Prevalence of *Staphylococcus aureus* Isolated from Mastitic Milk, Udder Surfaces and Milkers’ Hands from Different Farms in Bikaner, Rajasthan

Taruna Bhati1*, Kumar Gaurav2, Vikas Khichar3 and Anil Kumar Kataria1

1Department of Veterinary Microbiology, College of Veterinary and Animal Science, RAJUVAS, Bikaner, INDIA
2Department of Veterinary Microbiology, M.B. Veterinary College, Dungarpur, INDIA
3Department of Animal Husbandry, Government of Haryana, INDIA.

*Corresponding author: T Bhati; Email: vetcvas.bhati@gmail.com

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ABSTRACT

*Staphylococcus aureus* is recognized worldwide as one of the most important contagious mastitis pathogen and is frequently isolated from mastitic milk and extramammary sites. The present study was undertaken to study prevalence of *S. aureus* strains isolated from mastitic milk, udder surfaces and milkers’ hands from organized (n=5) and unorganized dairy farms (n=2). For this, a total of 197 samples (80 mastitic milk samples, 66 udder swabs and 51 swabs of milkers’ hands) were collected from different places. A total of 107 isolates including 51 from mastitic milk samples, 35 from udder and 21 from milkers’ hands, were presumptively identified as *S. aureus* on the basis of cultural and biochemical properties and then genotypically confirmed using 23S rRNA ribotyping followed by PCR amplification of *nuc* gene. An overall recovery rate of *S. aureus* was 54.3% with highest (63.8%) recovery from mastitic milk samples followed by udder (53.0%) and milkers’ hands (41.2%). The unorganized dairy farm showed highest prevalence (65.4%) of *S. aureus* than that in other farms.

Keywords. Mastitis, *nuc* gene, ribotyping, *Staphylococcus aureus*

Mastitis is one of the most complex and multifactorial disease affecting dairy animals. *Staphylococcus aureus* is one of the chief causative agent of clinical mastitis in bovines in India (Padhy *et al*., 2014; Kutar *et al*., 2015; Bhagat *et al*., 2017; Choudhary *et al*., 2018) and worldwide (Abdel-Tawab *et al*., 2016; Hanon, 2017). The milk from infected mammary glands serves as the primary reservoir of the bacterium which may be transferred to other animals in the herd during milking (Capurro *et al*., 2010). In addition human handlers, milking equipment, environment, udder and teat skin of infected animals are possible sources of infection to the animals. In dairy workers, the hands may be considered the most important site of *S. aureus* because the hands are in intimate contact with the udder.

Although various PCR based detection systems have been developed so far for identification of *S. aureus* but not found sufficiently reliable to detect all strains of *S. aureus*. A PCR system developed by Straub *et al.* (1999) based on amplification of species-specific 23S rRNA allows specific detection of all strains of species. Thus this method is extensively used throughout the world for genotypic identification and confirmation of *S. aureus* from various clinical and subclinical infections (Salasia *et al*., 2004; Upadhyay *et al*., 2010; Khichar *et al*., 2014; Yadav *et al*., 2015).

Furthermore the molecular diagnosis of *S. aureus* infection is also carried out by amplification of *nuc* gene (~270 bp) that encodes for thermonuclease enzyme (Kateete *et al*., 2010; Vremera *et al*., 2011; Nazir *et al*., 2017).

*Staphylococcus aureus* is a contagious mastitis pathogen and its transmission is thought to occur from animal to animal principally through the mastitic milk, udder surfaces, milkers’ hands, milking utensils, or milking machine (Radostits *et al*., 2006). However, its epidemiological behaviour is not definite with strains
signifying infectious and/or environmental transmission patterns (Fernandez et al., 2013). Hence the present study was undertaken to study the molecular epidemiology of S. aureus strains in and around Bikaner, Rajasthan, India by 23S rRNA ribotyping and amplification of nuc gene.

MATERIALS AND METHODS

Animals and sample collection
In the present study, a total of 197 samples were collected from seven different locations (five organized and one unorganized dairy farm in and around Bikaner, Rajasthan, India and one unorganized dairy farm in Bhiwani, Haryana). Animals of selected locations were diagnosed with clinical mastitis by clinical symptoms. From each location, three types of samples were collected which comprised of milk samples from cows with clinical mastitis, swabs from udder of infected cows and swabs of milkers’ hands who were working in that farm or location. A total of 80 mastitic milk samples, 66 udder swabs and 51 swabs of milkers’ hands were collected in the morning hours and were immediately transported on ice to the laboratory for further processing as per standard procedures (Quinn et al., 1994). All the procedures have been carried out in accordance with the guidelines laid down by the Institutional Ethics Committee and in accordance with local laws and regulations.

Isolation and identification of bacteria
The samples were inoculated in nutrient broth over night and then swabbed on nutrient agar followed by overnight incubation at 37 °C. Bacterial colonies were closely observed for their morphology, color and consistency. Gram’s staining was used as primary identification test and suspected colonies were streaked on mannitol salt agar and incubated for 24 h at 37 °C under aerobic conditions.

Molecular identification of S. aureus

23S rRNA Ribotyping
The isolates were genotypically confirmed by 23S rRNA species specific PCR using forward primer-1 (5′-ACGGAGTTACAAAGGACGAC-3′) and reverse primer-2 (5′-AGCTCAGCCTTAACGAGTAC-3′) (Straub et al., 1999).

nuc gene typing
The amplification of nuc gene was carried out using forward primer (5′-GCG ATT GAT GGT GAT ACG GTT-3′) and reverse primer (R-5′-ACG CAA GCC TTG AAC TAA AGC-3′) as described by Brakstad et al. (1992). The 25.0 μl reaction mixture for nuc gene consisted of 5.0 μl 5X Go Taq® Flexi buffer, 3.0 μl MgCl2 (25mM),1.0 μl dNTP mix (25mM each), 1.0 μl Forward Primer(10 pM/μl), 1.0 μl Reverse Primer(10 pM/μl), 0.2 μl Taq DNA polymerase (5U/μl), 3.0 μl DNA template (30 ng/μl) and 10.8 μl nuclease free water to make 25.0 μl. Amplification was carried out in a Veriti thermal cycler (Applied biosystem) and consisted of PCR cycle of pre denaturation at 94°C for 5 min, followed by 34 cycles of amplification (denaturation at 94°C for 60 s, primer annealing at 55°C for 60 s and primer extension at 70°C for 60 s), and final extension at 72°C for 10 min. The PCR products, were resolved on 1.2% agarose gels prepared in 1.0X TBE buffer containing 0.5 μg/ml of ethidium bromide and 50 bp DNA ladder were used as molecular marker. The amplification products were electrophoresed for 50-60 min at 100 Volts. The gels were then visualized under gel documentation system (ENDURO GDS).

RESULTS AND DISCUSSION
Details of samples and recovery of S. aureus isolates in samples collected from different locations of Bikaner have been depicted in Table 1. On the basis of cultural and biochemical properties, out of 197 samples, 107 S. aureus isolates were presumptively identified of which 51 isolates were from mastitic milk samples, 35 from udder and 21 from milkers’ hands.

In the present investigation, all the 107 isolates produced an amplicon of 1250 bp following 23S rRNA based ribotyping (Fig. 1) and an amplicon of 270 bp upon PCR amplification of nuc gene confirming them to be S. aureus (Fig. 2). The overall recovery rate was 54.3%. Highest prevalence of S. aureus was observed in mastitic milk samples being 63.8% followed by 53% and 41.2% prevalence in udder and milkers’ hands, respectively.
Prevalence of *Staphylococcus aureus* isolated from mastitic milk...

Among the seven groups under study, the overall highest prevalence of *S. aureus* was found in group no. 5 (unorganized dairy farm) being 65.4% followed by group no. 7 (60.0%), group no. 3 (58.3%), group no. 2 (55.3%), group no. 4 (55.0%), group no. 1 (40.0%) and group no. 6 (35.7%). Further, all the groups showed higher occurrence of *S. aureus* in mastitic milk than in extramammary sites (Table 1).

Genotypic confirmation by 23S rRNA ribotyping using similar primers has been reported by Sanjiv et al. (2008); Upadhyay et al. (2010); Khichar et al. (2014); Yadav et al. (2015); Choudhary et al. (2018) from the same laboratory. Likewise, Salasia et al. (2004); Bhandari et al. (2009); Montaz et al. (2010); Yang et al. (2012); Parth et al. (2016); Hamid et al. (2017) obtained species-specific amplicon of 1250 bp in genotypic identification of this organisms from mastitic milk samples from different geographical locations.

Kateete et al. (2010) considered identification of *S. aureus* using PCR amplification of the *nuc* gene as a gold standard method. Detection of *nuc* gene for the identification of *S. aureus* strains isolated from intramammary infections has been adopted by many workers in India and from other parts of the world. Similar prevalence of 100% *nuc* gene carrying *S. aureus* isolates has been reported in previous studies on intramammary infections in cattle by Kalorey et al. (2007), Sarkar et al. (2014), Xu et al. (2015), Nazir et al. (2017) and Javid et al. (2018) in different parts of the world.

The overall occurrence of *S. aureus* (54.3%) from all the locations in present study is similar to that reported by Souza et al. (2012) who collected 446 samples from several sites in dairy farms and recovered 244 (54.7%) *S. aureus* isolates. Sarkar et al. (2014) also reported 73.6% prevalence of *S. aureus* in the farm from mastitic milk samples and nasal swabs of farm workers similar to present study.

Nathiya et al. (2018) reported recovery of 47.45% *S. aureus* isolates from milk of cows with clinical mastitis from same area of work as in present study. Similarly, Kutar et al. (2015) detected higher (56%) incidence of *S. aureus* in clinical mastitis cases in Uttar Pradesh, India. Likewise, Parth et al. (2016) found that 54.29% of clinical mastitic milk samples yielded *S. aureus* (cows 61.90% and buffaloes 42.85%) in Gujarat, India. A high prevalence of 63.8% of *S. aureus* isolates from mastitic milk samples observed in present study is similar to the findings of Sori et al. (2011) who isolated 86 (52.4%) *S. aureus* strains from 164 high CMT score milk samples in Ethiopia. Hanon, (2017) reported incidence of 47.69% *S. aureus* from bovine mastitis in Iraq and Baloch et al. (2018) isolated 46.2% *S. aureus* strains from bovine mastitic milk in China.

However some workers have reported low prevalence viz. 6.6% (Lee et al., 2012), 19.9% (Anderson et al., 2012), 15.5% (Mekuria et al., 2013) and 12% (Leigue et al., 2017) with similar study pattern of isolating *S. aureus* from mastitic milk samples and extramammary sites. While some workers have reported a low prevalence of *S. aureus* from mastitic milk samples collected from different farms i.e. 22.7% (El-Jakee et al., 2008); 29.16% (Abdeen et al., 2015); 22.5% (Hamid et al., 2017); and 28.1% (Srednik et al., 2018).
In the present study, the occurrence of *S. aureus* in mastitic milk, udder skin and milkers’ hands was found to be different in all groups owing to the difference in management practices of maintaining animal health as well hygiene of the farm and farm workers.

Similar to the present research work of isolating *S. aureus* from mastitic milk samples and extramammary sites, studies have been conducted in other parts of India (Sarkar *et al*., 2014) and world reporting the prevalence of *S. aureus* in milk, body sites and environment in varying degrees (Capurro *et al*., 2010; Souza *et al*., 2012; Mekuria *et al*., 2013; Schmidt *et al*., 2017). When compared to the prevalence rates detected by other workers worldwide, it was found that alike the prevalence rate of *S. aureus* in milk, the prevalence rate of *S. aureus* on the udder skin and in the hands of animal worker population varies depending upon the size, geographical area and management practices of the herd under study.

In conclusion, the results of the present study demonstrate that genotyping methods involving 23S RNA ribotyping and nuc gene amplification provides easy identification of *S. aureus* isolates in bovine mastitis. Furthermore this study also substantiates the previous reports of *S. aureus* being most prevalent pathogen causing clinical mastitis in dairy animals revealing higher prevalence in samples of bovine origin as compared to human samples. The isolation of *S. aureus* from milkers hands in fairly good numbers indicate transmission of this pathogen between animals and man on a farm. Hence it may be suggested from the present study that stringent management practices should be adopted to prevent the spread of these bacteria and of mastitis in a herd of animals.

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