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Bovine lactoferrin gene polymorphism and their association with mastitis in HF crossbred and Deoni cattle

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Abstract
A study was conducted to ascertain the polymorphism of Bovine lactoferrin gene and its association with mastitis in HF crossbred and Deoni cattle. Milk and blood samples were collected from 152 HF crossbred and 92 Deoni cattle. Milk samples were screened for subclinical mastitis using three indirect tests viz. California Mastitis Test (CMT), Electrical conductivity and Somatic Cell Count (SCC). Depending on these test animals were grouped into affected and healthy. Genomic DNA was isolated by high salt method from blood samples, the quantity and quality of DNA was ascertained by spectrophotometer and 0.8 percent agarose gel electrophoresis. An intron 6 region of BLTF gene was amplified by PCR using published primer. The PCR-RFLP method using E*coRI* enzyme revealed two genotypes AA and AB in both studied population. BB genotype was totally absent. Frequency of AA genotype 0.37, 0.71 and for AB genotype 0.71, 0.29 in HF crossbred and Deoni cattle respectively. The gene frequency was 0.69, 0.85 and 0.31, 0.15 for C and D in HF crossbred and Deoni cattle respectively. Chi-square (χ_2) analysis revealed that there is significant difference between these genotypes regarding mastitis incidence in Deoni cattle. AA genotyped animals were found to be less susceptible for mastitis, where as in HF crossbred there was no association was found.

Keywords: BLTF gene, Deoni, mastitis and polymorphism

Introduction
Mastitis is the most significant disease affecting dairy cattle (Shook and Schutz, 1994) [16], is a multietiological disease, which is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissue (Radostits *et al.*, 2000) [13]. Lactoferrin (formerly known as lacto transferrin) is a glycoprotein and a member of a transferrin gene family. Ogorevc *et al.* (2009) [12] mentioned that about 934 candidate genes are involved in mammary gland development, milk production, sensitivity and resistant to mastitis. They also pointed out 15 candidate gene (TLR4, CD14, LTF, bola-13, IL8, IFNG, IL6, IL8, LBP, SAA3,CCR2 IL1B, C5AR1, TLR2 and β-4 defensing) useful in monitoring the mechanism of the development of an infectious disease as well as natural resistance of cows to mastitis. Among these Bovine Lactoferrin gene was considered one of the candidate gene for mastitis. LF is present in milk and also in other exocrine secretions such as tears, semen, saliva, and cervical mucus (Wakabayashi *et al.*, 2006) [19]. Lactoferrin (LF) is a protein which exerts several functions related to innate immunity. At the same time lactoferrin also takes part in specific immune reactions but in an indirect way (Legrand *et al.*, 2004) [11]. Due to its strategic position on the mucosal surface lactoferrin represents one of the first defense systems against microbial agents invading the organism mostly via mucosal tissues and also affects the growth and proliferation of a variety of infectious agents including both Gram-positive and negative bacteria, viruses, protozoa, or fungi (Kirkpatrick *et al.*, 1971) [9]. LF was first known for its iron chelating properties. Its ability to bind free iron which is one of the essential elements for the growth of bacteria leads to bacteriostatic effect (Arnold *et al.*, 1980) [2]. The ability to keep iron bound even at low pH is important, especially at sites of infection and inflammation the pH may fall under 4.5 due to the metabolic activity of bacteria. In such a situation also lactoferrin binds to iron released from transferrin, which prevents its further usage for bacterial proliferation by bacteria (Valenti and Antonini, 2005) [18]. The antibacterial activity of lactoferrin especially against *E. coli*, *P. aeruginosa* and *S. aureus* has been proved in various *in-vitro* as well as *in-vivo* studies (Brock, 2002; Lacasse *et al.*, 2008) [4, 10].

Material and Methods

Animals and Screening tests

A total of 244 unrelated animals were selected for the study among, 152 are H.F. Crossbred and 92 were Deoni from different talukas surrounding Bangalore and Bidar district respectively. Milk samples were collected for this study which were screened for subclinical mastitis using three indirect tests viz. California Mastitis Test (CMT), Electrical conductivity and Somatic Cell Count (SCC). Depending on SCC, these test animals were grouped into affected and healthy because of its high sensitivity and specificity compared to other two test. Blood samples were collected from grouped animals.

DNA extraction and PCR amplification

Miller's high salt is employed for isolation of genomic DNA from venous blood. The DNA concentration and quality were assessed based on the absorbance of UV light at 260 (A260) and 280 nm (A280) using spectrophotometer and 0.8 per cent agarose gel electrophoresis. The genome DNA was diluted to 50 ng/µL and stored at -20 ^{0}C. PCR was used to amplify the 301 bp DNA fragments containing the polymorphism of interest. Each PCR reaction was carried out in a total volume of 25 µL solution containing a master mix system with 50 ng template DNA and 1µL each of forward and reverse primer. Sequences of the primers used in the PCR were reported previously by Chang Hong *et al*. (2009) [5]. The sequences of the forward and reverse primers were 5' GCCTCATGACAACTCCCACAC 3' and 5'CAGGTTGACACACATCGGTTGAC 3'respectively. PCR cycling conditions were as follows: initial denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 60 s , 62 ºC annealing temperature for 60 s, extension at 72 °C for 60 s, and a final extension at 72 ºC for 5 min. The fragments of PCR were detected on a 1.5 per cent agarose gel.

The PCR products were digested with 10 U of *Hinf* I enzyme (Thermo Scientific, Luthiania.) in 30 µL of reaction volume at 37 °C for 12 h. The digested products were separated by horizontal electrophoresis on 2.0 per cent agarose gel in 1 x TBE stained with ethidium bromide and visualized under gel document system.

Statistical analysis

The genotype was determined by scoring the bands under the gel documentation system. The allele number, allele frequency, genotype frequency and observed and expected heterozygosites were calculated as described by Snedecor and Cochran, (1967) [17] and association between genotypes and mastitis was canalized by using chi square test.

Result and Discussion

The milk samples were screened for mastitis by different diagnostic methods namely, CMT, EC and SCC. Electrical conductivity of milk can only give useful information about udder health status, but are considered not reliable when used alone in the diagnosis of mastitis (Hillerton and Walton, 1991) [7] Of the three tests, SCC is having high sensitivity and specificity as compared to CMT and EC (Badiuzzaman *et al*., 2015 and Kandiwa *et al.*, 2017) [3, 8], hence, the experimental animals were classified into mastitis free and mastitis affected solely based on SCC results (Table 1).

Table 1: Classification of animals based on SCC

Sl. No.	Breed	Total no.	Mastitis free	Mastitis affected
1.	H.F crossbred	152	52	100
2.	Deoni	92	73	19

PCR amplification of BLTF gene

The amplified product was approximately of 301 bp in length with no variation in size either within or between the breed studied (Figure.1). The sizes of the amplification products were identical in all the cattle breeds. The size of the amp icon was further confirmed by nucleotide sequencing.

RFLP analysis

The PCR amplified product (301 bp) was digested with restriction enzyme *Eco*RI in HF crossbred and Deoni cows. Restriction analysis revealed two patterns in both the breeds. One pattern showed only one fragment of 301bp length (undigested fragment). The other pattern showed three fragments of sizes 301, 201 and 100 bp. The animals with 301 bp fragment were classified as AA genotype. Animals with 301, 201 and 100 bp fragments were classified as AB genotype. Genotype BB was totally absent in both the studied populations (Figure 2). The present findings are in agreement with earlier reports (Anggraeni *et al.*, 2012; Hemati *et al.*, 2014) [1, 6], which also revealed the presence only two genotype *i.e*., AA and AB and absence of BB genotype in HF/ Holstein dairy cows. Contrary to the reports of Seyfert and Kuhn *et al.* (1994) [15] and Wojdak-Maksymiec *et al.* (2006) [20], reported the presence of all the three genotypes (AA, AB and BB) in HF dairy and Polish Black and White dairy cows. HF Crossbred cows showed two genotypes, AA and AB genotype with the genotype frequency was 0.37 and 0.63 respectively and the allelic frequency was 0.69 and 0.31 for A and B, respectively. However the genotype frequency was 0.71 and 0.29 for AA and AB, respectively. The allelic frequency was 0.85 and 0.15 for A and B, respectively in Deoni cows. However BB genotype was totally absent in both the breeds (Table 2).

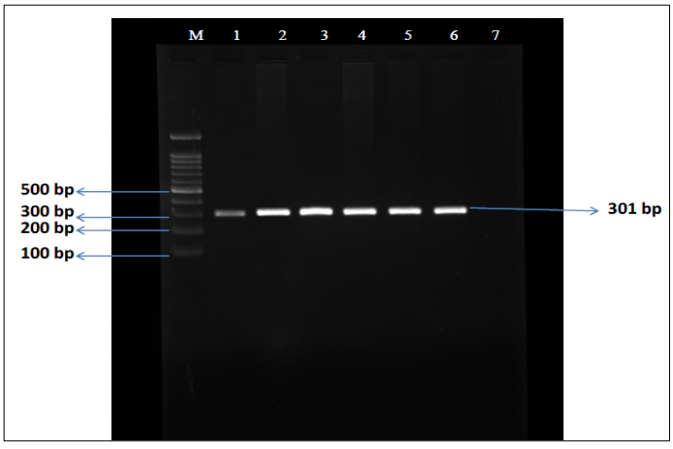

Fig 1: Agarose gel (1.5 %) electrophoresis showing PCR amplified product of BLTF (intron 6 region) gene. Lane M: Molecular marker (100 bp DNA ladder); Lanes 1, 2, 3: PCR amplified product 301 bp (HF crossbred); Lanes 4, 5, 6: PCR amplified product 301 bp (Deoni) and Lane 7: No Template Control

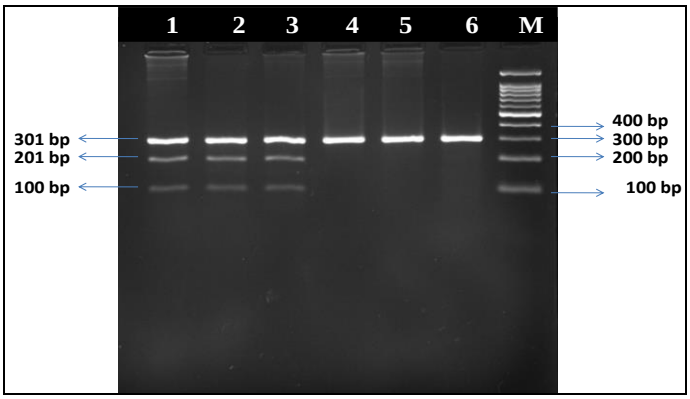

Fig 2: Agarose gel (1.5 %) electrophoresis showing PCR product after digestion with restriction enzyme *Eco*RI for detection of BLTF (intron 6) gene polymorphism in HF crossbred and Deoni cows. Lane M: Molecular Marker (100bp DNA ladder); Lanes 1, 2: Heterozygous genotype AB in HF crossbred (301, 201 & 100 bp); Lane 3: Heterozygous genotype AB in Deoni (301, 201 & 100 bp); Lanes 4, 5: Homozygous genotype AA in HF crossbred (301 bp) and Lane 6: Homozygous genotype AA in Deoni (301 bp)

Table 2: Observed genotypes of BLTF gene and their association with mastitis in HF crossbred cows

Groups	Total number of animals	Genotype frequency			χ2 Value
		AA	AB	BB	
Mastitis affected	100	41	59	-	1.528^{NS}
Mastitis free	52	16	36	-	
Total	152	57	95		

Table 3: Observed genotypes of BLTF gene and their association with mastitis in Deoni cows

Groups	Total number of animals	Genotype frequency			χ2 Value
		AA	AB	BB	
Mastitis affected	19	8	11	-	9.411^{**} (p<0.01)
Mastitis free	73	57	16	-	
Total	92	65	27	-	

HF crossbred cows had higher frequency of AB genotype which is in agreement with reports of Seyfert and Kuhn (1994) [15] and Wojdak-Maksymiec *et al.* (2006) [20] in HF and Polish Black and White dairy cattle, respectively. Whereas, it was lower than the reports of Anggraeni *et al.* (2012) [1] and Hemati *et al.* (2014) [6] in HF dairy cows.

Association Study

In the present study, no significant association was observed between the BLTF genotypes and mastitis in HF crossbred cows. In contrast, Hemati *et al*. (2014) [6] and Sender (2006) [14] reported association of AB and BB genotype to lower SCC in Holstein dairy cattle and Polish Black and White dairy cattle, respectively. However significant association (p<0.01) was observed between the genotypes and mastitis in Deoni cows, where in majority of animals with AA genotype were mastitis free. Therefore, it can be inferred that AA genotype/ A allele of BLTF gene may be favored for lesser incidence of mastitis in Deoni cows (Table2 & 3).

Conclusion

It can be concluded that the polymorphism of BLTF gene was observed in both HF crossbred and Deoni cattle. No significance association was observed between BLTF gene and mastitis in HF crossbred. The BLTF genes in Deoni cattle may be considered as candidate genes for selection of mastitis resistant animals, prior to which, suitable validation and confirmation in larger populations is a necessity.

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