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CHARACTERISATION OF *STAPHYLOCOCCUS AUREUS* OF CATTLE CLINICAL MASTITIS MILK ORIGIN FOR COAGULASE AND HAEMOLYSIN PROPERTIES

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ABSTRACT

In the present investigation 28 *S. aureus* isolates obtained from milk collected from matitic Holstein-Friesian crossbred and Rathi cattle of various parities confirmed by 23S rRNA based genotyping were studied for their coagulase and haemolysin properties. The coagulation reaction was evaluated against plasmas from 10 species of animals and from human in which the overall strongest coagulation reaction was recorded with plasma from rabbit followed by buffalo, cattle, camel, human, goat, sheep, dog, horse, chicken and pig in descending order.All the isolates were haemolytic on sheep blood agar of which 13 isolates showed both α- and β-hemolysis and 15 isolates produced only β-hemolysis which exhibited the phenomenon of Hot-cold lysis. The titre of α-haemolysin from H-F isolates ranged between 1:40 and 1:1280, whereas for Rathi isolates it ranged between 1:160 and 1:2560. The titre of β-haemolysin was much less for isolates both from H-F (between 1:5 and 1:30) and Rathi (between 1:5 and 1:60) cattle.

INTRODUCTION

Mastitis is the most common global disease of cattle causing great economical losses. The main causative agent of clinical mastitis is *Staphylococcus aureus* which is also responsible for food poisoning and many other human ailments. Though the organism can be identified by conventional methods but there is no single phenotypic test (including the tube coagulase) that can guarantee reliable results in the identification of *S. aureus* [1] as this organism shows variations in phenotypic expressions [2].

Coagulase is a collagen binding protein encoded by *coa* gene which has been demonstrated to be directly related with bovine mastitis [3]. Its production was believed to be a reliable index of pathogenicity [4] and as an important criterion for identification of *S. aureus* [5]. However, non-production of coagulase by *S. aureus* has also been reported by various workers [6-8]. Haemolysins produced by *S. aureus* have been considered true virulent factors in causation of the disease and typing and titration of these haemolysins may well be an indicator of pathogenicity of these organisms. Determination of virulence genes is important for animal health and food hygiene [9].

The present investigation was carried out with a view to characterize *S. aureus* isolates obtained from milk from Holstein-Friesian crossbred and Rathi cattle with clinical mastitis for their coagulase properties with respect to its production and comparison of coagulase reactions

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with plasmas obtained from different species of animals and from human, and for haemolytic properties with respect to haemolysis on blood agar and typing and titration of haemolysins.

MATERIALS AND METHODS

Isolation and identification of bacteria: The milk samples collected from Holstein-Friesian crossbred and Rathi cattle (a breed native to Bikaner area) with clinical mastitis were used to isolate and identify *S. aureus* [10, 11]. In the study 13 *S. aureus* isolates from H-F crossbred and 15 isolates from Rathi cattle were included.

Genotypic confirmation of organisms (Ribotyping) : The DNA was isolated from bacterial culture grown overnight in 25 ml nutrient broth in shaker incubator at 37^{o}C [12]. The integrity of DNA was checked by agarose gel electrophoresis and DNA quantification was carried out by spectrophotometric measurements [13]. The quantified DNA was diluted to a final concentration of 25 ng/µl in TE buffer and ribotyping based on 23S rRNA gene was carried out using primer-1, 5'-ACGGAGTTACAAAGGACGAC-3' and primer-2, 5'-AGCTCAGCCTTAACGAGTAC-3' [14].

Blood collection: The blood was collected for plasma separation to be used in coagulase test from cattle, buffalo, sheep, goat, horse, dog, camel, rabbit, chicken, pig and human volunteers, for blood agar preparation from sheep and for separation of erythrocytes to be used in haemolysin titration from cattle, horse and rabbit.

*Coagulase production***:** The test was carried out in tube for production of coagulase using plasmas from different animal species (viz. cattle, buffalo, sheep, goat, pig, horse, dog, rabbit, chicken, camel) and human. The reaction was read at 1, 3 and 5 hr of incubation [15].

Haemolysin production, typing and titration **:** To test haemolysin production by bacterial isolates and to confirm whether the isolates were able to produce zones of partial haemolysis, zones of complete haemolysis, both or none, sheep blood agar medium was used and typing and titration was done [16].

RESULTS AND DISCUSSION

23S rRNA gene based confirmation: In the present investigation all the isolates which were subjected to PCR amplification targeting 23S rRNA gene produced a species-specific amplicon of 1250 bp size confirming them to be *S. aureus*.

Coagulase production: All of the isolates produced coagulase and the overall strongest coagulation reaction in regards to early onset and firmness of clot was recorded with plasma from rabbit followed by buffalo, cattle, camel, human, goat, sheep, dog, horse, chicken and pig in decreasing order. Production of staphylocoagulase, an extracellular protein, by *S. aureus* forms an important criterion for identification of this organism. The staphylocoagulase forms a complex with plasma prothrombin leading to exposure of active site on the prothrombin molecule. This complex is capable of splitting fibrinogen into fibrin which then forms a clot.

Along with proper morphology and the organism being catalase positive, the four plus (++++) coagulase test reaction should stand alone as the definitive identification of *S. aureus* [16]. However, against this concept coagulase negative *S. aureus* isolates have also been obtained and reported by many workers. The affinity for certain plasma samples has also been suggested for the variation in coagulase reaction [17, 18]. The use of plasmas from several animal species whenever practicable has also been advocated as staphylococcal biotypes display variable ability to coagulate different plasmas [19, 20]. Considering the above facts in the present study the production of coagulase by *S. aureus*isolates was evaluated against plasmas from ten species of animals and against human plasma as well. The present observation on coagulation of plasmas from different species were in conformity to the earlier observations [21] who recorded rabbit plasma most suitable for coagulation reaction of *S. aureus* isolates.

In the present study the plasma did not clot spontaneously in contrast to other report [21], which recorded horse plasma unsuitable in coagulase test as it clotted spontaneously. The findings in the present study in regard to duration of incubation on coagulase test results also did not match with those of these workers who reported that duration of incubation had no significant effect on coagulase test results whereas in the present investigation the reaction started weak in many of the isolates at 1 h observation and then firmness of clot increased. With some of the isolates the clot later (5 h) dissolved in some of the plasmas but clot with buffalo and chicken plasma did not dissolve with any of the isolates.

In the present investigation *S. aureus* caused better coagulation of human plasma than that from sheep which is in accordance to the findings of other workers [22] who found that human plasma was more sensitive (91%) than sheep plasma (81%) for the tube coagulase test.

The early and strong coagulation of rabbit plasma and comparatively weak reaction with those of sheep and dog may be because of relatively less amounts of coagulase activators. In a study on the mode of action of *S. aureus* coagulase plasmas from cow, sheep, dog, guinea pig and mouse were found with relative deficiency in coagulase activator while plasma of human, monkey, horse, cat, pig, fowl and rabbit contained the most [23].

In contrast to the finding in our investigation dog plasma was found to show faster coagulation than rabbit plasma by other workers [24] who further suggested that rabbit plasma may not be the ideal coagulase test medium for *S. aureus* from all sources. Likewise human

prothrombin was recorded superior to bovine prothrombin in coagulase test [25] in contrast to our findings.

Haemolysis on blood agar: Of the 28 isolates, 15 produced incomplete (β) haemolysis which turned into complete haemolysis when the plates with partial haemolysis were incubated at 4°C overnight (Hot-cold lysis). Thirteen isolates showed complete zone (α-haemolysis) surrounded by incomplete zone of haemolysis (β-haemolysis).

The results of haemolysis on blood agar in the present study are in complete agreement with those of other workers [26] who also did not record non-haemolytic *S. Aureus* obtained from milk samples of cattle. On the other hand many workers recorded variable percentages of non-haemolytic *S. aureus* viz. 62.7% [27]; 61%; 57.13% [28]; 26% and 8.1% [29].

In the present study none of the isolates produced only α-haemolysis. These observations corroborate the earlier findings who recorded only 1.6% to 2.46% isolates to produce α-haemolysin [30]. However, others had recorded a high percentage of *S. aureus* from cattle milk samples showing α-haemolysis on sheep blood agar.

On blood agar, only complete (α-) haemolysis was not shown by any of the isolates in this study. When titration was carried out against rabbit, cattle and horse erythrocytes it was found that all the isolates produced α- and β-haemolysins and none of the isolates produced δ-haemolysin. It is possible that the effect of α-toxin in the blood agar plate could have been masked by β-toxin [31]. Our findings are in complete agreement to those of other workers who recorded conspicuous complete haemolysis on blood agar with isolates which either did not produce β-toxin or produced it in lower concentration. The non-production of δ-toxin by all the isolates could have been an additional reason in not causing complete haemolysis on blood agar as δ-toxin is also responsible for complete haemolysis [32].

Qualitative Haemolysin assay: All the isolates produced α- and β-haemolysins but none of the isolates produced δ-haemolysin. Production of haemolysin in various combinations has been demonstrated repeatedly by various workers and has been correlated with the pathogenicity of the isolates. The results in the present investigation in regard to production of α- and β-haemolysins by all the isolates and δ-haemolysin by none are in agreement to an earlier observation who also did not record production of δ-haemolysin by *S. aureus* isolates obtained from H-F cross and Rathi cattle with clinical mastitis. Similarly, a very small number of *S. aureus* isolates were recorded to produce δ-haemolysin by other workers [32].

Quantitative Haemolysin assay: The titre of α-haemolysin produced by *S. aureus* isolates from H-F cattle ranged between 1:40 and 1:1280, whereas for the isolates from Rathi cattle it ranged in between 1:160 to 1:2560. The titres of β-haemolysin were much less than that of α-haemolysin for all the isolates which ranged in between 1:5 and 1:30, and in between 1:5 and 1:60 for isolates from H-F and Rathi cattle respectively.

The present findings of α-haemolysin titres of *S. aureus* from Rathi cattle were in complete agreement to an earlier observation [26] in which similar titre were recorded. When the number of isolates producing α-haemolysin was analysed*vis-à-vis* titres it was recorded that overall the number of isolates producing higher titres was more. There was a degree of evidence suggesting that α-haemolysin is a virulence factor of this pathogen for the mammary glands and its production is the feature of bovine mammary isolates [22].

The lower titres of β-haemolysin than that of α-haemolysin in the present investigation were in complete agreement to the earlier reports from the same laboratory [8, 26] wherein comparatively lower titres of β-toxin were recorded.

CONCLUSION

In conclusion, all the *S. aureus* isolates from mastitic cattle were coagulase positive and showed variable degree of reaction with plasmas from different animal species and man. The best coagulation reaction was observed with rabbit plasma. All the isolates were haemolytic on blood agar and titration revealed production of α- and β-haemolysins only with higher titres of former haemolysin.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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