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Molecular detection of *Staphylococcus aureus* from bovine clinical mastitis

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Abstract
Mastitis is considered to be the most costly disease in dairy herds in terms of treatment costs, loss of milk production, early culling and death. *Staphylococcus aureus* is one of the most frequently encountered contagious pathogen responsible for mastitis. The present study was carried out in 83 clinical cases of bovine mastitis wherein, the majority of animals affected with mastitis were aged above five years (63.86 per cent) and had calved more than four times (26.5 per cent). Nearly half of the affected animals were in the mid stage of lactation (40.96 per cent). Milk samples collected from 83 affected quarters were subjected to cultural and biochemical characterisation and 49 isolates (59.04 per cent) were presumptively identified as *Staphylococcus* spp. Among 49 isolates obtained, all the isolates amplified the 756 bp fragment specific for the *16SrRNA* of *Staphylococcus* spp. whereas only 21 isolates amplified the 1318 bp fragment specific for the *23SrRNA* of *S. aureus*. In conclusion, a simple and rapid PCR-based assay was standardised for the molecular detection of *S. aureus* from bovine clinical mastitis.

Keywords: Contagious mastitis, polymerase chain reaction, *Staphylococcus aureus*, 16SrRNA, 23SrRNA

Introduction
Mastitis is a multi-aetiological complex production disease affecting dairy animals around the globe (Radostits *et al*., 2007) [1]. It causes huge economic losses in the global dairy industry by decreasing the quality and quantity of milk produced, as well as compromising the dairy cow health and welfare (Seegers *et al*., 2003) [2]. The occurrence of mastitis results from an interplay between the pathogenic, environmental and host factors that ultimately results in mammary incompetence against the invasion of infectious agents (Sudhan and Sharma, 2010) [3].

During mastitis, pathological modifications to the milk secreting epithelial cells occurs leading to a decrease in its functional ability. Depending upon the pathogen, functional losses may sometimes continue in to further lactations thereby compromising the productivity and potential weight gain by the offspring. While most infections caused relatively mild clinical or subclinical local inflammation, more serious cases lead to agalactia or even extensive systemic involvement ultimately leading to death (Hamadani *et al*., 2013) [4].

Numerous pathogens were responsible for mastitis with majority of the infections caused by bacteria. Among the diverse bacterial pathogens, *Staphylococcus aureus* (*S. aureus*) was frequently recognised worldwide as a major contagious mastitis pathogen with a prevalence ranging from 5 per cent to 70 per cent in cows and in 90 per cent of herds (Zecconi and Scali, 2013) [5].

Being an economically significant production disease of animals all over the world, early, rapid and accurate detection of mastitis pathogens is an absolute necessity (El-Razik *et al*., 2010) [6]. Early identification of the aetiological agent facilitates prompt treatment and thereby minimises the development of chronic disease and contributes to infection control in the herd. However, the identification of mastitis pathogens based on conventional culture and biochemical characterisation is often relative and not so reliable. It has reduced sensitivity to detect the low numbers of bacteria, especially in case of intermittent shedders or those inhibited by residual therapeutic antimicrobials or leukocytes. Moreover, the cumbersome and time-consuming isolation procedures together with the increased prevalence of environmental pollutants or intra-cisternal microorganisms and low sensitivity for species level differentiation acts as a barrier in its widespread use. It is therefore relatively common to replace or validate these conventional procedures using molecular methods such as PCR (Cai *et al*., 2003) [7]. Therefore, the present study was envisaged to investigate the efficacy of molecular

characterisation using polymerase chain reaction (PCR) targeting the *16SrRNA* and *23SrRNA* for identification of *Staphylococcus* spp. and *Staphylococcus aureus* respectively.

Materials and Methods

The present study was conducted in 83 cross bred dairy cows of varying age, parity and stages of lactation showing clinical signs such as changes in colour and consistency of udder or milk and enlargement of supra mammary lymph nodes, suggestive of clinical mastitis.

Teat was cleaned and swabbed with 70 per cent ethanol and about 10 mL of midstream lacteal secretions from infected quarters were aseptically drawn into sterile plastic screw-capped vials. Samples collected were placed securely in an insulated container having sufficient amount of ice packs and transported to the laboratory as quickly as possible for further identification.

Isolation and identification of bacteria

Isolation of bacteria was attempted from milk by direct streaking on to Brain Heart Infusion agar (BHIA; M211, Himedia, India) followed by incubation of the plates at 37 ºC for 24 hrs. Plates were examined after 24 to 48 hrs. A representative sample from the isolated colonies were selected for further identification.

The isolates were examined for their colony morphology and subjected to Gram's staining. The Gram positive colonies were inoculated on Mannitol salt agar (MSA), incubated at 37 °C for 24 hrs and observed for growth. Mannitol salt agar is a media with high salt concentration (7.5 per cent) that allows the selective proliferation of salt tolerant bacteria (Chapman, 1945) [8]. It could selectively differentiate *S. aureus* from coagulase negative staphylococci (CNS) and Micrococci. *Staphylococcus aureus* has the potential to ferment mannitol and changes the media acidic producing yellow colonies with yellow zones whereas other CNS and Micrococci being mannitol non fermenters, produced small pink or red colonies with no colour change in the media (Davies *et al*., 2005) [9]. Based on the cultural characteristics produced on Mannitol salt agar, the organisms were classified and subjected to further biochemical tests as per Barrow and Feltham (1993) [10] and Quinn *et al*. (2013) [11].

DNA Extraction

The heat lysis or snap chilling technique was used for the preparation of DNA template (Vijayakumar and Jose, 2021) [12]. Approximately one millilitre of overnight grown culture in mannitol salt broth (M383, Himedia, India) was centrifuged at 10,000 rpm for 10 min at 4 °C (Hispeed Centrifuge, KEMI). The pellet formed was resuspended in one ml of sterilised nuclease (DNAase & RNAase) free milli-Q-water (ML064, Himedia, India), followed by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellet was resuspended in 100µl tris EDTA buffer with pH 8, mixed in a vortex and kept in boiling water bath for 15 min. Then, snap chilled on crushed ice for 30 min, centrifuged at 10,000 rpm for 10 min at 4 ºC. The supernatant containing DNA was collected in sterile nuclease free centrifuge tubes and further checked for concentration and purity using a nanodrop spectrophotometer (Nanodrop™ 1000 Spectrophotometer) and stored at - 20 ºC.

Polymerase chain reaction

To confirm the presence of *Staphylococcus* spp. genus specific PCR was performed to amplify the *16SrRNA* gene of *Staphylococcus* spp. (Table 1). The polymerase chain reactions were carried out in 25µl reaction mixture (Table 2). The reactions were carried in thermal cycler (Bio-Rad Laboratories, USA) with initial denaturation at 94 °C for 5 min. followed by 30 cycles of each denaturation at 94 °C for 45 sec, annealing at 56.9 °C for 45 sec and extension at 72 °C for 90 sec. Final extension was allowed at 72 °C for 10 min. after the last cycle.

Amplification of *23S rRNA* gene by PCR

Species specific PCR targeting the *23SrRNA* gene was performed to confirm the presence of *Staphylococcus aureus* (Table 1). The amplification reactions were performed with initial denaturation at 94 °C for 5 min. followed by 30 cycles of each denaturation at 94 °C for 45 sec, annealing at 55.8 °C for 45 sec and extension at 72 °C for 90 sec, with final extension at 72 °C for 10 min.

Table 1: Oligonucleotide sequence of primers used in the study

Genus/Species	Target gene	Sequence	Reference
Staphylococci spp.	*16SrRNA*	F: AAC TCT GTT ATT AGG GAA GAA CA R: CCA CCT TCC TCC GGT TTG TCA CC	Ciftci *et al*., 2009 [13].
Staphylococcus aureus	*23SrRNA*	F: GGA CGA CAT TAG ACG AAT CA R: CGG GCA CCT ATT TTC TAT CT	El-Razik *et al*., 2010 [6].

Table 2: Components of a single PCR reaction mixture

Name of the reagent	Quantity (µL)
2 X PCR Master mix (SapphireAmp, Takara)	12.5
Forward Primer (10 pmol/µl) (Sigma Aldrich)	1
Reverse Primer (10 pmol/µl) (Sigma Aldrich)	1
Nuclease free water	5.5
Template DNA	5
Total	25

Five microliters of the PCR product were electrophoresed on 1.2 per cent agarose gel, at 400mA and 70 V for 60 min. (Bio-rad, USA). Three microlitres of 100 bp DNA ladder (MBT-049; Himedia, India) a positive control and negative control was added along with each set of samples.

Subsequent to electrophoresis, the gel was transferred to a UV trans illuminator (GeNei™, India) for visualisation of bands. The gel was documented in a gel documentation unit (Gel Doc™ EZ imager, Biorad, USA).

Sequencing and Procurement of Accession Number

Deoxyribonucleic acid sequence determination was carried out by the sequencing service, Agrigenome, Ernakulam with the same primers that were used for amplification. Sequences were edited and aligned by using emboss merger and global sequence comparisons were performed by using basic local alignment search tool (BLAST) hosted by the National Center for Biotechnology Information (NCBI). The new sequences

were submitted using the Bankit software and deposited in the GenBank database under the procured accession numbers.

Results and Discussion

Milk samples from 83 cross bred dairy cows suffering from clinical mastitis were collected over a period from March 2019 to July 2020. Being a multi aetiological complex disease, the occurrence and outcome of mastitis depends on numerous epidemiological risk factors such as host resistance, infectious agents and the environmental or managerial factors. These key epidemiological factors often interact together to compromise the cow's natural resistance against invasion of infectious agent. In the present study, age wise distribution of clinical mastitis revealed a higher incidence in animals aged above five years (63.86 per cent) and the least number of cases were found in animals less than three years of age (10.48 per cent). Some of the possible causes for the increased occurrence of mastitis with advanced age include the progressive suppression of the host immune system, age related anatomical changes in udder and teats, long term exposure to milking practises or resistance of pathogens to antibiotic that were indiscriminately used in previous lactations (Tegegne *et al*., 2020) [14]. In contrast to the present findings, Rathish (2014) [15] reported an increased occurrence of clinical mastitis in younger cows which might be acquired from subclinically infected herd mates. However, this cannot be generalised to the present situation, since the majority of samples were obtained from individual households.

The distribution of clinical cases with respect to parity revealed a higher incidence (26.5 per cent) in cows that calved more than four times whereas animals in the third and fourth parity comprised 24.1 per cent and 18.07 per cent, respectively. Similar findings were made by Abebe *et al*. (2016) [16] who opined that multiparous animals that had four or more calvings are 24.8 times more likely to get clinical mastitis than primiparous cows. Bangar *et al*. (2018) [17] also reported a substantially higher incidence of mastitis ranging from 30.34 to 40.16 per cent in multiparous cows compared to primiparous cows (16.36 to 22.00 per cent). This might be due to the decreased effectiveness of the teat sphincter and the teat canal of older cows, as a barrier to infection or due to the relaxation of the median ligaments that supports the udder. The increasing trend of infection with parity can also be due to the milk yield of animals, that would be high in multiparous animals relative to primiparous animals, thereby favouring the incidence of mastitis infections (Bhat *et al*., 2017) [18].

In the present study, cows in mid stage of lactation (40.96 per cent) were more likely to have mastitis than cows in early (33.74 per cent) and late stage of lactation (25.3 per cent) The present results are in concordance with the findings of Kayesh *et al*. (2014) [19] who recorded a higher prevalence of 32.05 per cent in cows during mid lactation. But, in contrary, Revathi (2018) [20] reported an increased occurrence of coliform mastitis towards early stages of lactation which were attributed to the infections picked up during dry period as well as due to the increased oxidative stress and reduced antioxidant defense mechanism during periparturient period and early lactation. This might also happen due to the increased udder pressure, weaning associated milking practices, negative energy balance, hyperketonaemia or non-adaptation to the milking methods. The differences in the impact of lactation stages between different studies may be due to discrepancies in the age, parity or the breed of animals sampled, as well as the nature of the causative pathogen.

Clinical examination

The supra-mammary lymph nodes were enlarged in 33 animals (39.76 per cent) whereas, the remaining animals had normal size and consistency of the supramammary lymph nodes. These findings were in accordance with Khoramian *et al*. (2015) [21] who reported a significant increase in the length and depth of ipsilateral supra-mammary lymph nodes, wherein one or two quarters were positive in bacteriological culture. Enlargement of supramammary lymph nodes were also reported in tuberculous (Radaelli *et al*., 2011) [22] and mycotic mastitis. This might be due to the increased recruitment of CD^{4+} and CD^{8+} T lymphocytes in to the mammary gland and consequently to milk in response to infection (Souza *et al*., 2020) [23].

In the current study, swollen udder and varying degree of fibrosis were observed among 92.77 per cent and 74.69 per cent of the total animals examined, respectively. The increased frequency of udder fibrosis is an indication of untreated or therapy refractory infections that might have resulted in the replacement of soft infected mammary tissue with fibrous tissue ultimately resulting in pathogen persistence (Chakraborthy *et al*., 2019) [24]. One of the most convincing hypotheses to explain pathogen persistence and thereby the presence of fibrosis is the ability of most microorganisms to survive in biofilms in the infected tissues, thus developing an innate resistance to almost all therapeutic agents (Melchoir *et al*., 2006) [25]. Hussain *et al*. (2012) [26] reported similar findings, stating that biofilm producing bacteria can survive in the udder by establishing encapsulations and micro abscesses, which protect them from the immune system and antimicrobial drugs.

Variations in the colour and consistency of milk was evident in all the animals examined. Out of the 83 cases studied, 30 showed cream coloured milk; 23 were curd like; 12 were watery and 14 samples had flakes. Two animals each produced straw coloured and blood tinged milk respectively. The results of this study were in agreement with the findings of Radostits *et al*. (2007) [1] who stated that the colour of infected lacteal secretions varied from creamy to deep or greenish yellow and blood tinged. These changes often occur due to the inflammatory response as well as due to the destructive action of invading pathogens leading to a loss of integrity of the blood milk barrier.

Isolation and identification of bacteria

Out of the 83 milk samples which were collected, 57 samples yielded bacterial colonies whereas 26 (31.3 per cent) samples revealed no bacteriological growth. The potential reasons for this finding could be the presence of antibacterial substances or drug residues from previous treatment in the milk that contribute to a decrease in bacterial viability in the culture or the inherent limitations of conventional culture when compared to the detection of bacteria using a real-time PCR (Taponen *et al*., 2009) [27].

The 57 bacterial isolates obtained were identified based on their morphology, colony characteristics on selective media and biochemical characteristics. The present study revealed that among the 57 bacterial isolates, Gram positive cocci were the most numerous (88.06 per cent). This was in consonance with the findings of Kulangara *et al*. (2017) [28] who reported that Gram positive cocci (70.4 per cent) was the major pathogen responsible for persistent infections in dry bovine

udders. Furthermore, the present study showed a substantial increase in the prevalence (40.3 per cent) of coagulase negative staphylococci (CNS) compared to those of Sebastian (2001) [29] and Rathish (2014) [14], who could isolate only 6.4 per cent and 23.81 per cent CNS from all bacterial isolates, respectively. High prevalence of staphylococcal infections in the mammary glands of dairy cattle has previously been recorded in large dairy farms, but high prevalence in isolated small holder dairy systems practising hand milking by individual milkers indicates that staphylococcal infection is endemic among the cattle population in the region under research.

Molecular characterisation of *Staphylococcus* spp.

According to Lange *et al*. (2015) [30] the *16S rRNA* was found to be an important molecular target for the detection of staphylococcal isolates from 95 per cent of the bovine mastitis cases studied and in the present study the primer pair targeting the same *16S rRNA* is employed. Amplification of *16SrRNA* gene of *Staphylococcus* spp. was evident in PCR from all the 49 presumptively identified Gram positive cocci (Fig. 1). These findings were in accordance with Raj (2018) [31] and Amrithapriya (2019) [32] who targeted the *16SrRNA* for identification of *Staphylococcus* spp. from nasal swabs of healthy pigs and bovine mastitic milk. However, Ghebremedhin *et al*. (2008) [33] reported 90 to 99 per cent sequence similarity in the *16S rRNA* sequence of the 29 different *Staphylococcus* spp. and questioned its usefulness in phylogenetic study at species level. They concluded that the partial sequencing of the gap gene, a constitutive housekeeping gene that encodes a transferrin binding protein is a better molecular tool for the taxonomical analysis of staphylococcal isolates at species level as it has minimum sequence similarities when compared to other commonly employed genes such as the *hsp60* gene, the *sodA* gene, the *rpoB* gene, and the *tuf* gene. But this could not be extrapolated in to our study since we attempted only the genus level identification of staphylococcal isolates using *16S rRNA*.

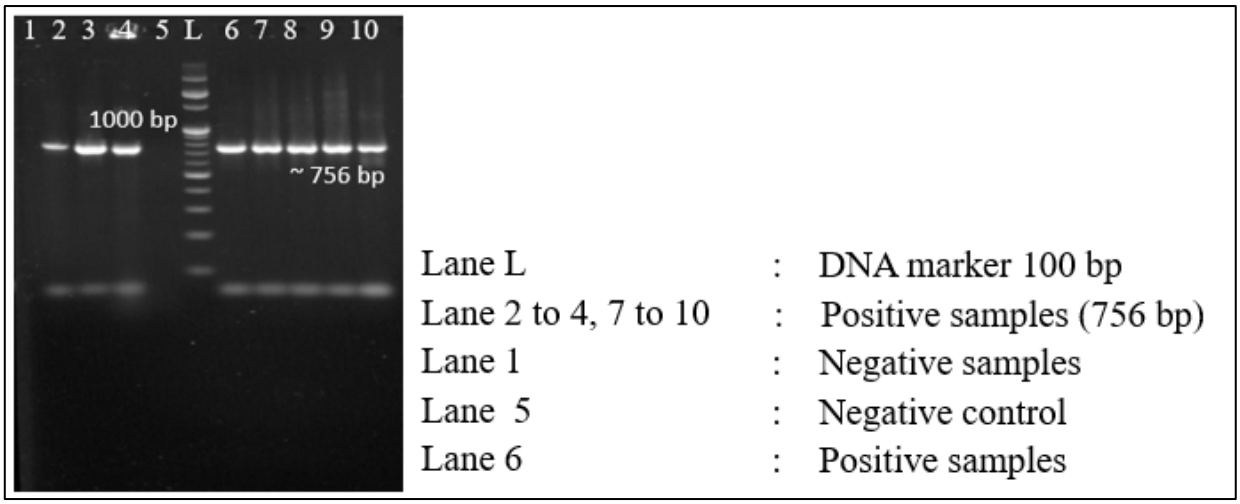

Fig 1: Agarose gel electrophoresis of *16SrRNA* specific PCR of *S. aureus* isolates

Molecular characterisation of *S. aureus* isolates

Numerous PCR-based systems targeting different genes such as the *nuc* gene (Ciftci *et al*., 2009) [12], *femA* gene (Gandhale *et al*., 2017) [34], *coa* (Guler *et al*., 2005) [35], *spa* (Koreen *et al*., 2003) [36] have been documented in literature for detection of *S. aureus*. In the current study, molecular characterisation of *S. aureus* was done using primers targeting the *23S rRNA*. The primers targeting the *23S rRNA* gene amplified the 1318 bp fragment specific for *S. aureus* (Fig 2) in 21 out of the 49 DNA samples and confirmed the presence of *S. aureus*, a contagious mastitis pathogen. This was substantiated by the findings of Ludwig *et al*. (1992) [37], who determined the complete *23S rRNA* primary structure of Gram positive bacteria and proposed that in addition to the functional constancy of *rRNA*, its structural elements contain evolutionarily less conserved to highly variable regions whose freedom of variation increases with the length of variable parts and is most pronounced in the large subunit RNA. Thus, there is more variation among *23S rRNA*, compared to that of *16S rRNA*, making it as excellent target for construction of species-specific primers. Out of the 49 isolates that were positive for *Staphylococci* spp. by PCR, 22 isolates yielded the PCR product with an amplicon size of 1318 bp corresponding to *23Sr RNA* and were confirmed to be *S. aureus.* This was consistent with the findings of Hamid *et al.* (2017) [38] who compared the efficacy of thermonuclease (*nuc*) gene with that of the *23S rRNA* and reported that eight *nuc* gene negative isolates were further confirmed as *S. aureus* by *23S rRNA.* However, Straub *et al.* (1999) [39] reported that even though highly sensitive, ambiguities can arise when using *23S rRNA* for detection of *S. aureus* from meat starter cultures and dairy products due to lack of DNA or inhibition of hybridization reactions due to the presence of various inhibitory substances such as calcium ions, plasmin and proteins in dairy products. They proposed that the limitation could be overcome by an enrichment step which increases the sensitivity and minimises the chance of false positives by diluting the non-viable cells of dried or heated milk products.

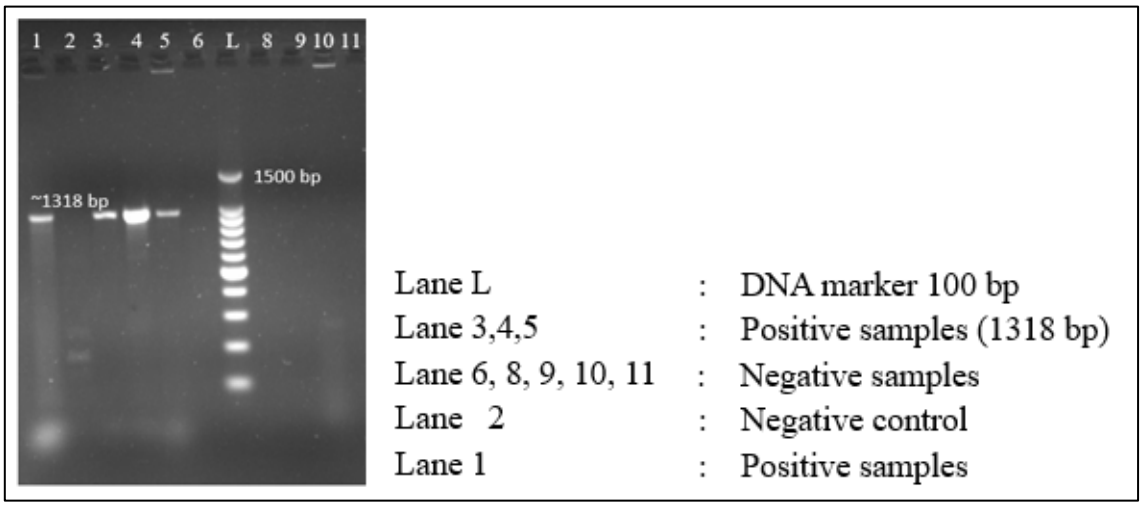

Fig 2: Agarose gel electrophoresis of *23SrRNA* specific PCR of *S. aureus* isolates

A BLAST search against GenBank revealed the highest similarity score (100 per cent) for sequences of *Staphylococcus aureus* with *Staphylococcus aureus* strain UMCG 578 complete genome, accession number CP077738.1 available in GenBank database. The nucleotide sequences of *Staphylococcus aureus* isolated from pure culture were deposited in GenBank database with the accession number MW195499.1.

Conclusion

In conclusion, a simple and rapid PCR-based assay for the molecular detection of *Staphylococcus spp. and S. aureus* is described here. The results of the study identified the *16S rRNA* and *23S rRNA* as reliable markers for the molecular characterisation of *Staphylococcus* spp. and *S. aureus* respectively. The assay was performed after the isolation of organism by conventional culture on specific media. However, when directly performed on milk samples without a culture step, the PCR would be less time consuming and takes less than 24 h to complete, while identification of bacteria to the species levels by conventional microbiological and biochemical methods requires more than 48 h. Therefore, the present study suggests the need to develop and validate PCR assays for direct detection of the pathogen from milk sample.

Ethical approval and consent statement

There is no specific law in India that requires the permission from ethics committee for collecting milk samples as a part of disease investigation. However, animals were examined and samples were collected as per standard examination and sample collection procedures, after taking consent from the owner.

Conflicts of interest

There were no conflicts of interest reported by the author (s).

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