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Identification of *Staphylococcal aureus* isolates from bovine mastitis origin through polymerase chain reaction

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Abstract
Among the various mastitis pathogens, *Staphylococcus aureus* (*S. aureus*) is identified as chief etiological agent responsible for clinical, sub clinical and chronic mastitis and most challenging for the Veterinary clinician to treat such cases. In the present study 435 cows were screened for mastitis by California mastitis test and Somatic cell count from the Northern tropical region of India. On the basis of cultural and biochemical properties 61 isolates were presumptively identified as *S. aureus.* Further genotypically identified as *S. aureus* by Polymarase chain reaction (PCR) and 52 isolates were confirmed by species specific 16S rRNA and for thermonuclease gene (nuc). The present study might help to understand the distribution of prevalent *S. aureus* infection in the dairy farms and comparison between standard morphological tests and molecular characterization techniques.

Keywords: Bovine mastitis, *Staphylococcus aureus,* 16S rRNA, thermonuclease gene

Introduction

Staphylococcus spp. is the chief etiological agent of mastitis in cattle and buffaloes (Sharma *et al.*, 2007) [26]. The organisms are ubiquitous and can colonize the skin as well as the udder. They are capable of causing per acute, acute, sub-acute, chronic, gangrenous and subclinical types of mastitis. The prevalence of *S. Aureus* increases with parity of cow. Currently, *staphylococci* other than *S. aureus* comprise of 39 characterized species. More than 10 CNS species have been isolated from mastitis, but only few species predominate in herds. Mastitis caused by CNS was very mild, and usually remains subclinical (Taponen *et al.*, 2006). However in many countries it became the most common mastitis-causing agents (Pitkala *et al.*, 2004; Tenhagen *et al.*, 2006).

The bacterial culturing of the raw milk is the standard procedure for diagnosis of the bacterial flora of a particular dairy farm causing intramammary infection, but the method is time consuming. Hence molecular identification of *S aureus* by polymerase chain reaction (PCR) based on their 16S rRNAs is preferred because 16S rRNA genes are highly conserved throughout bacterial evolution (Kumar MK et al., 2020). Both 16S rRNAs and nuc gene can be used for species identification of *S. aureus* (Ciftci et al., 2009 [5]; Krimmer et al., 1999 [10]; Zhang et al., 2004) [27]. Therefore, the objective of this study is to characterize *S. aureus* phenotypically and genotypically with species specific 16S rRNA and also by thermonuclease gene (nuc).

Moreover, the sensitivity of PCR-based assays tends to be superior to bacterial cultures (Forsman *et al.*, 1997 [28]; Phuektes *et al*., 2003 [29]) allowing the detection of small number of microorganisms. Staphylococcal thermonuclease is considered as an important virulence factor and a unique marker widely used in the detection of *Staphylococcus aureus* from food samples and clinical specimens (Sandel and McKillip 2004 [30]; Alarcon *et al.,* 2006 [31]).

Material and methods

Collection of milk samples

Four hundred and thirty five lactating crossbred cattle were screened for intramammary infection (IMI) from northern part of India. The initial diagnosis of clinical and subclinical mastitis was based on clinical signs and California Mastitis Test (CMT) as described earlier (Schalm and Noorlander, 1957) [20]. Somatic Cell Count (SCC) was performed as per the guidelines of Schalm et al. (1971) [19]. The animals' positive by CMT point scores were selected for collection of milk sample.

The milk samples from affected quarters from each cow were collected after proper disinfection of hand and teat surface with 70 % ethyl alcohol. After teat preparation, about 5-10 ml of milk samples were collected in sterile vials, kept in ice box and carried to the laboratory, where the samples were kept at 4 ºC in refrigerator for further studies on SCC, and bacterial isolation.

Isolation and phenotypic characterization of *S. aureus*

Bacterial isolation: The isolation procedures were carried out under strict sterile environment. The identification of causative organism in collected milk samples was carried as per the method described by Griffin et al. (1977) [8]. The causative organism of the milk samples were identified initially on the basis of colony morphology, zone of haemolysis on 5 % blood agar after 24 hrs post incubation at 37 ºC (Cruikshank, 1964) [32].

Culture in selective media: The suspected colonies from 24 to 48 hrs. old culture grown in 5 % bovine blood agar were further grown on Mannitol Salt Agar (MSA). The colony characteristics were observed after 24-48 hours of incubation at 37 ºC. Further the colonies picked from MSA were again streaked on Baird Parker media. The suspected samples were selected for biochemical testing.

Biochemical test of *S. Aureus*

The biochemical characterization of *Staphylococcus aureus* was performed by HiStaph™ Identification Kit (Himedia Lab Pvt. Ltd., India), the test contains Voges Proskaeuer's test, Alkaline Phosphate, ONPG, Urease, Arginine utilization and ability to ferment carbohydrate including Mannitol, Sucrose, Maltose, Arabinose, Raffinose, Trehalose, and Maltose (Quinn et al., 2002) [33].

Catalase test, Coagulase test and Oxidase test

Catalase test producing bacteria were identified as per method of Thomas (1963) [23]. Catalase production was detected on nutrient agar slants after 24-72hrs of incubation by adding 3 % hydrogen peroxide over culture slants. Coagulase test was performed as per procedure described by Gillespie (1943) [7]. Oxidase test was performed by filter paper spot method as described by Kovacs (1956) [9].

Genotypic characterization of *S. aureus*

Identification of *S. Aureus* by PCR

The isolates were identified as *S. aureus* by amplification of 16S ribosomal RNA (rRNA). Briefly, for DNA preparation isolates were incubated overnight in 10 ml Brain Heart Infusion broth (BD, USA), centrifuged (5000 *g*, 15 min) and resuspended in 5ml TE (10mM Tris, 1mM EDTA, pH 8). Total cellular DNA was extracted from 1ml TE with QIAamp DNA Mini Kit (Quiagen, Netherlands) for G + ve bacteria according to manufacturer's protocol. From each sample, 5 µl of total cellular DNA were than evaluated by PCR with primers used previously described by (Lovseth et al., 2004) [11] (Table 1). Cyclining conditions for amplification: 1 × 5 min at 95 ºC, 1 min at 95 ºC, 0.5 min at 55 ºC, 1.5 min at 72 ºC, and a final extension at 72 ºC for 7 min.

Thermonuclease (*nuc*) gene

Species specific published primer sequence of (Brakstad et al., 1992) [2], (Table 1) was employed for the amplification of nuc gene for molecular identification of *S. aureus*. Cyclining conditions for amplification: 1 × 5 min at 95 ºC, 1 min at 95 ºC, 0.5 min at 55 ºC, 1.5 min at 72 ºC, and a final extension at 72 ºC for 7 min.

Results

Out of 435 cattle screened by CMT, 186 cattle were found positive for IMI. The SCC ranged from 2.81 ± 1.29 x 10^5 and to 20.22 ± 1.43 x 10^5 cells per ml of milk samples isolated from normal healthy cows and mastitic cows respectively.

Bacterial isolation

Out of 186 milk samples, 173 samples revealed growth on 5 % sheep blood agar plates and of which 112 samples with zone of hemolysis. Grams staining of 112 isolates, 96 numbers of samples were found as Gram's positive cocci. Isolation on MSA, on 48 hrs. post incubation out of 96 isolates 69 samples were positive as lactose fermenting colonies, which were positive for catalase and negative for oxidase tests. Baird Parker agar plates revealed characteristic black colonies in 63 samples on 24 hrs. post incubation. The isolated 63 cocci were further screened by coagulase test which revealed viscous clot of rabbit plasma in varying proportions in 61 samples.

Biochemical confirmation of *Staphylococcus aureus*

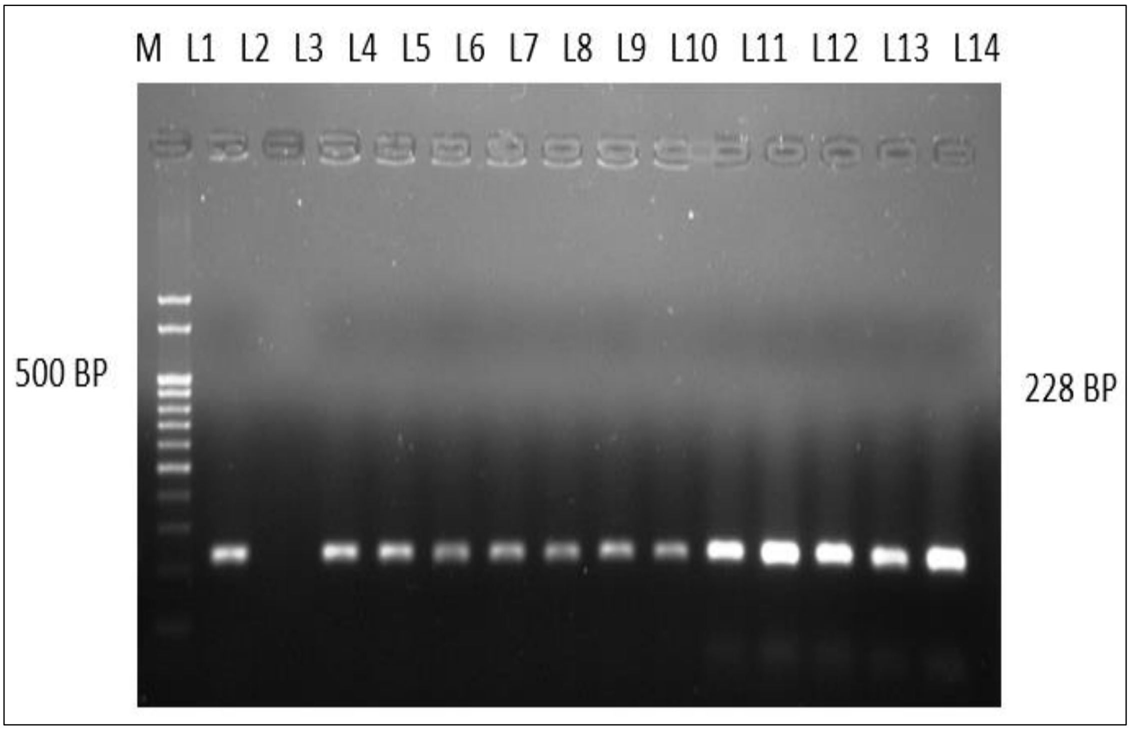
Fifty micro liter of overnight grown BHI broth cultures of characteristic coagulase positive isolates (n=61) were inoculated into each well of the kit (HiStaph™ Identification Kit) and kept at 37 ºC for 24 hours. Sixty one numbers of isolates showed characteristic color change before and after reagent addition the isolates were positive for VP, Alkaline Phosphate, Urease, Arginine utilization and ability to ferment carbohydrate including Mannitol, Sucrose, Maltose, Trehalose, and negative for ONPG, Arabinose and Raffinose. They were (n = 61) considered as *Staphylococcus aureus* based on cultural characteristic and biochemical tests.

Genotypic identification of *Staphylococcus aureus*

Isolation of Bacterial Genomic DNA

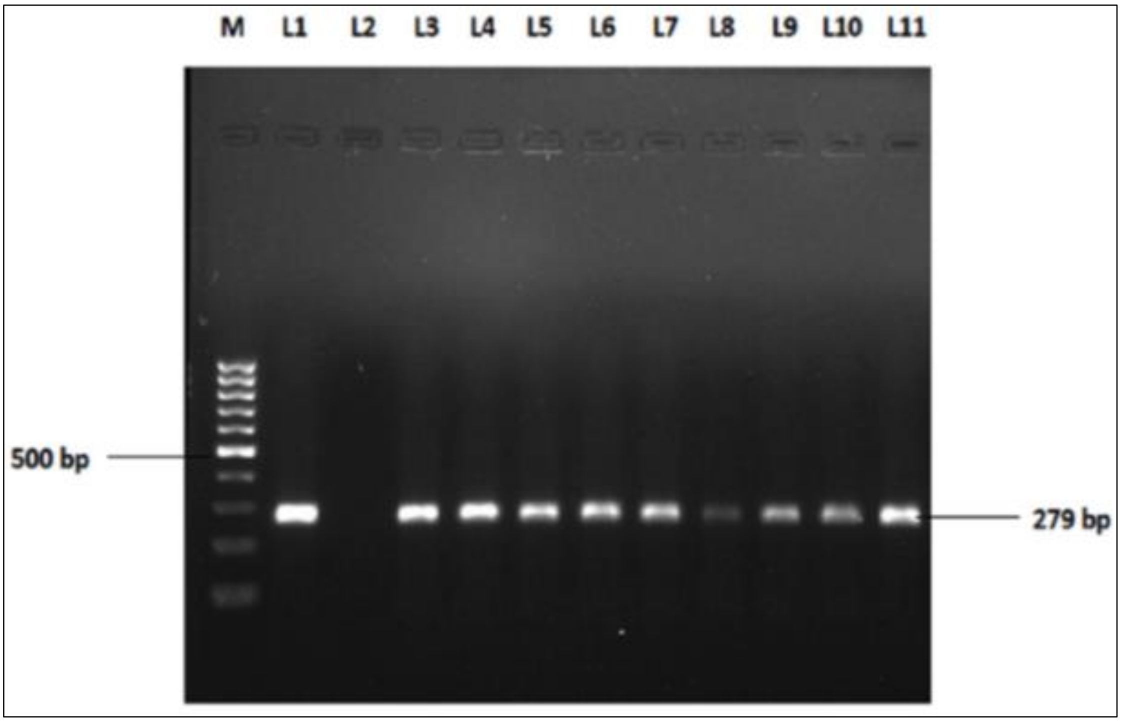
Isolation of bacterial genomic DNA from Staphylococcal isolates (n= 61) yielded varying concentration of DNA ranged from 45 ng/µl to 1606ng/µl with purity ranging from 1.50-1.97 as measured by Nanodrop Spectrophotometer. Amplification was observed in all 52 isolates using MTCC 2940 Staphylococcal strain as positive control.

Amplification for 16s rRNA was observed in 52 solates .The amplified products were of nearly 228 bp when resolved in gel electrophoresis. Amplification for *nuc* gene was observed in 52 solates. The amplified products were of nearly 279 bp when resolved in gel electrophoresis. One of the representative *nuc* gene PCR product was sequenced. The sequence on annotation and subsequent BLAST analysis in the NCBI database matched with the sequences from *S. aureus*. The phylogenetic analysis and sequence characterization revealed 100 % identity at the nucleotide level and deduced amino acid level with nuc isolates from India, China and Switzerland. The sequence was submitted to the NCBI Gen Bank and assigned with the accession number KR920356 for *S. aureus nuc* gene (Annexure I). On contrary, one *nuc* isolate from India 71.1 % divergent from our isolate. The *nuc* gene amplified in this study was species specific to *S. aureus* but still variation at the nucleotide level was observed among the isolates.



Lane M: 100 bp DNA ladder (Thermo-scientific, USA)
Lane 1: 16s *r*RNA gene of MTCC – 2940 as +ve control
Lane 2: Negative control (NFW)
Lane 4 to 14: PCR amplification for 16s *r*RNA gene in mastitis milk samples affected with *S. Aureus*

Fig 1: Agarose gel (2 %) showing PCR amplified 16s *r*RNA gene from mastitis milk samples



Lane M: 100 bp DNA ladder (Thermo-scientific, USA)
Lane 1: *Nuc* gene of MTCC – 2940 as +ve control
Lane 2: Negative control (NFW)
Lane 4 to 11: PCR amplification for *nuc* gene in mastitis milk samples affected with *S. Aureus*

Fig 2: Agarose gel (2 %) showing PCR amplified *nuc* gene from mastitis milk samples

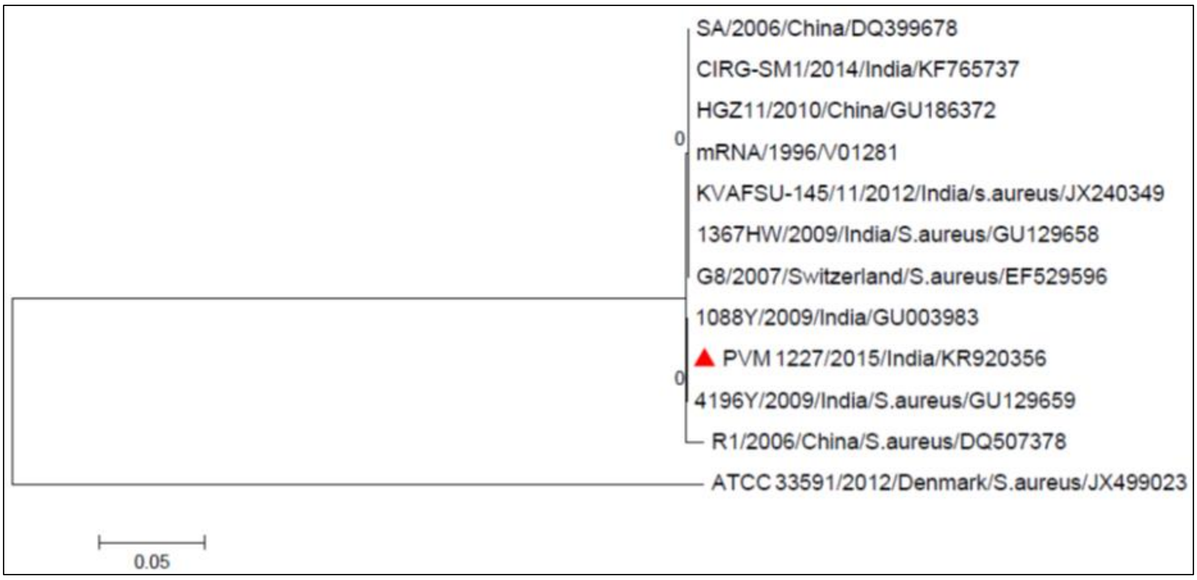


Fig 3: Phylogenetic analysis of *nuc* gene of *S. aureus* derived from mastitis milk samples with other isolates

Discussion

Mastitis is caused by several pathogens but *S. aureus* is a prime etiologic agent causing intramammary infection in dairy animals in most parts of the world (Sutra and Poutrel, 1994) [22]. Many researchers (De and Mukherjee 2013 [6]; Mukherjee, 2006 [34]; Singh et al. 1995 [35]; Sharma et al., 2015 [36]) reported staphylococci causing bovine mastitis from northern Indian states. *S. aureus* can be recognized phenotypically by a number of ways like Staphylocoagulase test and clumping factor test etc. (Chesneau et al., 1993) [3] and direct bacteriological examination of milk samples. Moreover these tests may give positive results for some other species also; hence molecular identification is recognized to be superior to earlier mentioned methods (Ahmadi et al., 2010) [1]. The identification of *S aureus* based on their 16S rRNAs is preferred because 16S rRNA genes are highly conserved throughout bacterial evolution. They consist of regions which are common to all eubacteria and other regions which are extremely species specific and for species identification of *S. aureus* has been used by many workers (Ciftci et al., 2009 [5]; Krimmer et al., 1999 [10]; Zhang et al., 2004) [37]. In present study 83.8 % (52 out of 61 isolates) of the isolates were identified as *S. Aureus* on the basis of amplification of 16S rRNA gene. Similarly, 83.8 % (52 out of 61 isolates) of the isolates were identified as *S. Aureus* basing on the amplification of *nuc* gene which depicts 9 numbers (14.76 %) of the IMI were caused by other coagulase positive staphylococcus like *S. Intermedius*, *S.hyicus* and others.

The exoenzyme thermonuclease coded by *nuc* gene is considered as one of the virulent determinants of *S. Aureus*. The pathogenicity of *S. Aureus* prompts the researcher to develop some quick means of characterization of the pathogen. The organism can be recognized phenotypically by a number of ways like Staphylocoagulage test, TNase test, clumping factor test etc. (Chesneau *et al.*, 1993) [3]. Whilst the exact pathogen could not be recognized as these tests gave positive results for some other species also. Hence molecular identification is recognized to be superior to earlier mentioned methods. Brakstad *et al.* (1992) [2] opined amplification of *nuc* gene for identification *of S.aureus*, with the amplicon size of 279 bp, located from 49 to 327 bp on the sequence. Chesneau *et al.*, (1993) [3] had modified the procedure of molecular identification of *S. Aureus* successfully using species specific probe by choosing much conserved region of *nuc* gene. Recently, some advanced studies with genomic and proteomic approach have also been conducted worldwide for confirmation of etiology and gene expression [13].

Conclusion

In the present study 435 cows were screened for mastitis from the Northern tropical region of India. 52 isolates confirmed as *S. Aureus.* This study also resulted that molecular characterization is superior to conventional morphological and biochemical tests for detection of *S. Aureus* which can be substantiated by further study.

Acknowledgments

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Appendix-XI

>seq1 [Staphylococcus aureus] isolate PVM 1227 Staphylococcal Thermonuclease gene, partial cds
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DEFINITION [Staphylococcus aureus] isolate PVM 1227 Staphylococcal
Thermonuclease gene, partial cds.
Accession KR920356
Version
Keywords
Source Staphylococcus aureus
Organism Staphylococcus aureus
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcus.
Reference 1 (bases 1 to 281)
Authors: Pati BK, Mukherjee R, Chaudhary P, Chakravarti S and Kar D
Title: Molecular characterisation of staphylcoccalthermonuclease gene isolated from bovine mastitic milk from India
Journal Unpublished
Reference 2 (bases 1 to 281)
Authors: Pati BK, Mukherjee R, Chaudhary P, Chakravarti S and Kar D
Title: Direct Submission
Journal Submitted (28-MAY-2015) Veterinary Medicine, Indian Veterinary Research Institute, Izatnagar, Bareilly,
Uttar Pradesh 243122, India
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