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IDENTIFICATION OF *NUC* GENE IN STAPHYLOCOCCAL SUB-CLINICAL MASTITIS IN CATTLE

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ABSTRACT

Mastitis, caused by multifactorial etiopathological factors, is one of the costliest disease of dairy animals across the globe. For the epidemiological study 550 lactating cows were screened by modified California mastitis test (MCMT). Clinical examination of animals and their udder/milk, phenotypic and genotypic characterization of *Staphylococcus* spp. was carried out. A total of 212 MCMT positive quarter milk samples were processed for bacteriological examination and molecular identification of *S. aureus* by *nuc* gene amplification. Synthetic oligonucleotide primers of 21 and 24 bases, respectively, were used in the polymerase chain reaction (PCR) to amplify a sequence of the nuc gene, which encodes the thermostable nuclease of Staphylococcus aureus. A DNA fragment of approximately 270 bp was amplified from isolated DNA. The PCR product was detected by agarose gel electrophoresis. Amplification was not recorded when other staphylococcal species were tested. Some of the non-S. aureus staphylococci produced thermostable nucleases but were PCR negative. The overall occurrence of Staphylococcal SCM was reported to be 16.36% (90/550) animal wise and 6.07% (127/2092) quarter wise as identified on the basis of colony morphology, characteristic colour changes on Mannitol salt agar, positive catalase test, haemolysis pattern on blood agar and DNase activity on DNase agar. The occurrence recorded using PCR for identification of *nuc* was 14.62%.

Keywords: Mastitis, MCMT, *Staphylococcus* spp., *nuc* gene.

Introduction

Mastitis is a general term which refers to the inflammation of the parenchyma of the mammary gland, regardless of the cause. Different kinds of microorganisms e.g. Staphylococcal species (spp.), Streptococcal spp., *Escherichia coli, Klebsiella* spp., *Enterobacter* spp., *Corynebacterium bovis, Corynebacterium pyogenes, Bacillus* spp., *Mycoplasma bovis, Micrococcus* spp., etc. are common etiological agents of mastitis in animals, however, the major part of microbial mastitis has been reported due to Staphylococci and Streptococci (Lee *et al.,* 2008). Amongst these causative agents, *Staphylococcus aureus* (here after referred to as *S. aureus*) has been recognized as the most common cause of intra-mammary infection in milch animal species, which often leads towards the damage and sometimes even complete loss of the gland. Staphylococcal mastitis has been reported as 25-30% of the entire mastitic infections and milk losses, which has been reported to vary between 10 to 25% (Sutra and Poutrel, 1994). In case of *S. aureus* there is variability in expression of phenotypic characters by strains of bovine origins. There is lack of proper phenotypic tests for identification between *S. aureus, S. intermedius* and S. *psuedintermedius*. Therefore, accurate identification of *S. aureus* by genotypic methods is

advocated by various workers. The nuclease (*nuc*) gene encodes the thermonuclease (Tnase) production which has species-specific sequences and amplification of the *nuc* gene has a potential for the rapid diagnosis of *S. aureus* (Brakstad *et al.*, 1992).

Materials and Methods

Animals

A total of 550 lactating cattle belonging to non descript, cross bred and exotic breeds were screened over a period of more than a year i.e. from February 2015 to February 2016 by modified California mastitis test (MCMT). The cattle belonged to the dairy farms in and around Jabalpur were screened. Different parameters, about individual animals i.e. breed, age, lactation number, stage of lactation, herd size and number of quarters affected were recorded.

Collection of milk sample

The milk sample was collected from each teat of cattle indicating MCMT positive for subclinical mastitis. The udder and teats were cleaned and washed with potassium permanganate 0.01% then wiped with clean cloth. First few streams of foremilk was discarded and then about 8 ml of milk from each affected quarter was collected in fresh, sterile, labeled screw cap test tubes and brought to the department in ice for further examination.

Bacterial isolation and characterization of *Staphylococcus* spp. in positive milk samples (MCMT positive)

Isolation of *Staphylococcus* spp.

Bacterial examination was done on the MCMT-positive milk samples. Pre enrichment of *Staphylococcus* spp. was done on Muller Hinton broth with 6.5 per cent sodium chloride. Pre enriched samples were cultured bacteriologically to isolate the *Staphylococcus* spp. on Mannitol salt agar. These isolates were incubated aerobically at 37ºC for 24 hours. The yellow or pink colony on Mannitol salt agar were indicating the presence of *Staphylococcus* (Markey *et al.*, 2012). Gram staining done to understand colony morphology (Gram positive violet colour *cocci* arranged in grape-like clusters).

Identification of *Staphylococcus* spp.

Biochemical characterization

Each bacterial isolate was further characterized by various biochemical tests such as Catalase, Coagulase test, Haemolysis on blood agar and DNase test.

Molecular detection

Extraction of DNA from culture:

The procedure of DNA extraction was performed by chelex based extraction of DNA using Insta Gene Matrix (Bio-Rad laboratories, India Pvt. Ltd.) as described by Giraffa *et al*. (2000). For extraction of DNA, 1.5 ml of broth culture was vortexed for 10 seconds and centrifuged for 1 minute at 10,000–12,000 rpm. The supernatant was removed and 200 micro liters

(µl) of Insta Gene matrix was added to the pellet. The suspension was incubated at 56°C for 15–30 minutes and again vortexed at high speed for 10 seconds. The cell suspension was heated in a boiling water bath for 8 minutes and again vortexed at high speed for 10 seconds and spun at 10,000–12,000 rpm for 2–3 minutes. Then 10 µl of the resulting supernatant was used per 25 µl PCR reaction and the remainder supernatant was stored at -20°C for further use.

Oligonucleotide sequence and PCR cycling condition

The primer (synthesized by Integrated DNA Technology) used in the study for amplification of *nuc* gene from 24 hour old BHI broth culture is listed below:

Details of the primer used in the study

Gene	**Primer**	**Oligonucleotide Sequence**	**Reference**
Nuclease (*Nuc*)	Forward Reverse	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	Brakstad *et al.* (1992)

PCR Conditions for detection of *nuc* gene

The PCR was carried out in 25 µl of reaction mixture which was composed of:

PCR mastermix	12 µl
Forward primer	1 µl
Reverse primer	1 µl
Nuclease free water	1 µl
Sample DNA	10 µl

The cycling conditions used for amplification of *nuc* gene were as follows:

Step 1	37 cycles of denaturation	94°C for 1 min
Step 2	Annealing	52°C for 0.5 min
Step 3	Extension	72°C for 1.5 min
Step 4	Final extension	72°C for 3.5 min

The PCR products were stored in the cycler at 4°C until they were collected. The amplification of specific PCR product was checked by electrophoresis of the PCR product in 1.5% agarose gel and viewed in UV transilluminator system.

Results and Discussion

Bacterial isolation and characterization of *Staphylococcus* spp. in milk samples found positive for SCM

A total of 2092 milk samples from functional quarters of lactating cattle were screened for udder health status. Milk samples were collected for bacterial isolation and identification on primary and selective media, respectively, from 212 quarters found positive on MCMT. As many as 127 samples were found positive for *Staphylococcus* spp. as identified on the basis of characteristic colour changes on Mannitol salt agar (yellow or pink colour colony) (Figure 01), colony morphology (Gram positive violet colour *cocci* arranged in grape-like clusters) (Figure 02), positive catalase test, haemolysis pattern on blood agar (alpha and beta haemolysis) and DNase activity on DNase agar (Figure 03).

Agarose gel electrophoresis showing amplification of 270 bp fragment specific for *S. aureus* with M: 100 bp molecular marker; Lane 1-6 shows positive bacterial culture; Lane 7-11 shows negative bacterial culture. The occurrence recorded using PCR for detection of *nuc* gene was 14.62 per cent (31 isolates were found positive for *nuc* gene). The results are shown in table and Figure 04.

Table: Detection of *nuc* gene based on PCR method of identification in bacterial culture positive for *Staphylococcus* spp.

Total no. of samples	Bacterial culture positive for *Staphylococcus* spp.	PCR for *nuc* gene
212	127	31 (14.62 per cent)

S. aureus is the most common cause of contagious mastitis in cattle and it is most significant bacterial pathogen associated with bovine mastitis. It generally causes chronic and sub clinical mastitis in lactating dairy animals. As the organism is intra cellular in nature, the resistance to antibiotics makes this pathogen to be one of the most difficult entities to treat.

Nucleic acid amplification by PCR has applications in many fields of biology and medicine, including the detection of viruses, bacteria, and other infectious agents. Nuclease gene is one of the virulence factor which decides virulence of *S. aureus*. The nuclease (*nuc*) gene encodes the thermonuclease (Tnase) production. The *nuc* gene polymorphism is frequently applied for epidemiological investigations of bovine *S. aureus* mastitis (Rusenova *et al.*, 2013). A total of 212 MCMT positive milk samples were collected aseptically for bacterial isolation and identification using phenotypic test, then PCR was performed for amplification of *nuc* gene. The *nuc* gene of *S. aureus* yielded a PCR product of 270 bp on amplification. Brakstad *et al.* (1992), Eswaran *et al.* (2011) and Rusenova *et al.* (2013) have also amplified the *nuc* gene to detect the *S.*

aureus. The occurrence of *nuc* gene was recorded in 14.62% (31 isolates were found positive for *nuc* gene) in subclinical mastitis. Rusenova *et al.* (2013) suggested that routine approach using a combination of phenotypic and molecular detection systems could improve *S. aureus* detection in milk.

Conclusions

Amplification of *nuc* gene yielded a single PCR product of 270 bp in culture found positive for *Staphylococcus* spp. when visualized in UV transilumilnator gel documentation system after amplification and electrophoresis in 1.5% agarose. The occurrence recorded using PCR for identification of *nuc* was 14.62%.

References:

Brakstad, O.G., Aasbakk, K. and Maeland, J.A. (1992). Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *Journal of Clinical Microbiology*, **30** (7): 1654–1660.

Eswaran, M.A., Poorni, K.E., Sukumar, K., Malmarugan, S., Manikandan, A. and Geethanjali, S. (2011). Detection of virulent gene for nuclease (*nuc*) in *Staphylococcus aureus* isolated from subclinical mastitis. *Indian Journal of Field Veterinarians*, **7** (1): 17-20.

Giraffa, G., Rossetti, L. and Neviani, E. (2000). An evaluation of chelex- based DNA purification protocols for the typing of lactic acid bacteria. *Journal of Microbiological Methods*, **42**: 175-184.

Lee, K.H., Lee, J.W., Wang, S.W., Liu, L.Y., Lee, M.F., Chuang, S.T., Shy, Y.M., Chang, C.L., Wu, M.C. and Chi, H.C. (2008). Development of a novel biochip for rapid multiplex detection of seven mastitis causing pathogens in bovine milk samples. *Journal of Veterinary Diagnostic Investigation*, **20**: 463-471.

Markey, B., Leonard, F., Archambault, M., Cullinane, A. and Maguire, D. (2012). Clinical Veterinary Microbiology, 2nd Edn., Elsevier Publishing Co., London, pp 118-344.

Rusenova, N., Gebreyes, W., Koleva, M., Mitev, J., Penev, T., Vasilev, N. and Miteva, T. (2013). Comparison of three methods for routine detection of *Staphylococcus aureus* isolated from bovine mastitis. *Journal of the Faculty of Veterinary Medicine, Kafkas University*, **19** (4):709-712.

Sutra, L. and Poutrel, B. (1994). Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus. Journal of Medicinal Microbiology*, **40** (2): 79-89.

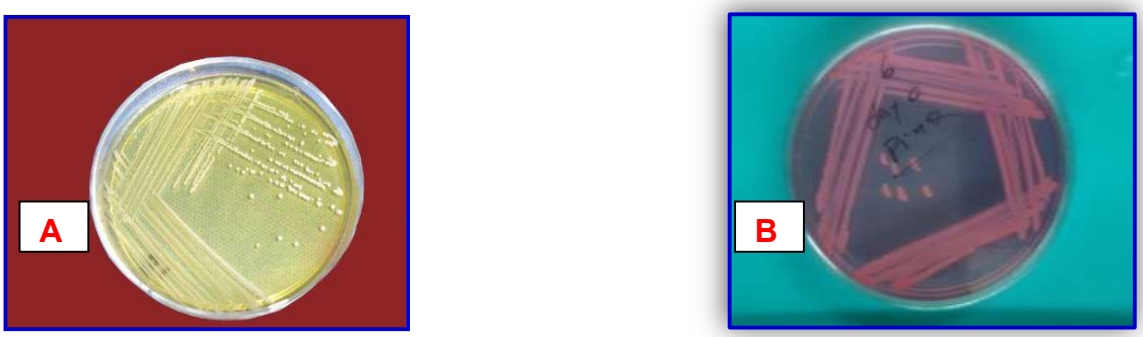

Figure 01: A. Yellow colony of Staphylococci on Mannitol salt agar
B. Pink colony of Staphylococci on Mannitol salt agar

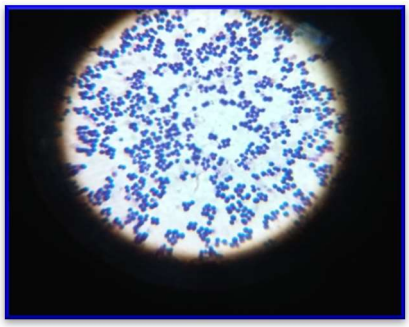

Figure 02: Gram-positive spherical cocci (Staphylococci*)* arranged in irregular clusters at 1000X magnification

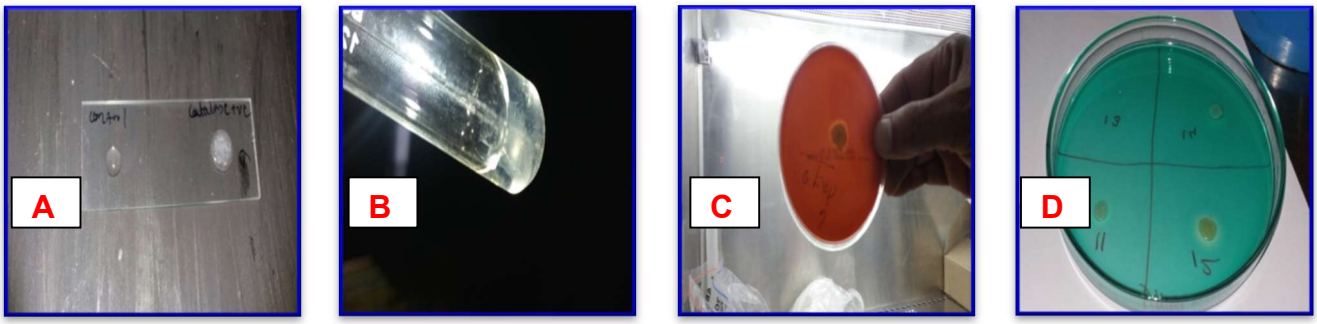

Figure 03: Various Biochemical tests for identification of Staphylococcus Spp.
A Catalase Test B Coagulase Test C haemolysis in blood agar D DNAse Test

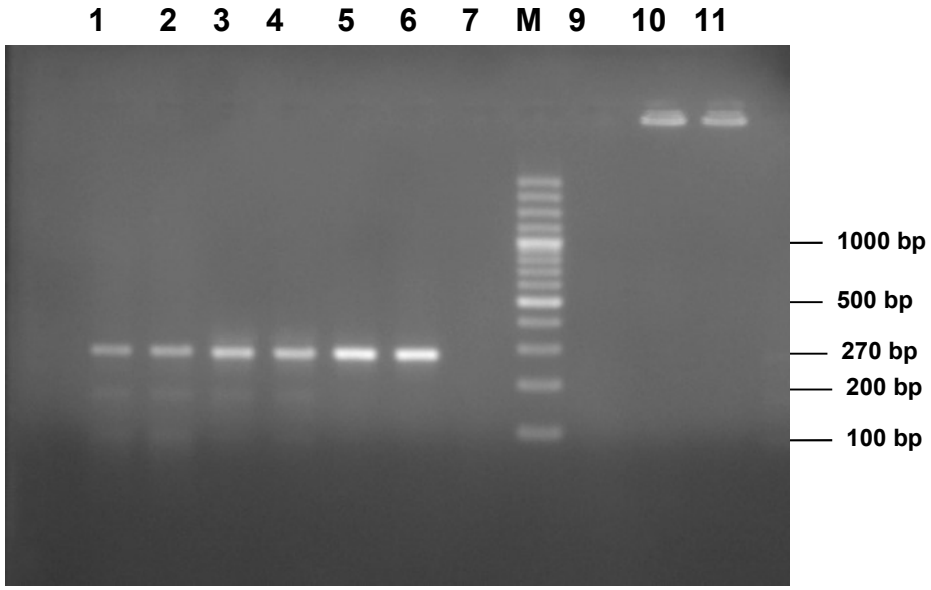

Figure 04: Molecular detection of *nuc* gene using PCR

Lane M : 100 bp DNA ladder

Lane 1-6 : Bacterial culture positive for *nuc*

Lane 7-11 : Bacterial culture negative for *nuc*