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Characterization of isolates of *Staphylococcus aureus* for biofilm formation in goats of Aizawl, Mizoram

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ABSTRACT

Staphylococcus aureus are the inhabitants of intricate biofilms holding multiple layers of bacteria embedded inside its extracellular matrix. The development of biofilms is a substantial factor that contributes to the failure of treatments. It is recommended to employ a combination of phenotypic and genotypic approaches in the study of biofilm formation in *S. aureus*. Among the 43 *S. aureus* isolates obtained from goats, both culturally and genotypically characterized, it was shown that 14 isolates (32.55%) exhibited the presence of the biofilm-producing gene *icaD*, while none of the isolates tested positive for the presence of *icaA*. However, out of the total 32 isolates, which accounts for approximately 74.41% of the sample, biofilm formation was seen using the crystal violet tube adherence method. The findings of this study demonstrate a notable prevalence of the ica genes among *Staphylococcus aureus* isolates derived from goats. Furthermore, it is seen that the presence of these genes does not consistently correlate with the in vitro development of biofilm. The capacity to produce biofilms can result in treatment inefficacy, posing a significant risk to public health and animal welfare, while also causing financial losses for farmers.

Keywords: *icaA*, *icaD*, Goat, Mizoram, *S. aureus*

Staphylococcus aureus is a prevalent opportunistic pathogen that has the ability to infect several hosts, including people, domestic animals, and wild animals (Monecke *et al.* 2016). *Staphylococcus aureus* is a significant zoonotic pathogen that causes substantial economic losses in the animal industry and poses a public health danger. The significance of the *icaA* and *icaD* genes in the formation of biofilms has been observed in *S. aureus* and *S. epidermidis* (Tormo *et al.* 2005). The development of biofilms is a substantial factor that contributes to the failure of treatments. The production of biofilm is considered a significant virulence component that not only shields bacteria from human defence mechanisms but also provides protection against antimicrobial agents (Flemming *et al.* 2016). It is recommended to employ a combination of phenotypic and genotypic approaches when studying the formation of biofilms in *S. aureus* (Vasudevan *et al.* 2003).

The presence of *S. aureus* in livestock is of great importance due to its potential to be transmitted to people, hence posing a considerable zoonotic risk. This transmission can occur through the consumption of contaminated animal products as well as through occupational contact, as highlighted by Smith (2015). Limited data is currently accessible about the occurrence of biofilm-producing *S. aureus* in goats within the Indian context. Currently, there is a lack of research about the documentation and comprehensive analysis of the isolation, molecular, and phenotypic characterization of *S. aureus* for biofilm formation in goats from Mizoram. The primary focus of this work was the isolation and molecular characterization of *Staphylococcus aureus* from goats that appeared to be in good health in the Aizawl region of Mizoram.

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MATERIALS AND METHODS

Sample collection and S. aureus isolation: A total of one hundred goat samples were obtained from various organized and unorganized farms as well as slaughterhouses located in different regions of Aizawl. Following the collecting process, each sample was appropriately labelled to indicate its code, date of collection, and other relevant details. Subsequently, all the samples were carefully stored in thermocol boxes that were equipped with ice packs. These boxes were then transported to the departmental laboratory to undergo further processing. The nasal swab samples were subjected to enrichment in peptone water enrichment broth for a duration of 24 hours at a temperature of 37°C. Subsequently, these samples were inoculated onto Baird Parker agar (BPA) plates for cultivation at a temperature of 37°C for a period of 48 hours. The colonies obtained from BPA were streaked onto a plate containing Mannitol Salt agar (MSA) and thereafter incubated at a temperature

of 37°C for a duration of 24 hours. The identification of the colonies presumed to be *S. aureus* was conducted through the utilization of Gram staining, catalase testing, and coagulase testing. Subsequent to the identification of presumptive colonies of *S. aureus*, subculturing was performed and confirmation was achieved through PCR amplification of the *nuc* gene (a gene specific for *S. aureus* encoding thermonuclease). PCR oligonucleotide primers utilized in this work is shown in Table 1. The PCR amplification was carried out under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was conducted at 72°C for 5 minutes.

Detection of biofilm-producing genes (ica genes): The bacterial DNA lysate of the *S. aureus* isolates exhibiting positive phenotypic characteristics was obtained through the utilization of boiling and snap chilling techniques. The PCR amplification of the *icaA* and *icaD* genes was conducted using the following conditions: an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 4 seconds, annealing at 59°C for 45 seconds, and extension at 72°C for 1 minute. The amplification process concluded with a final elongation step at 72°C for 5 minutes.

Detection of Biofilm-Production by the Tube-Adherence Method: The tube-adherence technique was employed to evaluate the formation of biofilms, as described by Dumaru *et al.* (2019). The bacterial strains were injected into glass tubes containing 1 mL of sterile trypticase soy broth after overnight cultures were prepared. Subsequently, the tubes were incubated under static conditions for a duration of 24 hours at a temperature of 37°C. Following the incubation period, the cells that had adhered were subjected to three consecutive rinses using phosphate buffer saline (PBS). Subsequently, the supernatant was removed, and the tubes were dried using a paper towel. The biomass that adhered to the tubes was stained using a 1 mL solution of 0.1% crystal violet (CV). The solution was applied to the contents of the tubes and left to incubate for a duration of 3 hours at ambient temperature. Following the decision to exclude the CV solution, the tubes were subjected to an additional series of three rinses using PBS. Subsequently, the tubes were dried using a paper towel. In this experiment, the determination of a strain's biofilm production capability was conducted by the utilization of visual observation of biofilm formation. The identification of a biofilm-producing strain was based on the presence of a visible biofilm lining on the bottom and inner wall of the glass tube.

RESULTS AND DISCUSSION

The frequency of biofilm-forming *S. aureus* in ostensibly healthy goats residing in Aizawl, Mizoram, was investigated in the present study. Using biochemical and cultural methods, a total of 91 staphylococci strains were isolated from 128 samples. As illustrated in Fig. 1, nucleic acid amplification was employed to validate 43 isolates (47.25%) among the 91 strains that were examined. The confirmed isolates consisted of 38 samples obtained from nasal swabs, 3 samples obtained from preputial samples, and 2 samples obtained from goat udders (Table 2). Fig. 2. illustrates the identification of the *icaD* gene in 14 goat isolates. As shown in Table 3. in contrast, the *icaA* gene was absent from each of the isolates.

Concerning the development of biofilms, Tormo *et al.* (2005) have identified the importance of the *icaA* and *icaD* genes in *S. aureus* and *S. epidermidis*. Lira *et al.* (2016) recognized the existence of the *icaD* gene in 82% of the isolates procured from goat dairy plants and classified as biofilm producers via the Congo Red Agar (CRA) method, which contradicts the results of the present inquiry. The CRA technique and the identification of the *icaD* gene exhibited a significant degree of concurrence (K = 0.79), as evidenced by this observation. Additionally, Andrade *et al.* (2021) noted that one-fifth of the samples, or five isolates in total, displayed the simultaneous presence of the *icaA* and *icaD* genes. This discovery was consistent with the data presented in the present study regarding the frequency

Table 2. Prevalence of *S. aureus* from different samples of goats in Aizawl, Mizoram

Sample	No. of samples	No. of samples positive for *S. aureus* (*nuc* gene)
Goat nasal swabs	100	38 (38%)[a]
Preputial swabs	18	3 (16.66%)[b]
Udder swabs	10	2 (20%)[b]
Total	128	43 (47.25%)

(Figures in parenthesis are percentages) On Statistical analysis, (Chi-square test) there was a significant difference ($p<0.05$) between pair-wise comparison in the prevalence of *S. aureus* between goat nasal swabs and Preputial swabs while, there was no significant difference ($p>0.05$) between preputial swabs and udder swabs in Aizawl, Mizoram.

Table 1. Oligonucleotide PCR primers used in the study

Target gene	Primer sequence (5'-3')	Amplicon size	Reference
nuc	F-GCG ATT GAT GGT GAT ACG GTT R-AGC CAA DCC TTG ACG AAC TAA AGC	279 bp	Brakstad *et al.* (1992)
icaA	F- ACA GTC GCT ACG AAA AGA AA R- GGA AAT GCC ATA ATG ACA AC	103 bp	Satorres and Alcaráz (2007)
icaD	F- ATG GTC AAG CCC AGA CAG AG R- CGT GTT TTC AAC ATT TAA TGC AA	198 bp	

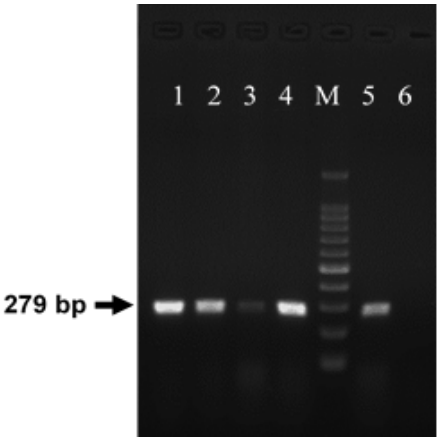

Fig. 1. Gel electrophoresis of amplified nuc in S. aureus isolated from goats in Aizawl, Mizoram. M: 100 bp marker; L1–L5: the nuc gene of some S. aureus isolates, amplified at 279 bp; L6: Negative control

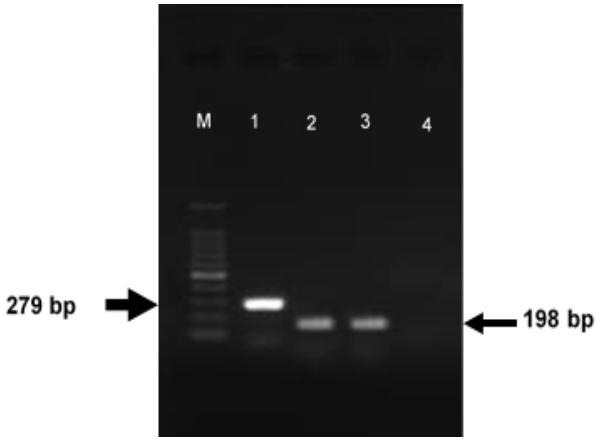

Fig. 2. Gel electrophoresis of amplified *ica*D in *S. aureus* isolated from goats of Aizawl, Mizoram. M: 100 bp marker; L1: the *nuc* (279 bp) gene of *S. aureus* isolate; L2 & L3: *ica*D(198 bp) gene of *S. aureus* isolate; L4: Negative control

Table 3. Detection of *icaA* and *icaD* genes from goats

Biofilm producing gene	No. of isolates positive for biofilm-producing genes (n=43)
icaA	0 (0%)
icaD	14 (32.55%)

of the *icaD* gene. The present investigation, similar to the results reported by Xu *et al.* (2015), confirmed that the *icaA* gene was not present in any of the staphylococcal isolates examined. Additionally, in comparison to the results of the current inquiry, the authors of the previously mentioned study documented a considerably higher prevalence of the *icaD* gene, amounting to 71.43%. (Tormo *et al.*, 2005) attribute the discrepancy in prevalence rates to variations in DNA sequences, which may result in inadequate gene amplification and erroneous negative outcomes.

The omission of the results pertaining to the genes *ica*A and *ica*D was observed in a prior investigation carried out by Simojoki *et al.* (2012). The reason for this exclusion was that their primers were incapable of detecting these genes in *S. aureus* isolates but could discern them in confirmed *icaA* and *icaD*-positive strains of *S. epidermidis*.

A biofilm lining was observed on the inner wall and bottom of the glass tube for 32 isolates (74.41%), which indicates their capacity to produce biofilms, as determined by the present study. Likewise, a phenotypic trait of biofilm formation was observed in 24 strains (68.57%) of the 35 *S. aureus* isolates that were responsible for causing mastitis. As indicated by the positive amplification of the *ica* locus and the presence of the *icaA* and *icaD* genes, additional research revealed that these strains contained the genetic elements associated with biofilm formation (Vasudevan *et al.* 2002). The outcomes developed in the current investigation are in opposition to this discovery. Salimena *et al.* (2016) determined that the genes *icaA* and *icaD* were present in every single isolate under investigation. As demonstrated, however, biofilm formation capability was observed in only 97.5% of these isolates.

As determined by the tube-adherence method, the correlation between *icaA* and *icaD* detection and biofilm formation was insufficient. An independent control of the *ica* gene on slime generation and adhesion mechanism, as demonstrated by Liberto *et al.* (2009), can explain the presence of *icaA* or *icaD* negative biofilm positive isolates.

On the contrary, the failure of *Staphylococcus* isolates to form biofilm under laboratory conditions despite testing positive for the *icaA* and/or *icaD* genes may be a result of point mutations within the locus or other unidentified factors that prevent the synthesis of polysaccharide intercellular adhesion or the biofilm formation process (Cramton *et al.* 1999). Additionally, prior studies have demonstrated that the lack of biofilm phenotypic expression may be a result of *icaA, icaD*, or both mRNAs being lost (Liberto *et al.* 2009). In conclusion, it can be stated that the goat population of Aizawl, Mizoram are infested with biofilm-producing multidrug resistant *S. aureus*. Among the *S. aureus* strains isolated, majority of them were positive for biofilm associated *icaD* gene responsible for production of polysaccharide intercellular adhesin (PIA). As the same *S. aureus* strains are equally pathogenic for human, it is important to maintain good hygienic practices during slaughter and marketing of chevon to prevent further transmission of such zoonotic pathogen among the human population.

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