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Biotyping and PCR Based Detection of *Staphylococcus* Aureus in Mastitic Large Ruminant Population of District Faisalabad

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ABSTRACT

S. aureus is considered one of the most important economical disease of dairy enterprise worldwide which affect the quantity and quality of the milk. The present study aimed at detection of S. aureus in mastitic cows and buffaloes using PCR based and biotyping techniques. For this purpose, a total of 374 lactating animals (cows n= 169; buffaloes n= 205) belonging to five small holder and three institutional dairy herds were examined for clinical and subclinical mastitis. Using Surf Field Mastitis Test (SFMT), 14 (8.28%) out of 169 cows and 35(17.07%) out of 205 buffaloes were clinically mastitic while 157 (23.22%) out of 676 were apparently mastitis free quarters. While 205 buffaloes were found positive for subclinical mastitis. Biotyping with the aid of Staphytect plusTM kit, 83 of the recovered isolates of S. aureus were assigned to six patterns. Predominant pattern embraced 28, 17, 15, 9, 7 and 7 of pattern I, IV, III, V, II and VI, respectively showing fairly strong biotype herd association. From these 83 biotypes, coagulase gene was confirmed in 79 isolates. It was hence concluded that coagulase gene primer PCR was found to be the most efficient and sensitive diagnostic technique for detection and identification of clinical and subclinical mastitis.

INTRODUCTION

Mastitis is the inflammation of parenchyma of mammary glands adversely affecting milk production and milk composition. It can be classified into two main classes including clinical and subclinical mastitis. Former class manifests clinical signs including inflammation of udder, fever, pain and decline in milk yield while the later class does not exhibit visible signs which can only be identify using diagnostic tools (Sharun *et al*., 2021). Mastitis is the costliest disease of livestock. In Punjab, Rs.240 million losses are caused due to clinical mastitis annually. These losses argue to decrease production, discard milk, premature culling and replacement of animals (Hogeveen *et al*., 2019).

The degree of severity of the disease depends upon pathogenicity of causative agent, breed, age, lactation state and immunological health of the animal (Vlasova *et al*., 2021). The major pathogens causing mastitis worldwide are; *S. aureus*,

Streptococcus agalactiae, Streptococcus uberis (Strep uberis) and *Streptococcus dysgalactiae* (Kabelitz *et al*., 2021). *S. aureus* is the most important in producing toxins Initially *S. aureus* damages gland cistern and teat linings of the quarter which leads to the tissue damage (Piepers *et al*., 2017). It is mainly associated with a decrease in milk production, early exclusion or production of cows, low-quality milk, veterinary expenses and medicines (Girma *et al*., 2022).

Various techniques can be implied for the diagnosis of clinical and subclinical mastitis including bacteriological examination of milk and increased somatic cell count. In developed countries California Mastitis Test (CMT) is used for the detection of subclinical mastitis. An ideal, easy and economic animal-side test for detection of subclinical mastitis is Surf Field Mastitis Test (SFMT) consisting of sodium alkyl aryl sulfonate reagent (Muhammad *et al*., 2010). Moreover, use of molecular techniques has been increased over the past decades, in which the most sensitive is PCR (Algharib *et al*., 2024).

Thus, the objective of present study was to investigate the diversity of *S. aureus* isolates on the basis of DNA analysis and the discriminatory powers of different methods used for the detection and identification of *S. aureus* isolates from mastitis milk.

MATERIALS AND METHODS

The study was carried out on three institutional dairy farms (Herd I: Department of Animal Breeding and Genetics, University of Agriculture, Faisalabad crossbred dairy herd, Herd II: Department of Livestock Management, University of Agriculture, Faisalabad cow and buffalo dairy herd, Herd III: Ayub Agriculture Research Institute Jhang Road, Faisalabad) and 5 peri-urban private dairy herds (Herd IV: Mahar Zafar Dairy Farm, Herd V: Suddhupura cattle farm, Herd VI: Rana Irfan Suddhupura Livestock Farm, Herd VII: Raja Asghar Dairy Farm and Herd VIII: Bilal Rasheed Dairy Farm).

Collection of Milk Samples from Cows and Buffaloes for Mastitis

374 lactating animals (cows n= 169; buffaloes n= 205) were sampled and then clinically mastitic cows and buffaloes and those who are suffering/affliction from subclinical mastitis were identified using Surf Field Mastitis Test (Muhammad *et al*., 1995**)** in Mastitis Research Laboratory, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad. To this end, quarter milk samples were mixed with an equal volume of 3% solution of a household detergent (Surf Excel, Lever Brothers, Pakistan) in the four receptacles of the Surf Test paddle, mixture was spin for about 15 seconds and looked for gel formation (i.e. thickening of the mixture).

Isolation and Identification of *S. aureus* from Mastitic (Clinical and Subclinical) Milk Samples

Procedures depicted by National Mastitis Council Inc. (1987) were followed for culturing the mastitic milk samples and for identification of *S. aureus*. The samples were gently shaken 8 times to get a uniform distribution of the pathogens. Using a platinum-rhodium inoculation loop, 0.01 ml of each quarter foremilk sample was streaked, each onto blood agar and Staph 110 agar plate. Four quarter milk samples were cultured on a 100 mm plate by plating individual quarter sample on one quadrant of plate and were incubated at 37˚C for 48 hours. The representative colonies of the microorganisms were isolated and purified by streaking onto fresh blood agar plates. Catalase positive coccal isolates were presumptively identified as Staphylococci and were subjected to tube coagulase test using citrated rabbit plasma. Latex Slide Agglutination Test using Staphytect plus™ kit (Oxoid, UK) was conducted for determination of clumping factor, protein A and certain polysaccharides found exclusively in *S. aureus*. The final confirmation of *S. aureus* was based on determination of 7-digit numerical biochemical profiles using a commercially available identification kit of Genus Staphylococcus (api Staph, bioMerieux, France).

Biotyping of *S. aureus* Isolates (n= 83) Recovered from Clinically and Sub-clinically Mastitic Milk samples of Cows and Buffaloes

S. aureus isolates were biotyped using a commercially available identification kit (api Staph; bioMerieux, France) as per manufacturer's directions. Staphytect plus™ (Oxoid, Basingstoke Hampshire, UK) is a latex slide agglutination test for the identification of *S. aureus* by detection of clumping factor, Protein A and certain polysaccharides.

Coagulase Gene Specific PCR

The Coagulase gene specific PCR (Mello *et al*., 2020) was performed in a 20µl reaction with the following components and PCR temperature profile, d_3H_2O (12.8 µl), 10 X PCR buffer (2 µl), dNTPs (2 µl), Primer 1 (1 µl), Primer2 (1 µl), DNA (1 µl) and Taq (0.2 µl). The steps involved 30 cycles including denaturation at 94˚C, annealing at 54˚C, extension at 72˚C for 45 sec, 60 sec, 120 sec respectively.

RESULTS AND DISCUSSION

Mastitis is one of the notorious causes that result in shortage of milk supply in Pakistan (Khan, 2019). It adversely affects the milk production as well as the quality of the milk and leads to premature culling and termination of the lactation of dairy cows and buffaloes (Singh, 2022). Screening for real mastitis in dairy cows is a very important step in combating the disease. Various surveillance studies are currently underway to determine the frequency of un-labeled atypical disease of udder. *S. aureus* is the most important causative agent of udder hygiene. In present work, 374 lactating animals were examined and screened through SFMT. 8.28% cattle and 17.07% buffaloes were found clinically mastitis positive. While 23.22% cows and 18.29% buffaloes were suffering from subclinical mastitis (Table 1). Similar results were found by Hashemi *et al*. (2011) in which 21.6% cattle were positive for subclinical mastitis using CMT while percentage of clinical mastitis was different i.e. 0.71%. Biotyping with api Staphytect plus™ kit (Biomereux, France) assigned the 83 *S.aureus* to six patterns. Pattern-I was the most predominant pattern embracing 28 of 83 isolates. This was followed by pattern-IV with 17, pattern-III with 15, pattern-V with 9, pattern-II and pattern-VI with 7 isolates from each. A fairly strong of biotype-herd association (restriction) was observed. Thus, isolates of biotype-I were recovered from Herd I, II and V. While biotype II, IV, V and VI were limited to herd II, herd I, herd IV and herd V, respectively.

As PCR is used to amplify and detect the coagulase (coa) gene of S. aureus isolates in order to confirm its pathogenicity, since the presence of the coagulase gene is an indicator of pathogenicity (Stephan *et al*., 2001). So, 83 identified *S. aureus* (API-STAPH) were subjected to coagulase gene PCR and thus 79 (95.18%) isolates in total were confirmed via this molecular method. Out of which 27 isolates recovered from pattern I, 15 from pattern III and IV, 9 from V, 7 from II and 6 isolates from pattern VI. All of the 79 coagulase positive DNA samples from isolates were amplified through PCR for the coagulase gene using the primers COAG2 (50CGAGACCAAGATTCAACAAG30) and COAG3 (50AAAGAAAACCACTCACATCA30). Similar results were obtained in the study conducted by Enany *et al*. (2008) in which 34 out of 34 (100%) positive milk samples identified through bacterial culture were confirmed for *S. aureus* using coagulase gene primer of PCR (Figure 1). Our results coincide with Enany *et al*. (2013) in which 100% isolates were confirmed for coagulase gene after bacteriological examination and biotyping.

Table 1

Clinical and Sub-clinical mastitis status of lactating cows using surf field mastitis test belonging to institutional and small holder dairy herds sampled in the study

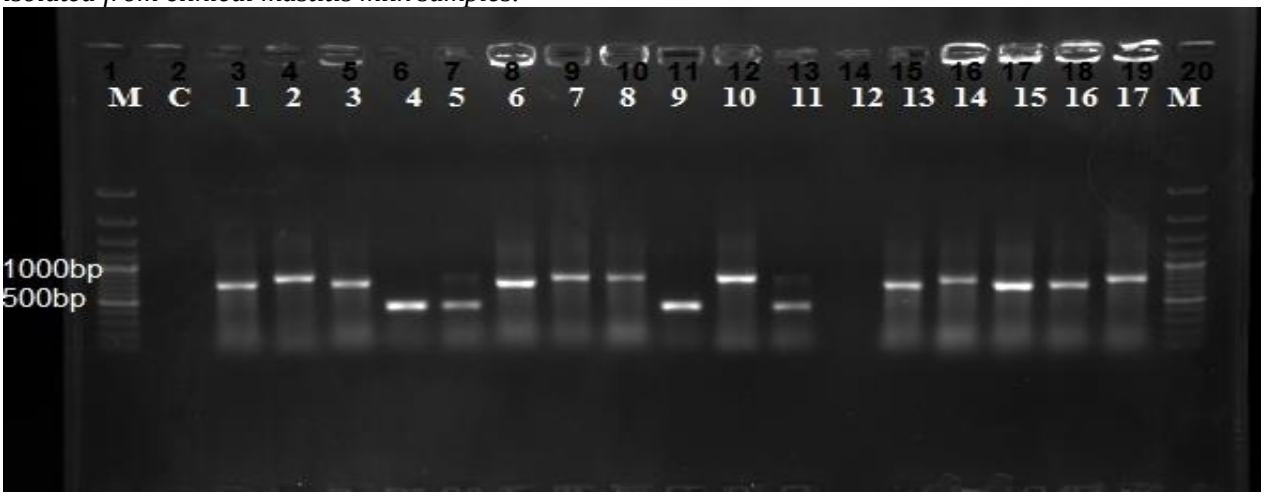
Owner		Department of Animal Breeding and Genetics, University of Agriculture, Faisalabad crossbred dairy herd	Department of Livestock Management, University of Agriculture, Faisalabad cow and buffalo dairy herd	Ayub Research Jhang road	Mahar Zafar, Dairy farm	Suddhupura. Cattle farm	Rana Irfan Suddhupura Livestock farm	Raja Asghar Dairy farm	Bilal Rashed Dairy farm	Total
Herd Designation		Herd I	Herd II	Herd III	Herd IV	Herd V	Herd VI	Herd VII	Herd VIII	8
	Total No. in the herd	29	48	0	0	10	6	27	49	169

Cows	Clinically Mastitic	Cows n=2; Quarters n=2	Cows n=3; Quarters n=6	0	0	Cows n=2; Quarters n=2	Cows n=1 Quarters n=1	Cows n=5; Quarters n=5	Cows n=3; Quarters n=5	Cows n = 16; Quarters n = 21
	Sub-clinically mastitic (SFMT* Positive)	Cows n=15; Quarters n=31	Cows n=17; Quarters n=42	0	0	Cows n=6; Quarters n=12	Cows n=3; Quarters n=4	Cows n=14; Quarters n=23	Cows n=24; Quarters n=45	Cows n = 79; Quarters n = 157
Buffaloes	Total No. in the herd	0	28	29	14	35	9	22	68	205
	Clinically Mastitic	0	Buffaloes n=4; Quarter n=4	Buffaloes n=5; Quarter n=7	Buffaloes n=2; Quarter n= 2	Buffaloes n=6; Quarters n=8	Buffaloes n=2; Quarter n=2	Buffaloes n=2; Quarter n=2	Buffaloes n=12; Quarter n=12	Buffalo n = 33; Quarters n = 37
	Sub-clinically mastitic (SFMT* Positive)	0	Buffaloes n=9; Quarters n=17	Buffaloes n=12; Quarter n=19	Buffaloes n=8; Quarters n=17	Buffaloes n=13; Quarters n=25	Buffaloes n=3; Quarters n=5	Buffaloes n=7; Quarter n=13	Buffaloes n=27; Quarter n=54	Buffaloes n = 76 ; Quarters n = 150

SFMT* = Surf Field Mastitis Test (Muhammad *et al.*, 2010)

Figure 1

The detection of coagulase gene in S.aureus through PCR in samples of different isolates. Lane 1and 20 100bp DNA ladder, Lane 2 and 14: Negative control, Lanes 3-13 and 15 to 19: showed coagulase positive isolated from clinical mastitis milk samples.



CONCLUSION

It was concluded that the PCR primer of the coagulation enzyme gene is the most effective and sensitive diagnostic method for detecting and identifying clinical and subclinical mastitis.

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