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Isolation and Molecular Confirmation of *Staphylococcus aureus* from Bovine Mastitis in Various Locations of Tamil Nadu, India

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Abstract

This present investigation was done for the Isolation and Molecular confirmation of *Staphylococcus aureus* from Bovine mastitis-infected cows in various Tamil Nadu, India locations. Two hundred milk samples, ranging from 15 to 20 ml, were collected aseptically from cows in farms in and around Tamil Nadu and surrounding areas after discarding the first few streaks of milk from 2021 to 2023. We used the CMT test to check the milk samples. Following a thorough mixing of the milk samples delivered to the laboratory, one loop containing the infected milk sample was plated on Nutrient agar and incubated at 37 ˚C for 18 to 24 hours. To identify the *Staphylococcus aureus*, the isolated bacterial colonies were preliminarily identified by Microscopic examination (Gram staining and Motility test), Plating in Selective medium (Mannitol Salt Agar) and Biochemical tests. The DNA of the bacteria was extracted and Polymerase Chain Reaction (PCR) was done to detect the presence of the *nuc* gene. Of the 200 milk samples obtained from nursing cows, 37.5 % were from Mastitis positive animals; of these, 61.3 % were in the sub-clinical stage, while 38.7 % were in the clinical stage. Only 33 (16.5 %) of the 200 milk samples tested positive for *Staphylococcus aureus*. A total of 26 out of 75 animals (34.7 %) with Mastitis, and 7 out of 125 animals (5.6 %) without Mastitis. Fifteen (57.7 %) of the twenty-six *Staphylococcus aureus* isolates found in cows with mastitis were from clinical cases, whereas eleven (42.3%) were from sub-clinical cases. All of the *Staphylococcus aureus* isolates examined contained the 279 bp *nuc* gene, according to the conventional PCR findings. In conclusion, the research has shown that sub-clinical mastitis is more common than clinical mastitis, and that *Staphylococcus aureus* was substantially more often isolated from mastitis milk, particularly in clinical instances.

Keywards: *Staphylococcus aureus*, Mastitis, Cow, Polymerase Chain Reaction (PCR) and *nuc* gene.

العزل والتأكيد الجزيئي لبكتريا *S. aureus* من التهاب الضرع البقري في الهند

الخلاصة

تم إجراء هذا البحث من أجل العزل والتأكيد الجزيئي لبكتيريا *S. aureus* من التهاب الضرع البقري في الهند. تم جمع مائتي عينة حليب، تتراوح من 15 إلى 20 مل، بطريقة معقمة من الأبقار في المزارع في ولاية تاميل نادو وما حولها، وكذلك من المناطق المحيطة، بعد التخلص من الشرائط القليلة الأولى من الحليب من عام 2021 إلى عام 2023. استخدمنا اختبار CMT لفحص عينات الحليب. بعد الخلط الدقيق لعينات الحليب التي تم تسليمها إلى المختبر، تم وضع حلقة واحدة تحتوي على عينة الحليب المصابة على أجار أولي وحضنت عند درجة حرارة 37 درجة مئوية لمدة 18 إلى 24 ساعة. لتحديد البكتيريا إيجابية وسلبية لصبغة الجرام، تم تلوين المستعمرات المعزولة بمحلول جرام و وبعد ذلك تم اجراء اختبارات كيمياحيوية. تم استخلاص الحمض النووي للبكتيريا وإجراء تفاعل البلمرة المتسلسل (PCR) للكشف عن وجود جين nuc. ومن بين 200 عينة حليب تم الحصول عليها من الأبقار المرضعة، كانت 37.5% منها من حيوانات مصابة بالتهاب الضرع؛ من بين هذه الحالات ، 61.3% كانت في المرحلة تحت السريرية، بينما 38.7% كانت في المرحلة السريرية. بينما سجلت 33 (أو 16.5%) من 200 عينة حليب إيجابية للمكورات العنقودية الذهبية. ان إجمالي 26 حيوانًا من أصل 75 (34.7%) كان مصاب بالتهاب الضرع و7 من أصل 125 حيوانًا (5.6%) غير مصاب بالتهاب الضرع. واظهرت النتائج ان خمسة عشر (57.7٪) من أصل ستة وعشرين عزلة من المكورات العنقودية الذهبية الموجودة في الأبقار المصابة بالتهاب الضرع كانت من الحالات السريرية، في حين كانت إحدى عشرة (42.3٪) من الحالات تحت السريرية. جميع عزلات المكورات العنقودية الذهبية التي تم فحصها تحتوي على جين *nuc* 279 bp، وفقًا لنتائج PCR. أظهرت نتائج البحث أن التهاب الضرع تحت السريري أكثر شيوعًا من التهاب الضرع السريري، وأن المكورات العنقودية الذهبية يتم عزلها في كثير من الأحيان من حليب الضرع المصاب، خاصة في الحالات السريرية.

Introduction:

Annual milk output in India is 146.3 million metric tons, putting it first globally (1). An enormous amount of money is lost by the dairy sector in India and throughout the globe due to mastitis, a disease that affects many dairy cows (2). Mastitis consists of the inflammation that affects the udder parenchyma: the pathological conditions become established in the tissues of the mammary glands that include redness, swelling and gland temperature increase, and also other modifications of the constituent properties in the milk appearing as a secretion (3). Depending on the manifestations of the disease, mastitis can happen in the form of Clinical or Subclinical mastitis (SCM). Milk colour and consistency can be the only signs of milk disorders in the case of clinical mastitis in the early stages. The inflammatory symptoms, such as udder oedema, reddening of the skin, udder, and pain, are the consequences of an ongoing disease process. The change is only clinically evident in SCM as a drop in milk yield, because the animal's milk and their udder remain unchanged. Hence, the problem can stay unnoticed for a long time (4). Consequently, SCM is thought to be a more widespread and striking form of the illness compared to clinical mastitis. According to estimates, SCM accounts for over 90 % of the overall milk loss in the dairy farms of some countries , such as Ethiopia (2). Mastitis in cattle may be brought on by diseases or trauma. Bovine mastitis may be caused by a variety of microorganisms, including bacteria, fungi, and viruses. *Staphylococcus aureus* is a kind of bacterium that may cause mastitis in animals and food poisoning when consumed in dairy products, particularly cheese and yogurt (5, 6). The persistence of the infection after treatment has begun is one of the distinguishing features of *Staphylococcus aureus* as a cause of mastitis. The fact that it has several pathogenicity and virulence factors including quorum sensing that allow it to evade immune defense systems and cause infections when immune cells are available could be the reason for this (7). Staphylokinase, lipase, and Hemolysin-producing enzymes are additional virulence factors, as is the rapid development of Antibiotic-resistance and enterotoxigenicity. The acquisition of mobile genetic components *via* gene transfer may be associated with the pathogen's antibiotic resistance developing nature (6, 8). For instance, *Staphylococcal* cassette chromosome mec (SCCmec) is a genetic component that Methicillin - Resistant *Staphylococcus aureus* (MRSA) strains of the bacteria have developed with (9). The virulence gene mecA is one of several in SCCmec; its codes for a variant of the penicillin-binding protein 2a. Considering that PBP-2a binds methicillin less strongly, mecA-positive the medicine of choice for treating infections caused by beta-lactam antibiotic-resistant *Staphylococcus aureus*, methicillin, has been produced by the bacteria. Further, the genetic cassette could include genes that code for resistance to different classes of antibiotics as well as other virulence factors, such as enterotoxins (6, 8). However, the synthesis of the penicillinase enzyme might be the reason why *Staphylococcus aureus* is resistant to penicillin. Penicillin and penicillin derivatives are hydrolyzed by breaking the β-lactam ring of penicillin, an action that is dictated by the blaZ gene (10 - 12). Due to the absence of alternative medicines, infections induced by these strains of the pathogen may be deadly (6, 13).

A heat-stable enzyme called thermonuclease is encoded by the *nuc* gene, which is also associated with *Staphylococcus aureus*'s thermostability. Thus, *Staphylococcus aureus* bacteria that are *nuc* gene positive may be able to live in foods that have been heated, and their toxins may be able to withstand high

temperatures as well. Despite efforts to identify species of *Staphylococcus aureus* by identifying the *nuc* gene, not all *Staphylococcus* strains have this gene (14).

Evaluating the frequency and antibiotic susceptibility traits of the bacterium in lactating cows can significantly aid in the implementation of treatment and control measures against the pathogen, *Staphylococcus aureus*. This bacterium is a major cause of disease in both animals and humans, either through infection or the production of toxins in food (6). This present research investigation was done for isolation and Molecular confirmation of *Staphylococcus aureus* from Bovine mastitis in Tamil Nadu, India.

Materials and Methods:

Sampling:

Two hundred milk samples, ranging from 15 to 20 ml, were collected aseptically from cows in farms in and around Tamil Nadu, as well as from surrounding areas, after discarding the first few streaks of milk. The time frame for the collection of these samples was 2021 – 2023. We used the CMT test to check the milk samples.

Bacterial isolation:

Following a thorough mixing of the milk samples delivered to the laboratory, one loop containing the infected milk sample was plated on Nutrient agar, MacConkey agar, and Mannitol Salt Agar (Hi Media). The plates that were cultured were then kept at 37 ˚C for 18 to 24 hours. To identify the Gram positive bacteria *Staphylococcus aureus*, the isolated bacterial colonies were preliminarily identified by Microscopic examination (Gram staining and Motility test), Plating in different medium (Mannitol Salt Agar). Subsequently, they were confirmed using a set of Biochemical tests, including Catalase, Oxidase, Indole, Methyl red, Voges Proskauer's, Citrate utilization, DNase test, and Coagulase test.

DNA extraction:

The extraction of genomic bacterial DNA was done using a kit from Genaid company and the extraction was done following the instructions of company.

Primer:

nuc gene F: 5′GCGATTGATGGTGATACGGTT

R: 5′AGCCAAGCCTTGACGAACTAAAGC
279 bp

PCR Reaction and condition:

The reaction mixture was adjusted to a volume of 25 µl by adding nuclease-free water, and it included 1µl of forward and reverse primers with concentrations of 20 pmol/ul, 12.5 µl of mastermix, and 2 µl of each template DNA. The following conditions were used to conduct PCR on a thermocycler: a 5 minutes initial denaturation at 94 ˚C, followed by 35 cycles of 94 ˚C for 45 seconds, annealing at 60 ˚C for 1 minute, and extension at 72 ˚C for 1 minute. After that, there was a last extension lasting 10 minutes at 72 ˚C. The gel documentation system was used to view the PCR results, which were performed on 1.5 % agarose with a 100 bp DNA molecular weight marker at 5 V/cm.

Results and Discussion

The 200 milk samples were obtained from lactating cows, 37.5% were from mastitis-positive animals; of these, 61.3 % were in the subclinical stage, while 38.7 % were in the clinical stage (Table - 1). Only 33 (or 16.5 %) of the 200 milk samples tested positive for *Staphylococcus aureus*. A total of 26 out of 75 animals (34.7 %) with mastitis and 7 out of 125 animals (5.6 %) without mastitis (Table - 2).

Fifteen (57.7 %) of the twenty-six *Staphylococcus aureus* isolates found in cows with mastitis were from clinical cases, whereas eleven (42.3 %) were from sub-clinical cases (Table - 3).

Table 1. Number and Percentage of Clinical and Sub-clinical mastitic samples

Type of Mastitis	Number	Percentage (%)
Clinical	29	38.7
Sub-clinical	46	61.3
Total (200)	75	37.5

Table 2. Number and Percentage of *Staphylococcus aureus* isolated from positive and negative Milk samples

Milk sample	Number	Percentage (%)
Positive	26	78.7
Negative	7	21.3
Total	33	100 %

Table 3. Number and Percentage of *Staphylococcus aureus* isolated from Clinical and Sub-clinical Mastitic samples

Type of Mastitis	Number	Percentage (%)
Clinical	15	57.7
Sub-clinical	11	42.3
Total	26	100 %

All of the *Staphylococcus aureus* isolates examined contained the 279 bp *nuc* gene, according to the conventional PCR findings (Figure - 1).

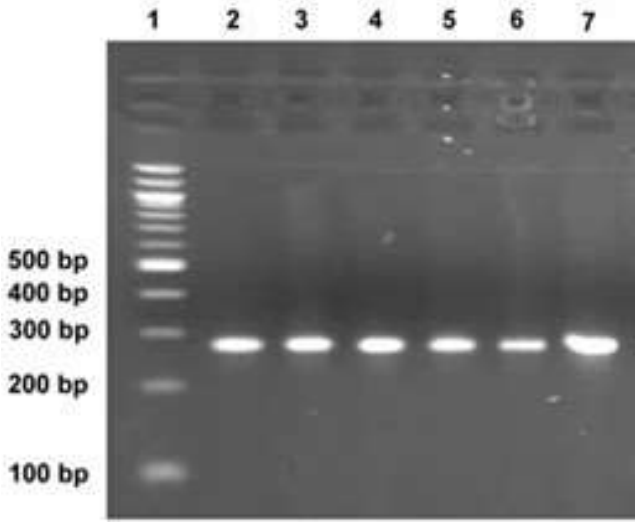

Figure 1. The PCR product was photographed using a UV-illuminator following Electrophoresis in a 1.5 % Agarose gel. The positive samples were *nuc* = 279 bp.

One of the main causes of infectious mastitis in cattle is *Staphylococcus aureus* (15). In addition to living in the teat canal, the bacteria can be found on the udder's and teats' surface areas (16 - 18). Among the microflora, it can infect animals as an opportunistic infection in response to mechanical trauma to the teat or other stressors (19).

Only 33 out of 200 (or 16.5 %) of the 200 milk samples tested positive for *Staphylococcus aureus* in this investigation. Compared to animals without mastitis, those with the condition had a considerably greater rate (34.7 % versus 5.6 %, $p < 0.05$). Several other investigations have similarly found different rates of *Staphylococcus aureus* isolation from cows that tested positive for mastitis. The

findings corroborated those of (20, 21). Nevertheless, it surpasses the results of (22 - 24), but it is less than the results of (2, 3, 25).

A total of 37.5 %, or 75 out of 200 cows, tested positive for mastitis. Sarba and Tola (26) also discovered that 41.7 % of cows in the Ambo area had mastitis, therefore our findings were in line with theirs. On the other hand, in certain zones, Dabele *et al*. (27) discovered a much higher illness prevalence rate of 30.5 %. This conclusion is lower than the majority of prior research on bovine mastitis, which has shown a prevalence rate of 46 % to 73 % in other regions of the nation (26, 28).

We also discovered that sub-clinical mastitis was far more common than clinical mastitis in the region where the research was conducted. Out of the cows that tested positive for mastitis, about 61.3 % had sub-clinical mastitis and 38.7 % had clinical mastitis ($p < 0.05$). More cases of subclinical mastitis than clinical mastitis have been documented (2). This is an area where the present result agrees with the majority of research (29, 30, 31). In Ethiopia, Argaw (32) found that sub-clinical mastitis was prevalent at 85 % and clinical mastitis at 23 %. Results from this research showed a higher incidence of clinical mastitis (14.73 %, 38/258) compared to previous studies (3, 27, 28). While other studies have found higher prevalence rates of sub-clinical mastitis (36.7 %, 59.2 %, 45.5 %, respectively), our findings are lower than those of (2, 33).

One of the most common ways to use Polymerase Chain Reaction (PCR) to identify *Staphylococcus aureus* is by looking for the *nuc* gene, which codes for thermonuclease (8, 9).

Girmay *et al*. (21) showed that all *Staphylococcus aureus* isolates were identified using standard techniques exhibited amplification of the *nuc* gene, a trait that was not seen in other bacterial species. Consistent with previous research (22), the current study confirms that the *nuc* gene can be used for more accurate Molecular detection of selected isolates (*Staphylococcus aureus*) through PCR amplification. Electrophoresis of the PCR products confirmed that all isolates had the *nuc* gene, and the results showed that the *nuc* gene's DNA strand had a molecular size of 300 base pairs.

Conclusion:

The research has shown that sub-clinical mastitis is more common than clinical mastitis, and that *Staphylococcus aureus* was substantially more often isolated from mastitic milk, particularly in clinical instances.

Conflict of interest

The authors declare, there are no conflicts of interest

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