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CULTURAL, BIOCHEMICAL AND HAEMOLYTIC PROPERTIES OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CASES OF SUBCLINICAL MASTITIS IN CATTLE

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ABSTRACT

In the present study 38 *Staphylococcus aureus* isolates from subclinical mastitis cases in Holstein-Friesian (H-F) crossbred and Rathi (a native breed) cattle were characterized on the basis of their cultural, biochemical and haemolytic properties after confirmation by 23S r RNA based genotyping. The overall incidence of subclinical mastitis was 44.70 % with higher incidence (51.61 %) in H-F crossbred cattle than in Rathi cattle (40.74 %). Among the cultural and biochemical properties, 35 out of 38 isolates produced golden yellow colonies and three isolates produced white colonies. In the sugar fermentation reactions using 11 different sugars, variations were observed in the results. Thirty five isolates produced coagulase and the overall strongest coagulation reaction was recorded with plasma from human followed by horse, cattle, rabbit, sheep, camel, goat, dog, buffalo and chicken. Thirty two isolates were haemolytic while six were non-haemolytic. All the isolates produced α-toxin of high titres (1:2560 and 1:5120) and β-toxin was produced by 34 isolates with lower titres (1:5 to 1: 160). None of the isolates produced delta toxin. In conclusion, incidence of subclinical mastitis was more in H-F crossbred cattle than in Rathi cattle. Not all the isolates were pigmented, coagulase producer and hemolytic.

INTRODUCTION

Staphylococcus aureus is a well recognized pathogen leading to both clinical and subclinical bovine mastitis (Suleiman *et al*., 2012). It has been implicated in more than 80% of subclinical bovine mastitis resulting in economic losses of about 300 $ per year per animal (Karahan *et. al*., 2011). Further milk from such animals with subclinical mastitis may legally be sold for human consumption which may lead to many different subsequent infections or food poisoning as such (Oliveira *et al*., 2011). *Staphylococcus aureus* can be easily isolated from subclinical mastitis cases using nutrient agar and Mannitol Salt Agar as selective media in the laboratory. Hemolysins produced by *S. aureus* have been considered true virulent factors in causation of the disease (Dinges *et al.,* 2000; Ariyanti *et al*., 2011) and typing and titration of these hemolysins may well be an indicator of pathogenicity factor because of its hemolytic, dermonecrotic and neurotoxic effects (Dinges *et al*., 2000). It produces coagulase, an extracellular enzyme that binds to protein to form a complex with thrombin-like activity which converts fibrinogen to fibrin (McDevitt *et al*., 1992).The production of coagulase by *S. aureus* has been related to pathogenicity of this organism and has also been used as an important criterion for the phenotypic identification of the organism by many workers. Hence the present study was undertaken for isolation, identification and phenotypic characterisation of genotypically confirmed *S. aureus* isolates from subclinical mastitis in cattle in terms of cultural and biochemical properties, coagulase production, haemolytic properties and toxin assays.

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MATERIALS AND METHODS

Sampling

Eighty five milk samples were collected during early morning hours in sterilized test tubes from Holstein–Friesian (H-F)

crossbred (n=31) and Rathi cattle (n=54) from different locations in Bikaner (Rajasthan, India). The samples were immediately taken to the laboratory for further processing on ice

Somatic cell counting (SCC)

A 0.1ml amount from each properly shaken milk samples was withdrawn with Pasteur pipette and spread evenly on a glass slide to count the somatic cell count as per the method of Prescott and Breed, (1910).

Ribotyping of organisms

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).

Identification of *S. aureus*

All the milk samples which showed SCC corresponding to subclinical mastitis were processed for isolation of *S. aureus* by conventional methods. Milk samples were streaked onto 5% sheep Blood Agar (BA), nutrient agar and DNase agar plates and incubated aerobically at 37^{o}C for 24-48 h. The tests of sucrose, D-mannose, D-manitol, maltose, sucrose, lactose, raffinose, dextrose, arabinose, dulcitol and inositol fermentation were carried out. The tube coagulase test was carried out for of free enzyme using plasmas from different animal species (*viz*. cattle, buffalo, sheep, goat, horse, dog, rabbit, chicken, camel) and human. All these tests were performed as described by Quinn *et al.* (1994).

Haemolytic properties and Haemolysin assays

The hemolytic activity was evaluated by inoculating *S. aureus* isolates in the form of streaks on the surface of triplicate plates of blood agar base supplemented with 5% sheep, bovine and horse blood for alpha, beta and delta hemolysin assays, respectively (Quinn *et al.,* 1994*)* and incubated at 37°C for 24 and 48 h. The criteria for hemolysin identification were: complete lytic zone (transparent) with blurred edges for α-hemolysin on ovine and incomplete (non-transparent) lytic zone, which became complete with sharp edges after overnight incubation at 4°C on bovine blood agar, for beta hemolysin. The delta-hemolysin production was determined as complete hemolytic zones on horse blood agar (Quinn *et al.,* 1994; da Silva *et al.,* 2005). Qualitative and quantitative assays for α, β and δ haemolysins were done using rabbit, cattle and horse erythrocytes respectively (Sanjiv and Kataria, 2007). The preparation of erythrocytes, typing and titration of haemolysins were done as per method described by (Sanjiv and Kataria, 2007).

RESULTS

Out of the 85 milk samples, 38 milk samples showed SCC in the range of 200×10^3 to 500×10^3 cells/ml corresponding to subclinical cases of mastitis as per the IDF 2005 criterion. All 38 isolates were also confirmed to be *S. aureus* by species specific PCR targeting *23S rRNA* and revealed an amplicon of 1,250 bp. In the present investigation, the incidence of subclinical mastitis associated with *S. aureus* was 44.7%. The recovery of *S. aureus* was 51.61% from H-F crossbred and 40.74% from Rathi subclinical mastitic milk samples. Following inoculation and culture on nutrient agar, 35 isolates produced golden yellow pigmentation, the intensity of which increased with passage of time whereas three (7.8%) isolates produced white colonies belonging to both the breeds of cattle (one to H-F crossbred and two to Rathi). All the 38 isolates were subjected to aerobic cultivation on mannitol salt agar the color of the medium changed to yellow from original pink by all the isolates but a variation in reaction time was observed for different isolates. In the sugar fermentation reactions using 11 different sugars only two sugars (mannitol and dextrose) were fermented by all the isolates obtained from crossbreds whereas six sugars (mannitol, sucrose, dextrose, fructose, lactose and mannose) were fermented by all the isolates from Rathi cattle. Of the total 38 *S. aureus* isolates,100% fermented mannitol and dextrose, 97.4% fermented fructose, sucrose and lactose, 94.7% fermented mannose, 92.1% fermented maltose, 13.1% fermented arabinose and 2.6% fermented raffinose sugar. Dulcitol and inositol were not fermented by any of the isolates from both the cattle breeds. Out of 38 isolates, 31(81.6%) *S. aureus* isolates showed a positive DNase test. In the tube coagulase test, 35 isolates showed positive coagulase test and three of the isolates showed negative reaction with plasma from all the species used. The overall coagulase reaction in the present study in descending order of superiority was human > horse > cattle > rabbit > sheep > camel > goat > dog > buffalo > chicken. In the present study, 32 (84.2%) isolates were hemolytic and six (15.8%) did not produce haemolysis on blood agar. Out of 32 isolates, six (18.75%) produced complete haemolysis, 17 (53.1%) produced partial haemolysis and nine (28.1 %) of the isolates showed both types of haemolysis on sheep blood agar. Only one isolate showed turning of partial hemolysis to complete hemolysis (hot-cold lysis) whereas other 16 isolates did not show hot-cold lysis. In the hemolysin assay, all the 38 isolates haemolysed rabbit erythrocytes indicating presence of alpha-toxin whereas beta-toxin was found to be produced by only 34 (89.5%) isolates and none of the isolates produced delta toxin. The titres of alpha toxin produced by most of the *S. aureus* isolates from crossbred and Rathi cattle were 1:2560. The titres of beta toxin were much below (1:5 to1:160) than that of alpha toxin.

DISCUSSION

The criterion of isolation of organisms along with SCC more than two lacs cells per ml of milk from normal udders has been followed in the present investigation to define subclinical mastitis (SCM). The SCC has been detected to be the most reliable test and closest to the bacteriological results for SCM in dairy cows by. Most of the workers (Sharma *et al*., 2010; Elango *et al*., 2010; Moges *et al*., 2011; Mosaferi *et al*., 2012) have detected a positive correlation between SCC and bacterial pathogen. The isolation of predominantly *S. aureus* from SCM cases in present study shows concordance between our observation and those from other workers *viz.* Abdel-Rady and Sayed, (2009) Moges *et al*. (2011) and Suleiman *et al*. (2012). The higher incidence of SCM in H-F crossbred than in the Rathi cattle supports the earlier observations of Abdel-Rady

and Sayed (2009) and Moges *et al*. (2011) who also reported higher prevalence of SCM in crossbred breeds than in the indigenous zebu cattle. Difference in colony pigmentation of *S. aureus* isolates which were recovered from cattle, human and other domestic animals was reported by many workers [Quinn *et al*. (1994), Adesiyun *et al.* (1999) Salasia *et al*. (2004)] as seen in our study. In the present study of the three isolates producing white colonies on nutrient agar, two were coagulase positive in tube test and possessed *coa* gene (as studied by genotyping) whereas the third one did not produce coagulase and was *coa* gene deficient. Our study revealed that coagulase production was independent of colony pigmentation. The observation of white colonies is in complete agreement to the observations of Sanjiv *et al*. (2008) who reported one isolate and white colonies and absence of *coa* gene.

The fermentation reactions in the present study for sucrose, mannose, fructose and lactose were also almost similar to those observed by Khichar (2011). When the results for two cattle breeds were compared it was concluded that isolates of Rathi origin showed fermentation of more sugars included in the study than the isolates from crossbred cattle. Our observations in regards to fermentation of glucose by all the isolates and of inositol by none of the isolate is in complete agreement to the findings of Morandi *et al*. (2009) who carried out biochemical profiling or metabolic fingerprinting of the *S. aureus* isolates from dairy products using biologue GP-2 microplate. DNase activity is important to distinguish between pathogenic staphylococci and nonpathogenic resident flora. DNase is as important as coagulase for pathogenesis (Pfaller & Herwaldt, 1988). In the study of Devriese and Oeding (1975), it was found that there was a strong association of DNase and coagulase production for *S. aureus*. Both of these tests were positive 96% of the time. Contrarily, our study revealed that that there is no correlation between DNase activity and coagulase production with pathogenicity in *S. aureus* isolates from both the breeds. In the tube coagulase test using plasma from nine species and human, our results were in conformity to those of Adesiyun and Shehu (1985) who also recorded strongest reaction with human and rabbit plasma followed by pig, donkey, chicken, cattle, duck and goat. They also reported spontaneous clotting of horse plasma but it was not recorded in the present study. Our results also supported observations of Kateete *et al.* (2010) who found that human plasma was more sensitive (91%) than sheep plasma (81%) for the tube coagulase test. The variations in coagulase reaction is probably due to affinity for different plasma samples (Wilson and Miles, 1975). The prevalence of β-hemolysis in bovine *S. aureus* strains in the present study is in full agreement with Aarestrup *et al*. (1999); Larsen *et al.* (2002); Morandi *et al.,* 2009. Our study on pattern of hemolysis of 38 *S. aureus* isolates on sheep blood agar is in contrast to the observations of Singh (2006) ; Sanjiv and Kataria (2007); Upadhyay and Kataria, (2010); Khichar (2011) who did not record ahemolytic *S. aureus* from milk of cattle and goat. Similar to our study, Yadav *et al*., (2015) reported ahaemolytic *S. aureus* isolates from milk of cattle and buffalo with clinical mastitis. Production of α-toxin by all the isolates in the present study is similar to the findings of Upadhyay and Kataria (2010); Khichar (2011); Yadav*et al*., (2015) who also reported production of α-toxin by all the isolates from bovine and goat mastitic milk. The non-production of δ-toxin is in contrast to observations of Upadhyay and Kataria (2010) and Yadav *et al*., (2015) who demonstrated production of δ-toxin by some of the isolates of *S. aureus* from mastitic milk samples. The present findings of α-toxin titres of 1:2560 of *S. aureus* from Rathi and H-F crossbred cattle are in agreement to those of Khichar (2011), Upadhyay and Kataria (2010) who also recorded similar α-toxin titres. The highest titres of 1:5120 for α-toxin are similar to the findings of Sanjiv and Kataria (2007) and Yadav *et al*., (2015). The highest titres for β-toxin in *S. aureus* isolates from both the breeds was same being 1:160 which is in complete agreement to the findings of Upadhyay and Kataria (2010) who also reported similar highest titres for β-toxin in isolates from cattle and goat mastitis. In the present study on 38 *S. aureus* isolates from subclinical mastitic cases, a lot of variations were observed in the biochemical properties since the biochemical reactions of staphylococci have been shown to vary within the same gland over time (Maisi and Riipinen, 1991). In conclusion, incidence of subclinical mastitis was more in H-F crossbred cattle than in Rathi cattle and not all the isolates were pigmented, coagulase producer and hemolytic. Hence different biotypes were observed on the basis of cultural, biochemical and hemolytic properties.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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