Sequence characterization and polymorphism detection in lactoferrin gene of Deoni (Bos indicus) cattle

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ABSTRACT
Lactoferrin is an iron binding glycoprotein which plays an essential role in antimicrobial defense and a potential candidate gene in dairy cattle breeding. The present study was undertaken with the objective of sequence characterization and identification of polymorphisms within exons 2, 3 and 14 and their flanking intronic regions in lactoferrin gene of Deoni (Bos indicus) cattle by single strand conformation polymorphism (SSCP). Amplicons of exons 4, 5 and 16 showed monomorphic patterns. PCR-SSCP analysis revealed a total of eight different variants in three investigated regions of the lactoferrin gene. The locus LtfE2 revealed four different variants, viz. LtfE2-A, LtfE2-B, LtfE2-C and LtfE2-D with the frequency of 0.42, 0.26, 0.22 and 0.10, respectively. Analysis of exon 3 and 14 revealed two unique SSCP patterns with the frequencies of 0.54 and 0.46 and 0.86 and 0.14 respectively. Comparison of nucleotide sequences of lactoferrin gene of the Deoni cattle with taurine cattle revealed a total of 12 point mutations, 11 of which were found to be in coding region with 10 transitions. Conceptualized translation of nucleotide sequence revealed 5 amino acid changes. The SNPs identified in the coding region of lactoferrin gene may serve as potential genetic marker(s) in cattle for disease resistance.

Key words: Deoni cattle, Exon polymorphism, Lactoferrin gene, PCR-SSCP.

INTRODUCTION
India has one of the largest livestock sector in the world and ranks first in milk production with annual milk production of 132.4 million tonnes (NDDB, 2012-13). Selection and multiplication of genetically superior germplasm is one of the most important steps in any development programme. However, there is large number of constraints hampering these programmes including prevalence of diseases like mastitis, which hamper the expression of true genetic merit of production of these animals. The prevalence of mastitis put a burden on huge farming community of India. Annual economic loss due to clinical mastitis was about Rs 3014.35 crores and while due to sub clinical mastitis was Rs 4151.16 crores (Bansal and Gupta, 2009).

Lactoferrin is a glycoprotein which shows high affinity for iron which was isolated for the first time from milk by Sorensen and Sorensen (1939). It has a molecular weight of about 80 kDa and comprises of a single polypeptide chain containing 708 amino acids folded into two globular lobes. Concentration of lactoferrin in the milk varies from 80 to 500 mg/L in cow milk (Drackova et al., 2009). It can keep the iron in bound form even in low pH which is important at bacterial infection sites where the pH reduces (Valenti and Antonini, 2005) and is well known for its function as a general antibacterial, antiviral, antitumor and immunomodulatory molecule during infections of mammary gland (Gonzalez-Chavez et al., 2009). The antibacterial activity of lactoferrin especially against Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus had been proved in various in vitro as well as in vivo studies (Lacasse et al., 2008).

It was also reported that the polymorphisms in lactoferrin gene has association with susceptibility/resistance to mastitis (Wojdak-maksymiec et al., 2006; Zhao et al., 2008). The bovine lactoferrin gene mapped to bovine chromosome 22 (Schwerin et al., 1994), contains 17 exons and spreads over about 34.5 kb of a genomic DNA. The above information clearly indicates the importance of lactoferrin gene as a candidate for selection of mastitis resistant cows. It is believed that indirect selection based on candidate gene markers can help to increase the efficiency of breeding.
programs. However, information regarding the polymorphisms within the *Bos indicus* lactoferrin gene is very scanty. In the present study, we describe the locus specific polymorphisms within exons and their flanking intronic regions of bovine lactoferrin gene through PCR-SSCP and DNA sequencing of Deoni cattle.

**MATERIALS AND METHODS**

**Experimental animals and their management:** A total of 182 Deoni cattle maintained under semi-intensive system of management at Southern Campus of National Dairy Research Institute, Bangalore, Karnataka and Marathwada Agricultural Research Institute, Parbhani, Maharashtra were used in the study. Deoni cattle are a medium sized dual purpose *Bos indicus* cattle found in Latur district in Maharashtra and neighbouring talukas of Karnataka and Telangana states in India. Blood (8-10 ml) was collected aseptically by jugular vein puncture using vacutainer tubes containing EDTA as anticoagulant.

**DNA isolation:** Genomic DNA was isolated by high salt method as described by Miller *et al.* (1988). The quality and quantity of DNA was checked by agarose gel electrophoresis and UV spectrophotometer. The stock solutions were stored at -20°C and used for further analysis. The working solution was prepared by diluting the stock to 100 ng/µL for utilizing as DNA template in PCR.

**Primers:** Six sets of the oligonucleotide primer pairs were designed based on published sequences of *Bos taurus* cattle (Ensembl Ref Seq: ENSBTAG00000001292) using primer 3 (http:/www.genome.wi.mit.edu/ cgi-bin/primer/primer-3www.cgi) software. The details of the oligonucleotide sequence, their annealing temperature and expected product size are presented in Table 1.

**PCR amplification:** The Polymerase Chain Reaction (PCR) was carried out on about 50-100 ng of genomic DNA in 25 µl per reaction volume. The PCR reaction mixture consisted of 200 µM of each dNTPs, 10X Taq Pol assay buffer, 1U Taq polymerase enzyme, 20 pM of each primer. The thermocycler conditions included with an initial denaturation at 94°C for 2 min, followed by 35 cycles with denaturation at 94°C for 30 sec with varying annealing temperature from 58°C to 60°C based on primer set (Table 1), extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR products were electrophoresed at 100 V in 1.5% agarose gel in 1X TBE buffer containing 0.5 µg/mL ethidium bromide along with a DNA molecular size marker. The gels were visualized and documented using Gel documentation system (Gel doc 1000, Bio-Rad, USA).

**SSCP analysis:** The concentration of acrylamide and bisacrylamide concentrations (29:1) viz 10% was utilized for native PAGE gel preparation. Amplified PCR products (10 µL) further diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% Xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA) and heat denatured at 94°C for 8 minutes followed by rapid chilling on ice block for 20 minutes and loaded on 10% acrylamide: bisacrylamide (29:1) in 1X TBE buffer for 8 hours (200 V). The gels were silver-stained as described by Sambrook and Russell (2001). Band patterns were characterized by the number of bands, mobility shifts and scored manually.

**SNP identification:** Representative PCR products giving unique SSCP patterns were custom sequenced using automated ABI DNA Sequencer (Amnion Biosciences Pvt. Ltd., Bangalore, India) to confirm the mobility shift in each pattern. Sequence data were analysed using Bio edit software Clustal W multiple alignments for detecting single nucleotide polymorphisms (SNPs) by comparing the observed sequence with the bovine lactoferrin gene reference sequence (EnsemblRefSeq:ENSBTAG00000001292).

### RESULTS AND DISCUSSION

In present study, different exonic regions of lactoferrin gene were generated using six sets of primers. SSCP patterns of lactoferrin gene fragments were sequenced

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequence</th>
<th>Location (bp)</th>
<th>Amplicon length (bp)</th>
<th>Annealing Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>F- CCTCCATCAGAGGAGAGTTG G- RCTGGGATCCTCCTCCCTT</td>
<td>5304 – 5467</td>
<td>164</td>
<td>60°C</td>
</tr>
<tr>
<td>3</td>
<td>F- TCTGGGCTCAACCACCTCT R- TCTGGGCTGTGACCCTCG</td>
<td>8444 -8552</td>
<td>109</td>
<td>60°C</td>
</tr>
<tr>
<td>4</td>
<td>F- ATTCAAGGGAGGCTGTTTCT T- AGCTGTGTTAAGAGTG ACC</td>
<td>8848 -9030</td>
<td>183</td>
<td>60°C</td>
</tr>
<tr>
<td>5</td>
<td>F- CTTGCTAGGCTCTGCTTGG T- CAGTTGCCACACTAGCTCACC</td>
<td>9647 -9794</td>
<td>148</td>
<td>59°C</td>
</tr>
<tr>
<td>14</td>
<td>F- CATTGCTGTCAGTTATGG C- TGGGACTGCCACAACAGTA</td>
<td>29510 -29577</td>
<td>68</td>
<td>58°C</td>
</tr>
<tr>
<td>16</td>
<td>F- TGCCCTCCCAAGTTCCAATA TC R- CCCACATCACCCTCATAATG</td>
<td>32813 -33002</td>
<td>190</td>
<td>59°C</td>
</tr>
</tbody>
</table>
and aligned to identify the SNPs using Clustal W program. PCR-SSCP analysis of amplicons of exons 2, 3 and 14 showed polymorphism while the remaining exons 4, 5 and 16 were monomorphic. Single strand conformation polymorphism analysis was performed on the amplified fragments of lactoferrin gene, viz. LtfE2, LtfE3, LtfE4, LtfE5, LtfE14 and LtfE16, covering exons 2, 3, 4, 5, 14 and 16, respectively. The results revealed a total of eight different variants in three investigated exonic regions of LtfE2, LtfE3 and LtfE14. The representative gel pictures showing the band patterns for various SSCP variants are presented in Figures 1, 4 and 7 respectively. The frequencies of SSCP variants for each exon in 182 Deoni cattle genotyped in the present study reveals four different variants, viz. LtfE2-A, LtfE2-B, LtfE2-C and LtfE2-D, in the locus LtfE2 with the frequency of 0.42, 0.26, 0.22 and 0.10 respectively (Fig. 1). The locus LtfE3 revealed two SSCP variants, viz. LtfE3-A and LtfE3-B, with the frequency of 0.54 and 0.46, respectively (Fig. 3). Two different variants, viz. LtfE14-A and LtfE14-B, were observed in the locus LtfE14 with the frequency of 0.86 and 0.14, respectively (Fig. 5). Representative samples were custom sequenced to confirm the mobility shift in each pattern. The retrieved sequences representing each of the unique PCR-SSCP patterns were further analyzed by comparing these sequences with the bovine lactoferrin gene reference sequence for Bos taurus cattle using Clustal-W multiple sequence alignment tool and DNA Baser for detecting Single Nucleotide Polymorphism (SNP) and their respective deduced amino acid variations (Table 2). Perusal of Table 2 indicates high degree of mutation in lactoferrin gene in Deoni breed of cattle. Out of the 11 SNPs observed in exonic regions 5 were brought about change in amino acid in the transformed products where as remaining 6 were silent mutations. In the intronic region one SNP was detected (Table 2). Sanger Trace Figures of SNPs observed in exon 2, exon 3 and exon 14 are shown in Fig. 2, Fig. 4 and Fig. 6, respectively.

Polymorphisms within exons 7 and 13 of lactoferrin gene have been reported earlier in humans (Liu et al., 2002). Li et al. (2004) found polymorphisms in exons 4, 8, 9, 11, 15, and in intron 4 in Holstein dairy cattle by using PCR-SSCP. Kaminski et al. (2006) reported that polymorphism in +32 position (G/C) played an important role in the determination of milk protein yield and milk protein content, however, it was not related to the somatic cell count. Raja (2007) reported that each of exons 3, 4, 5 and 8 of lactoferrin gene revealed 3 unique PCR-SSCP patterns and exons 2, 7, 11, 12 and 16 revealed two unique SSCP patterns, whereas, remaining exons showed monomorphism in Sahiwal cattle. O’Halloran et al. (2009) identified 47 SNPs in lactoferrin coding sequences of six different breeds of dairy cattle, out of these 27 SNPs were associated with amino acid changes. Polymorphism of bovine lactoferrin gene promotor was determined by using restriction fragment length polymorphism (PCR-RFLP) and three genotypes were identified viz. AA, AB and BB in Karan Fries cattle and two genotypes AA and AB in Sahiwal cattle (Chopra et al., 2013).

The present study revealed reasonable genetic polymorphism within investigated regions of Deoni lactoferrin...
gene. In order to identify the single nucleotide polymorphism, two representative samples from each SSCP variant of different investigated loci were sequenced. The nucleotide sequences were submitted to NCBI-Gene Bank and are available at accession nos. KM213016, KM213017, KM213018, KM213019 and KM213028. Comparison of nucleotide sequences of different regions of the Deoni lactoferrin gene with those of taurine cattle revealed a total of 12 point mutations. Among these, 11 were found to be in coding DNA sequence while the remaining one was in flanking intronic regions (Table 2). The sequence of Deoni lactoferrin exon 2 along with flanking partial intronic regions (Fig. 2) revealed a total of six point mutations, among which all were found to be transitions. Most of these mutations were non-synonymous leading to change in amino acid. The sequence of exon 3 (Fig. 4) revealed a total of three point mutations, among which two were found to be transition type while one was transversion. Most of these mutations were silent except one, which was non-synonymous leading to change in amino acid (Arg → Leu, G8494T). Three transitions were observed in exon 14 of the Deoni lactoferrin gene when compared to taurine cattle (Fig. 6). However, all these transitions were found to be synonymous in nature without affecting the sequence of amino acid. Thus, the conceptualized translation of nucleotide sequence revealed five amino acid changes in Deoni as compared to taurine cattle. Among the partial intronic regions, one nucleotide change was found to be in intron 2. SSCP analysis is based on the principle that a single base mutation can cause a change in the folded conformation of single strand DNA (ssDNA) leading to differences in electrophoretic mobility patterns (Hayashi, 1991 and 1992). In ssDNA, different nucleotide changes have various effects on the internal base pairing. Moreover, it is possible that nucleotide changes may not result in significant single strand conformational differences and vice versa. Thus, the polymorphism revealed by SSCP analysis in the present study could be independent of sequence variation. It has been reported that the variations in the lactoferrin gene had associations with somatic cell count (Wojdak-Makysmiec et al., 2006) and production traits (Kaminski et al., 2006). Pawlik et al. (2014) found association between four SNPs, localized in the 5′-flanking region and in exons 4 and 9 of the lactoferrin gene, and dairy performance traits in Polish Holsteins. This preliminary study reports the variations that exist in different exonic regions of the lactoferrin gene in Deoni cattle. SSCP variants observed in the present study need to be studied further in large samples and could be exploited for their possible association with mastitis resistance in cattle. Identification of genetic markers associated with SCC might be helpful in improving cows’ health by implementing appropriate cattle-breeding programmes.

**CONCLUSIONS**

The present study revealed high degree of genetic variation as indicated by different SSCP patterns which resulted in 11 SNPs in different exonic regions of the lactoferrin gene in Deoni cattle. The lactoferrin has important relation with the innate immunity, thus this protein gene is supposed to be a promising candidate gene for the mastitis-resistance trait. Our findings in the present study indicated that there is high variability in lactoferrin gene. Further studies on the association between SNPs in lactoferrin gene with lactoferrin content and SCC using large population could result in identification of markers for udder health and milk lactoferrin content. Present study could be a step towards identification of genetic markers for selecting cattle for udder health and immunity. However, further studies using large number of animals need to be carried out to validate the marker data before using them in the Marker Assisted Selection (MAS).

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REFERENCES