf*A, *cna*, *fnb*A, and *bap* genes, with percent positivity of 27.02%, 21.62%, 21.62%, 18.91%, and 13.51%, respectively. Also, it was observed that the biofilm formation-associated genes were amplified more in number in human-origin isolates compared to isolates of animal origin. Further, the presence of these genes in PCR amplification was found to be more diversified among the human isolates as well. A comparison of the biofilm assessment by the microtiter method and PCR amplification of biofilm formation-associated genes is mentioned in Table 5.

The prevalence of *S. epidermidis* among human samples was 10.0%, and a 34.0% prevalence was observed among animal samples, with an overall 14.8% prevalence among the total pus samples under investigation. Similarly, Wojtyczka *et al.* [31] investigated samples collected from hospital environments, and among the 122 samples positive for CoNS culture, 32 isolates were identified as *S. epidermidis*. Villari *et al.* [32] found 56 isolates out of 184 infections from the NICU over three years, comprising 35 (39.80%) isolates from bloodstream infection and 14 (29.80%) isolates of *S. epidermidis* from surface infection. However, Deplano *et al.* [33] have reported a high prevalence of *S. epidermidis* (76.0%) among bloodstream infections. Furthermore, Ayeni [34] reported the isolation of *S. epidermidis* in 33% of neonates' blood samples. *S. epidermidis* can adhere to plastic surfaces and medical devices by producing exopolysaccharides, forming biofilm, which is one of the emerging challenges for clinicians [35, 36]. The biofilm formation ability of the bacteria can be assessed by several methods, such as the CRA method, the TA method, and the TCP method. Previous research has found that the *S. epidermidis* isolates recovered from catheter segments show extreme biofilm production compared to the isolates from urine samples [12]. Differing, Solati *et al.* [37] reported a higher percentage of high biofilm production in urinary isolates than that from dialysis catheters. In the present study, the *S. epidermidis* isolates recovered from different clinical conditions such as post-operative wounds, skin and soft tissue wounds, abscesses, etc. were also found to exhibit diverse biofilm formation abilities in different isolates. Similar to the present study, Agarwal and Chaudhary [38] investigated urine, pus, sputum, and blood samples from a medical college and found 59.6% biofilm-forming isolates, whereas Samant and Pae [39] observed 42.7% of *Staphylococcus* isolates to be biofilm producers. Jayachandran *et al.* [40] reported that 46% of *Staphylococci* spp. from various clinical isolates were biofilm producers. Similarly, Apurva *et al.* [41] reported 52.6% of *S. aureus* with biofilm formation ability.

The present study revealed about 80% of *S. epidermidis* isolates as biofilm producers (CRA, 78.37%; TA and TCP, 81.08%) with varying intensities (43.24% strong, 35.13% moderate, 21.63% weak/non; 54.05% strong, 27.02% moderate, 18.52% weak/non, and 35.14% strong, 45.94% moderate, 18.92% weak/non-biofilm producers in CRA, TA, and TCM methods, respectively) among isolates by all three methods employed for phenotypic assessment of biofilm formation. Similarly, Saising *et al.* [42] found 65.38%, 55.76%, and 51.92% biofilm-forming isolates in the

TCP, CRA, and TA methods, respectively. Fadl *et al.* [12] found 56.6%, 30.2%, and 13.2% of staphylococcal strains, and Farran [43] reported 12%, 79.4%, and 8.6% of isolates as high, moderate, and non-biofilm producers, respectively. Likewise, Ansari *et al.* [44] observed that 30% of the *S. epidermidis* isolates were strongly positive for biofilm production, while the remaining 40% were moderate and 30% were weak or non-biofilm producers by the TCP assay method. Kaiser *et al.* [27] found 10% moderate biofilm producers in the CRA and modified CRA methods, while 4% in the new modified CRA method. However, Khudhur [45] reported no difference between CRA and modified CRA methods. Furthermore, Hassan [46] reported 49% of isolates as biofilm producers and 51% as non-biofilm producers in the TA method. In the TCP method also, the findings of the present study corroborate with the findings of Deka [28], who reported around 83% of isolates to be biofilm producing, and Nasr [47], who reported 88.6% of *S. epidermidis* isolated from catheters to have biofilm formation ability, while Mathur *et al.* [48] reported 36% strong producers and 16% moderate producers. It is reported that *ica*ABCD operon genes' presence in the staphylococcal genomes is associated with biofilm formation ability [49]. In the present study, only the *ica*D gene was amplified in the *S. epidermidis* isolates (27.02%). However, about 80% of the isolates were found to be biofilm-forming in the phenotypic methods (CRA, 78.37%; tube adherence method; and TCP, 81.08%). Similarly, Wojtyczka *et al.* [31] found 37.5% (12/32) *S. epidermidis* isolates as biofilm formers by the TCP method, but none of the isolates were positive by the CRA method, whereas 49.9% of isolates (15/32) were positive for the presence of the *ica*ADBC operon, among which 34.4%, 28.1%, 37.5%, and 21.9% were positive for the *ica*A, *ica*D, *ica*C, and *ica*B genes, respectively.

Tektook *et al.* [50] found the presence of the IcaA gene in 34% of blood cultures and 20% of catheter urine specimens, while the *ica*D gene was found in 38% of blood cultures and 20% of catheter urine specimens. Unlike this, Frebourg *et al.* [51] found some *ica*-positive strains failed to produce biofilm. Apart from the *ica*ABCD operon, various other genes are also associated with the proteins responsible for the formation of biofilm. In the present study, four more genes, *viz. cna*, *fnb*A, *clf*A, and *bap*, were PCR screened, which revealed the presence of the *cna* gene in 24.32%, the *fnb*A gene in 18.91% of the isolates, whereas *clf*A was found in 21.62%, and the *bap* gene was found in 13.51% of the isolates. Similarly, Kumar *et al.* [52] found the presence of *clf*A in 84% and *clf*B in 52%, while *fnb*A was found in 60% and *fnb*B was found in 40% of the isolates of Staphylococcus. Achek *et al.* [53] have reported the presence of the *fnb*A gene in 40% of the *S. aureus* isolates, while none of the isolates showed *cna* gene amplification in samples from sheep mastitis cases. Similarly, Mohammadi *et al.* [54] found 74.7% *cna*, 50.6% *clf* A, and 42.1% *fnb*A genes among the *S. aureus* isolates from hospital-acquired infections. The different percentages of biofilm-forming isolates reported in different studies can be attributed to the multiple factors responsible for the expression of different proteins responsible for biofilm, in parallel with the different investigation methodologies, species, and sources of clinical samples used in different studies.

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