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# Profiling of clumping factors A and B of *Staphylococcus aureus* isolated from caprine mastitis

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## Abstract
Mastitis is a common and economically important disease in goats, causing significant losses in the dairy sector due to reduced milk production, lower milk quality, and higher treatment costs. *Staphylococcus aureus* is a well-known pathogen responsible for both symptomatic and asymptomatic mastitis in goats worldwide. In this study, we investigated 34 *S. aureus* isolates from 57 milk samples collected from untreated goats with clinical mastitis in and around the Bikaner city region. The recovery rate of *S. aureus* isolates was found to be 59.64%.

We found that 20 isolates (58.82%) carried both the *clfA* and *clfB* genes, while two isolates (8.82%) lacked either of these genes. Among the total isolates, nine (26.41%) showed a 205 bp amplicon, indicating the presence of the *clfB* gene, and three isolates (8.82%) showed a 1000 bp amplicon, suggesting the presence of the *clfA* gene. The prevalence rates of clfA and *clfB* genes were 67.64% and 85.29%, respectively. Notably, 94.11% of the isolates harbored clumping factor genes. No variations were observed in the *clfA* and *clfB* genes among the studied samples. These findings provide valuable insights into the genetic characteristics and prevalence of clumping factors A and B (*clfA* and *clfB* genes) in *S. aureus* isolates associated with goat mastitis in the studied region.

**Keywords:** Mastitis, *Staphylococcus aureus*, goat, *clfA* and *clfB* genes

## Introduction
Goats have been domesticated since before 7000 B.C. and have the widest geographical range among livestock species (Banerjee, 1998) [5]. Goat milk causes fewer allergic reactions compared to milk from other animals (Acharya, 1992) and has several nutritional benefits, including lower fat content and higher protein and mineral levels (Bhattacharya, 2002) [1]. Goats play a crucial role in the agriculture of developing nations, providing meat, milk, fiber, skin, and contributing significantly to the economy. In Rajasthan, particularly in the arid western region, goats are of significant importance. They are raised for milk consumption locally and meat export to other parts of the nation (Nathawat *et al.*, 2015) [22]. Mastitis is a significant disease affecting goats in this region, leading to losses in milk yield, quality, and increased production expenses (Nickerson *et al.*, 1995; Dego *et al.*, 2002) [23, 10]. Untreated or poorly treated mastitis can render goats ineffective for milk production.

*Staphylococcus aureus* has been identified as the main pathogen responsible for both subclinical and clinical mastitis in goats worldwide (Bergonier *et al.*, 2003; Ibrahim *et al.*, 2009; Aras *et al.*, 2012) [6, 3, 14]. Severe cases can lead to gangrene, characterized by abscesses, pus drainage, and systemic toxemia symptoms (Sarker *et al.*, 2015) [33]. *S. aureus* produces various virulence components contributing to mastitis development, but not all strains produce the same virulence factors (Balaban and Rasooly, 2000) [4]. The adherence of *S. aureus* to the teat canal epithelium depends on two clumping factors, *clfA* and *clfB*, present on the bacterial surface (Stutz *et al.*, 2010) [37]. Clumping factor A (*clfA*) increases virulence and colonization during animal infection, making it a potential option for vaccination (Karahan *et al.*, 2011) [16]. Antibodies against *clfA* can enhance defense against infection, and the *clfA* gene has been linked to the pathogenicity of mastitis in cattle (Stephan *et al.*, 2001; Salasia *et al.*, 2004) [35, 31].

## Materials and Methods
In this research, a total of 57 milk samples were collected from goats with a mastitic condition. These goats were owned by different farmers living near Bikaner city. Each milk sample was about 5-10 milliliters and was promptly transferred to clean test tubes. The samples were then kept on ice and taken to the laboratory for further examination.

The scientists isolated and identified the organisms present in the samples using specific methods described by Cowan and Steel's and Quinn *et al.,* (1994) [29].

To make sure the presence of a particular bacterium called *Staphylococcus aureus* and conduct a genetic analysis, the researchers used a method called ribotyping. They used a specific primer that targeted the 23S rRNA gene, following the guidelines outlined by Straub *et al.,* (1999) [36]. This technique helped them accurately identify and distinguish between different strains of the *Staphylococcus aureus* bacterium.

**(i) *clfA* gene amplification:**

We followed a method described by Stephan *et al.,* (2001) [35] to amplify the *clfA* gene. This involved using two specific sequences called Primer 1 (5' GGC TTC AGT GCT TGT AGG 3') and Primer 2 (5' TTT TCA GGG TCA ATA TAA G 3'). For the amplification process, we prepared a mixture with a total volume of 25 µl. The mixture contained 12.8 µl of de-ionized water, 5 µl of 10x buffer, 2.5 µl of $MgCl_2$, 0.5 µl each of Primer-1 and Primer-2 (both 10 pM/µl), 3 µl of DNA (25 ng/µl), 0.5 µl of dNTP mix (10 mM), and 0.5 µl of Taq DNA polymerase. To conduct the PCR, we used a Veriti Thermal Cycler (Applied Biosystems) and followed specific temperature and time cycles. These cycles included an initial denaturation step at 94 °C for four minutes, followed by 35 cycles of denaturation at 94 °C for 60 seconds, annealing at 57 °C for 60 seconds, and extension at 72 °C for 60 seconds. After the cycles, there was a final extension incubation at 72 °C for ten minutes. We then separated the amplified products on a 1.2% agarose gel with 0.5 g/ml ethidium bromide in 1x TBE buffer. Electrophoresis was conducted at 100 V for 60 minutes, and we used the Endura GDS gel documentation system to visualize the gel. To determine the size of the amplified DNA, we used a 500 bp ladder as a molecular marker.

**(ii) *clfB* gene amplification**

We amplified the *clfB* gene using a method described by Tristan *et al.,* (2003) [38]. To do this, we used two specific sequences called Primer 1 (5'-ACA TCA GTA ATA GTA GGG GGC AAC-3') and Primer 2 (5'-TTC GCA CTG TTT GTG TTT GCA C-3'). To prepare the mixture for the amplification process, we took a total volume of 25 µl, similar to the clfA gene amplification. The mixture was carefully assembled following the same steps. The PCR amplification was conducted using a Veriti Thermal Cycler (Applied Biosystems). The process involved several cycles with specific temperature and time conditions. First, there was an initial denaturation step at 94 °C for five minutes. Then, we performed 30 cycles of denaturation at 94 °C for 60 seconds, annealing at 55 °C for 60 seconds, and extension at 72 °C for 60 seconds. After the cycles, there was a final extension incubation step at 72 °C for ten minutes. We separated the products of the PCR on a gel using a 100 bp molecular marker and followed the same electrophoresis gel parameters used for the clfA gene amplification.

**Results and Discussion**

We conducted a study on 57 milk samples taken from goats with clinical mastitis in and around the city of Bikaner. From these samples, we found 34 *S. aureus* isolates, which accounts for about 59.64% of the total samples. *S. aureus* is a harmful germ that can stick to various parts of the body, like proteins found in the extracellular matrix. This sticking ability is helped by certain proteins on the bacterial cell wall, specifically clumping factors A and B, which are controlled by genes *clfA* and *clfB*, respectively.

Our analysis of the *Staphylococcus aureus* isolates showed that about 58.82% of them had both *clfA* and *clfB* genes, while a small number (8.82%) lacked either of these genes (Fig. 1, 2). Additionally, some isolates (26.41%) had a particular gene called *clfB*, as shown by an amplicon of 205 base pairs, and a few (8.82%) had another gene called *clfA*, as indicated by an amplicon of 1000 base pairs (Fig. 1, 2). We found that the *clfA* gene was present in 23 isolates, which accounts for about 67.64% of the total isolates. Similarly, the *clfB* gene was found in 29 isolates, making up around 85.29% of the total isolates. In total, about 94.11% of the isolates had these clumping factor genes. This information helps us understand how *S. aureus* can cause infections and may aid in finding ways to prevent or treat such infections in goats and other animals.

**Table 1:** Details of clumping factor associated genes in *S. aureus* isolates

| S. No | Target gene | Isolates ID | Prevalence of gene | Amplicon size (bp) of gene |
|---|---|---|---|---|
| 1. | *clfA* | GM4, GM20, GM22 | 3 (8.82%) | 1000 |
| 2. | *clfB* | GM2, GM6, GM9, GM12, GM13, GM26, GM28, GM32, GM33 | 9 (26.47%) | 205 |
| 3. | *clfA* and *clfB* both | GM1, GM3, GM5, GM7, GM8, GM10, GM11, GM14, GM16, GM17, GM18, GM19, GM21, GM23, GM24, GM25, GM27, GM30, GM31, GM34, | 20 (58.82%) | 1000 and 205 |
| 4. | *clf* gene negative | GM15, GM29 | 2 (5.88%) | - |

Our study's findings on *clfA* genes' prevalence align with other researchers who have reported similar observations in *S. aureus* isolates from various sources. For example, Yang *et al.,* (2012) found the *clfA* gene in 62% of *S. aureus* strains from bovine clinical mastitis, while Memon *et al.,* (2013) [20] reported a prevalence of 59% in *S. aureus* isolates associated with bovine mastitis. Fitzgerald *et al.,* (2000) [13] discovered the *clfA* gene in 75.5% of Irish *S. aureus* isolates from bovine intramammary infection and 71.4% of American isolates. Similarly, Momtaz *et al.,* (2010) [21] identified the *clfA* gene in 74.11% of *S. aureus* isolates from cattle's clinical and subclinical mastitic milk, whereas Yadav *et al.,* (2015) [41] reported a prevalence of 84.37% in *S. aureus* isolates from bovine milk with clinical mastitis.

Akineden *et al.,* (2008) [2] found the *clfA* gene present in 93.75% of *S. aureus* from goat milk cheese, while Nathawat *et al.,* (2015) [22] detected the gene in 92.59% of *S. aureus* isolates from goat clinical mastitis. Higher prevalences (100%) of *clfA* gene were reported by Stephan *et al.,* (2001) [35], Salasia *et al.,* (2004) [31], and Pereyra *et al.,* (2016) [26]. However, Li-li *et al.,* (2012) found the *clfA* gene in only 55.47% of the 137 bovine mastitis isolates they examined,

indicating a lower prevalence. Similarly, Klein *et al.,* (2012) [17] reported the gene in only 50.6% of bovine mastitis isolates, and observed a prevalence of 19.2% in *S. aureus* isolates from raw milk from Brazilian dairy farms.

As for *clfB* genes, our research found a prevalence of 85.29%, which is consistent with Xu *et al.,* (2015) findings, who reported an 85.7% prevalence among *S. aureus* isolates from cow mastitis milk samples. Machuca *et al.,* (2013) [19] found *clfB* genes in 87% of *S. aureus* isolates from human sources, while Klein *et al.,* (2012) [17] reported a higher prevalence of 91.80% in bovine mastitis isolates, and Ote *et al.,* (2011) [25] identified the gene in 96.9% of strains. Ionescu *et al.,* (2015) [17] reported a prevalence of 97.91% in *S. aureus* isolates from human sources. In contrast, Li-li *et al.,* (2012) found the *clfB* gene in only 36.49% of the 137 bovine mastitis isolates they examined.

Regarding the presence of polymorphism in the *clfA* and *clfB* genes, our study found no variation. Similar results were obtained in several other studies, where researchers obtained amplicons of comparable sizes without polymorphism. For instance, Stephan *et al.,* (2001) [35], Salasia *et al.,* (2004) [31], Bhanderi *et al.,* (2009) [7], Momtaz *et al.,* (2010) [21], Proietti *et al.,* (2010) [28], Nathawat *et al.,* (2015) [22], and Salem-Bekhit *et al.,* (2010) [32] all observed a single amplicon of specific sizes in their respective *S. aureus* isolates. However, there were some instances of variation found in the *clfA* gene. El-Sayed *et al.,* (2006) [11] identified two variable-sized amplified products in samples of mastitic milk from cows. Fitzgerald *et al.,* (2000) [13] observed *clfA* gene amplicons of varying sizes in *Staphylococcus aureus* isolates from bovine intramammary infections. Akineden *et al.,* (2008) [2] found differing amplicons in goat milk cheese-derived *S. aureus*, and Reinoso *et al.,* (2008) [30] reported variations in the *clfA* gene from strains obtained from different sources. Karahan *et al.,* (2011) [16] also identified variable-sized amplicons in their *S. aureus* isolates from cattle with subclinical mastitis. Similarly, Memon *et al.,* (2013) [20] reported *clfA* gene amplicons of varying sizes in *S. aureus* isolates associated with bovine mastitis, as did Yadav *et al.,* (2015) [41] in isolates from buffalo and cow milk with clinical mastitis. Overall, our findings demonstrate the variable distribution of *clfA* and *clfB* genes among *S. aureus* isolates from different sources and geographic regions, with some instances of polymorphism and others showing consistent amplicon sizes.

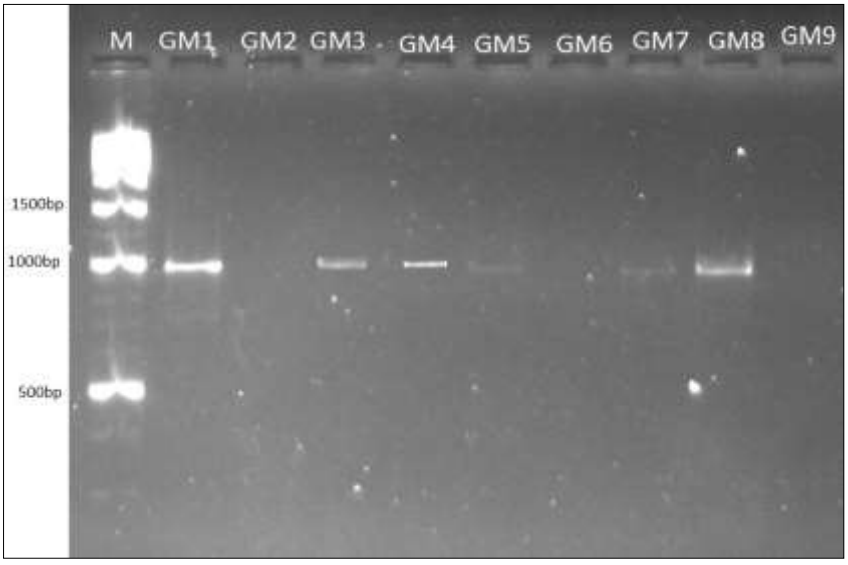


**Fig 1:** On an agarose gel, we observed the amplified segments of the *clfA* gene from *S. aureus* isolates

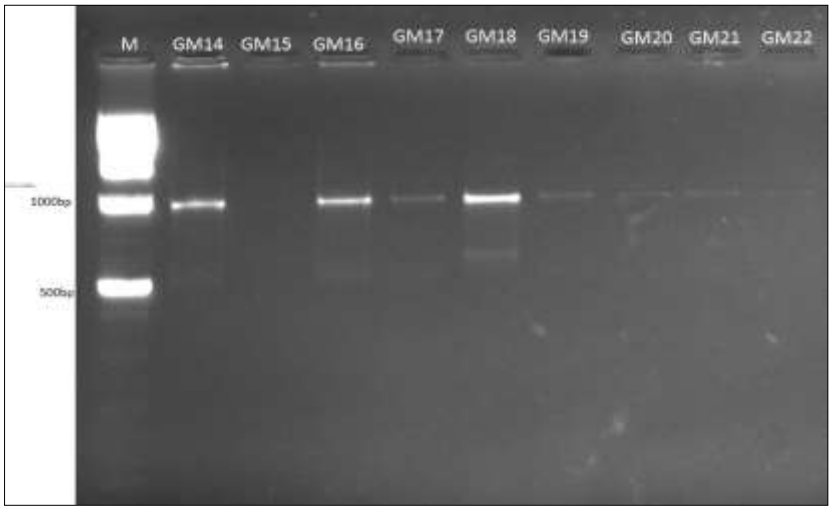


**Fig 2:** On an agarose gel, we observed the amplified segments of the *clfA* gene from *S. aureus* isolates

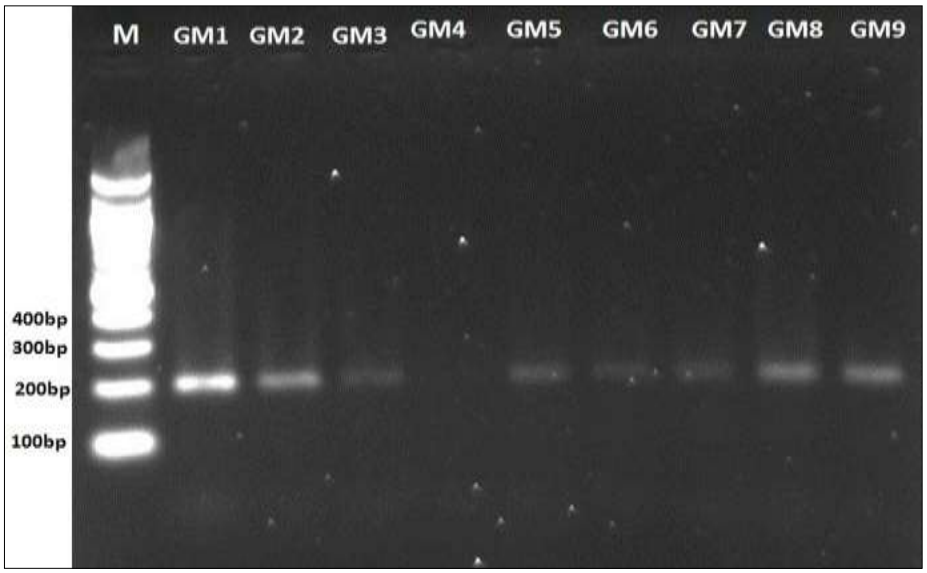


**Fig 3:** On an agarose gel, we observed the amplified segments of the *clfB* gene from *S. aureus* isolates

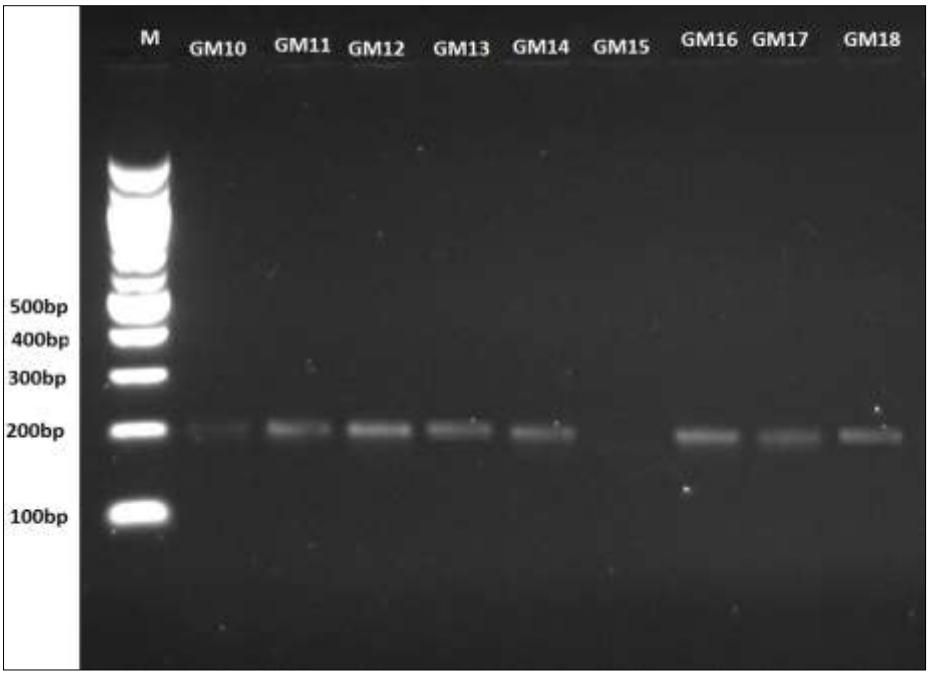


**Fig 4:** On an agarose gel, we observed the amplified segments of the *clfB* gene from *S. aureus* isolates

## Conclusions

In summary, our research highlights the importance of certain proteins found on the bacterial cell wall, specifically the genes *clfA* and *clfB*, in helping the *S. aureus* bacteria stick to host proteins. These genes play a crucial role in controlling the production of binding proteins that contribute to the disease-causing ability of *S. aureus*. Our study discovered that a large majority (94.11%) of the tested isolates contained these *clfA* and *clfB* genes, and they remained consistent without any observable changes. This suggests that these genes are widespread and stable in the *S. aureus* strains associated with caprine mastitis (a type of inflammation in goat's udders). Further research is needed to better understand how these clumping factor genes affect the development and management of mastitis in goats.

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## Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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