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Short Communication

Occurrence of *nuc* gene in *Staphylococcus aureus* from milk samples of different regions of West Bengal

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Abstract

***Staphylococcus aureus* is a Gram positive commensal of the mammalian body, commonly found in the respiratory tract, skin and mammary gland. Bovine milk is a major source of *S. aureus*. Various factors play a role in the production of pathogenicity of the bacterium including enzymes like thermonuclease, encoded by *nuc* gene. The present study was carried out for the isolation of *S. aureus* in bovine milk and determination of the occurrence of *nuc* gene in them. Out of 168 milk samples, 146 *S. aureus* strains (86.90%) were isolated, of which 49 *S. aureus* strains were positive for *nuc* gene (32.88%) in polymerase chain reaction (PCR). In the study, highest occurrence of *nuc* positive *S. aureus* was found in new alluvial zone (55.8%) and least in red laterite zone (3.77%). This indicates wide variation in the presence of *nuc* gene containing *S. aureus* in different agro-climatic regions of West Bengal that need further studies to reveal its implication, if any.**

Keywords: Cattle milk, *nuc* gene, PCR, *Staphylococcus aureus*, Virulence

Highlights

- Screening of *nuc* gene might be carried out to identify *Staphylococcus aureus* in milk samples.
- Wide variation in the presence of *nuc* gene containing *S. aureus* detected in different agro-climatic regions of West Bengal.

A natural commensal and a usual member of the microbiota of body, *Staphylococcus aureus*, the Gram positive, round shaped bacteria and a member of Bacillota, is frequently found in the upper respiratory tract and on the skin (Boldock *et al.*, 2018). Being a commensal, it can act as an opportunistic pathogen and can become a common cause of skin infection including abscesses, folliculitis, respiratory infections and food poisoning. Studies showed that *Staphylococcus aureus* could be isolated from milk samples of various lactating animals like cattle, buffalo, goat and sheep (Sharma *et al.*, 2011). There are several medical reports establishing the bacteria responsible for food poisoning. Due to increasing reports of the pathogenicity and antimicrobial resistance of these bacteria, detection of the bacteria has become essential. The most common tests for the identification of *S. aureus* include certain biochemical characteristics such as coagulase test, thermonuclease production test etc. Development of molecular biology and polymerase chain reaction (PCR) techniques contributed in easy detection of the bacteria genotypically. Initially, some typical *S. aureus* specific genes were targeted for those harbor some characteristics, viz. pathogenicity (Brakstad *et al.*, 1992). Thermonuclease specific gene viz. *nuc* was screened for the detection and characterization of *S. aureus*, but those were not highly convincing as *nuc* negative/deficient methicillin resistant *S. aureus* strains were also detected (Leeuwen *et al.*, 2008). A total of 150 mastitic milk samples were collected from Kashmir, and 80 numbers of *S. aureus* isolates were recovered (53.33%) (Shan *et al.*, 2019). Methicillin resistant, *nuc* negative staphylococci were also recovered from cattle, their handlers and their environment in Karnataka, India (Venugopal *et al.*, 2019). Although *nuc* gene is still not an indispensable gene for *Staphylococcus aureus* detection, it has several significances. Originally identified in 1956 by Cunningham, it was named as "micrococcal nuclease", before it was named as "thermonuclease" (Kiedrowski *et al.*, 2014). The role of *nuc* gene in pathogenicity is sketchy. However, previous studies have found that it affects biofilm formation. Occurrence of Staphylococcus nuclease prevents biofilm formation (Tang *et al.*, 2011). It was reported that *S. aureus* biofilm formation was simultaneously impacted by high levels of *nuc* and extracellular proteases

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(Kiedrowski *et al.*, 2014).

Bovine milk is a common source of *Staphylococcus aureus* species. A lot of previous works showed the occurrence of the concerned bacteria in milk. Milk samples collected from apparently healthy cattle of different European countries like Austria, Belgium, France, Italy and Germany showed positive results for *S. aureus* populations. A total of 1685 milk samples were collected, out of which 456 *S. aureus* strains were identified using *S. aureus* specific *nuc* gene approach (Cosandey *et al.*, 2016). A total of 12 *S. aureus* isolates were recovered from milk samples collected from 47 apparently healthy cows in Bangladesh (Jahan *et al.*, 2014). The present study was conducted to detect the occurrence of *Staphylococcus aureus* in bovine milk samples collected from three different agro-climatic zones (New alluvial, coastal saline and red laterite area) in West Bengal. The isolated *S. aureus* strains were screened genotypically for the characterization of *nuc* gene, specific for thermostable nuclease by PCR.

A total of 168 milk samples were collected from different agro-climatic zones of West Bengal, viz., new alluvial (Mohanpur, Dankuni), coastal saline (Diamond Harbour) and red laterite (Bankura). The samples were collected in sterile vials and were kept at 4ºC overnight and then incubated at 37ºC for 24 hours. After incubation, the samples were inoculated aseptically at sterile Mannitol salt agar (MSA) and incubated at 37ºC for 48 hours for characteristic Staphylococcal growth. The colonies in MSA appearing in yellow colour were selected and inoculated in nutrient agar slants for further testing. The isolated bacteria of the slants were subjected to biochemical tests viz., catalase, oxidase, Voges-Proskauer, citrate, methyl red, indole and urease for characterization of *Staphylococcus* species.

The chromosomal DNA of the bacteria was extracted and subjected to polymerase chain reaction (PCR) of 16S rRNA of *Staphylococcus aureus* species for the molecular confirmation of the bacteria (Ali *et al.,* 2014). The 16S rRNA was amplified with 25 nucleotide forward primer, 5'-GGAATTCAAAGGAATTGACGGGGGC-3' and a 29-nucleotide reverse primer 5'-CGGGATCCCAGGCCCGGGAACGTATTCAC-3'. The amplified product size was 479 bp. In brief, the following reaction mixture was added to each sample: 2 µL DNA (100 ng), 2 µL primer (100 pmol), PCR mixture (1.5 µL $MgSO_4$, 2.5 µL 10% PCR buffer, 0.5 µL dNTPs, 0.2 µL Taq polymerase) and completed to 25 µL volume by nuclease-free water. The PCR was conducted with Thermocycler (BioRad, India), using PCR protocol that included initial denaturation at 95ºC for 5 min, followed by 37 cycles of amplification of each cycle comprised of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 60 s, with final extension at 72ºC for 10 min. The PCR products were stored in the cycler at 4ºC until they were collected. The amplified products were further gel electrophoresed in 1% agarose gel (w/v) containing ethidium bromide (0.5 µg/mL) in 1X TAE buffer and were visualized under UV in a Gel documentation system (Labnet, USA).

The *nuc* gene was screened for the determination of virulence factor of the *S. aureus* isolates, specific for thermostable nuclease. The sequences of the two primers of 21 and 24 bases, respectively, were 5'-GCGATTGATGGTGATACGGTT-3' (forward primer) and 5'-AGCCAAGCCTTGACGAACTAAAGC-3' (reverse primer) (Brakstad *et al.*, 1992). The amplicon size was 279 bp. In brief, the PCR amplification was performed using the reaction mixture consisting of 5 µL of DNA sample, 5 µL of 10X PCR amplification buffer, 1.0 µL of each primer (100 µM stock solution), 0.5 µL of the deoxynucleoside triphosphates (1 mM each in stock solution), 0.25 µL of Taq polymerase (5 U/ µL of stock solution), and rest of nuclease-free water to make up the final volume of 25 µL. A total of 37 PCR cycles were run under the following conditions: DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s and DNA extension at 72°C for 1.5 min. After the final cycle, the reaction was terminated by keeping it at 72°C for 3.5 min. The PCR products were stored in the cycler at 4°C until they were collected. The amplified product was electrophoresed in 1% agarose gel (w/v), containing ethidium bromide as fluorescent dye, and was visualized under UV in a gel documentation system.

Out of 168 samples collected from different agro-climatic zones of West Bengal (viz. new alluvial, coastal saline and red laterite), 146 *S. aureus* isolates were generated that were tested by biochemical tests as well as confirmed by PCR (86.90%) (Table 1), a total of 48 *S. aureus* isolates were positive for *nuc* gene (48/146, 32.88%) which was confirmed by PCR (Table 1) (Fig. 1). When agro-climatic zone-wise occurrence of *nuc* positive *S. aureus* was assessed, it was observed that highest occurrence of *nuc* positive *S. aureus* in New alluvial zone (55.8%) followed by 25% in coastal saline zone and least in red laterite zone (3.77%) (Table 2).

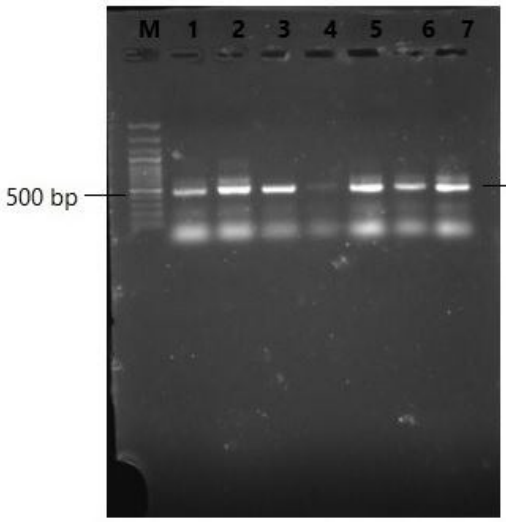
Being commensal to the animal body, the occurrence of virulent strain among the normal *Staphylococcus aureus* flora in cattle is a major concern to animal health. Moreover, the *nuc* PCR may be applicable for testing non-clinical samples like food (e.g., milk). Although virulence related to *nuc*, i.e., thermostable nucleases, is not significantly hazardous, but it cannot be neglected. A previous study on the determination of *nuc* specific *S. aureus* isolates from cattle of the same region (West Bengal) reported the generation of 94 (20.8%) isolates

Table 1. Occurrence of *nuc* gene in *Staphylococcus aureus* isolates

Number of samples	Number of *S. aureus* isolates as assessed by biochemical tests	Number of *S. aureus* isolates as assessed by PCR	Percent (%) of *S. aureus* isolated (Confirmed by PCR)	Number of *nuc* positive *S. aureus* as assessed by PCR	Percent (%) of *nuc* positive *S. aureus* isolated
168	132	146	86.90	49	33.56

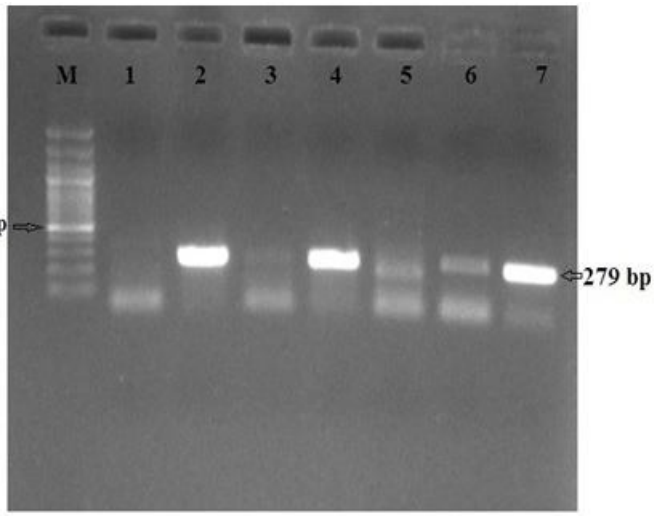
Table 2. Occurrence of *nuc* positive *Staphylococcus aureus* in different agro-climatic zones of West Bengal

Number of nuc positive *S. aureus*	Occurrence at New Alluvial zone	Occurrence at Coastal Saline zone	Occurrence at Red Laterite zone
49	43/77 (55.84%)	4/16 (25%)	02/53 (3.77%)



[Lane M: DNA ladder (100 bp ladder), Lane 1,2,3,5, 6,7: Positive samples, Lane 4: Negative Samples]

Fig 1. Agarose gel showing PCR amplified product (size 479 bp) of 16S rRNA gene of *Staphylococcus aureus* isolates



[Lane- M: DNA marker (100 bp ladder), Lane- 2,4,6,7: Positive samples, Lane- 1,3,5: Negative samples]

Fig. 2. Agarose gel showing PCR amplified product (size 279 bp) of *nuc* gene of *Staphylococcus aureus* isolates

from 450 milk samples (Mahanti *et al*., 2020), whereas in the present study, 48 *nuc* positive *S. aureus* could be recovered from 146 isolates (33.56%), this difference in observation might be due to the difference in the location and time of sample collection. The results of the present study corroborate with one previous study where 38 out of 118 *S. aureus* (32.20%) isolates recovered from milk samples collected from cattle of Kolkata and North 24 Parganas were *nuc* positive (Singh *et al.*, 2019).

Although sufficient milk samples could not have been collected from the coastal saline zone in the present study, and the sample size was limited to 16, a low trend of *nuc* positive *Staphylococcus aureus* in milk samples from red laterite zone (3.77%) was noted. The present study showed that the highest occurrence of *nuc* positive *S. aureus* was in the new alluvial zone (55.8%) and least in red laterite zone (3.77%), indicating the area-wise significance of *nuc* positive *S. aureus* in milk samples ($p<0.01$). However, one previous study carried out in West Bengal showed more number of *nuc* positive *S. aureus* from bovine milk (55.55%) compared to the isolates recovered from goats (44.83%) and dogs (29.63%) (Gurung *et al.*, 2017). This variation in observation might be due to the variation of sample collection sites as well as different animal species used in the study. Albeit detection of *nuc* gene has been limited to the genotypic characteristics of *S. aureus*, detection of 16S rRNA in *S. aureus* by PCR has revealed not all *S. aureus* harbor *nuc* gene. Detection of *nuc* gene cannot confirm the bacteria as virulent since there are

several other virulent specific genes like *femA* and *femB* (specific for expression of methicillin resistance in *S. aureus*) (Kobayashi *et al*., 1994), *icaA* and *icaD* (intracellular adhesion proteins) (Vasudevan *et al.*, 2003), *hlg* (specific for haemolysin) (Kumar *et al*., 2009) *sea, seb, sec, sed, see, seg, seh, sei* and *sej* (Staphylococcal enterotoxin genes, specific for toxic shock syndrome toxin, i.e., TSST) (Mahanti *et al*., 2019). However, *nuc* gene may be targeted to screen *S. aureus* in the samples as it is one of the important pathogenicity markers.

In short, screening of *nuc* gene might be carried out to identify pathogenic *S. aureus* in milk samples. A wide variation in the presence of *nuc* gene containing *S. aureus* was observed in different regions of West Bengal that need further studies to ascertain its implication, if any.

Conflict of interest: Authors declare that they have no conflict of interest.

Author's contribution: RB: Involved in the investigation, data collection and preparing the original draft manuscript; IS: Engaged in data interpretation and manuscript correction; KB, SD: Involved in data analysis, supervision and manuscript updating; SNJ: Engaged in conceptualization, fund management and final editing of the manuscript.

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