Lactoferrin gene polymorphisms associated with clinical mastitis in Honduran Holstein cattle

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Short Report

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Abstract

Background

Lactoferrin (LTF) is an iron-binding glycoprotein found in milk and other exocrine secretion with antibacterial activity proposed as an alternative to mastitis treatment or prevention. LTF has been proposed as a candidate gene for mastitis resistance selection. The aim of this paper was to assess LTF promotor to explore variations with potential association to mastitis resistance in dairy cows from Honduras.

Methods

A resequencing of promotor and Exon I of LTF gene in extreme mastitis susceptibility cows (126 Holstein and Holstein crossbred) was performed.

Results

Eight polymorphisms were found in promotor region, four of them were novel variations. Two were important by frequency among extreme groups, but a polymorphism in -421 A/T position was significantly (P=0.0188) associated to mastitis susceptibility.

Conclusion

Results support the key role of regulatory region of LTF gene. Some candidate genes are proposed in association with mastitis traits and implications are discussed.

Introduction

Dairy farming in Honduras is based mainly in Holstein cattle (*Bos taurus*) and reproductive management includes insemination with semen from North America, mostly considering genetic merit for milk yield in sire selection [Cañizares-Martínez et al., 2021] and protein and fat breeding values.

Mastitis is a prevalent issue in dairy production systems [Martínez-Matamoros et al., 2021] and is one of the most important current health problems in dairy industry, causing huge financial damages by decreasing the milk yield and increasing treatment costs. Mastitis cows are commonly treated with antibiotics, however, antibiotics can contaminate dairy products and causes problems when the milk is processed [Shimazaki and Kawai, 2017]. Additionally, antibiotic resistance cases have been reported in Honduras dairy farms [Martínez-Matamoros et al., 2021].

Among the most studied alternatives for treating mastitis for its antibacterial activity, is the Lactoferrin (LTF), an iron-binding glycoprotein found in milk and other exocrine secretion [Shimazaki and Kawai, 2017]. The bacteriostatic effect of LTF in endocrine form has been attributed to the ability to bind Fe3 +

ion, limiting availability for pathogen bacteria requiring it as an essential factor for growth and virulence factors expression [Orsi, 2004].

The LTF gene is located on the BTA22 chromosome and consists of 17 exons, 1122 bp of the promoter region [Huang et al., 2010]. Some polymorphisms have been reported in the bovine LTF gene [Li et al., 2004; Daly et al., 2006, O'Halloran et al., 2009; Huang et al., 2010], suggesting the potential for Lactoferrin as a candidate gene for mastitis resistance selection and pointing out the promoter of the LTF gene as a critical region for gene transcription since polymorphisms in these regions can alter gene expression.

Henceforth, the aim of the present study was the re-sequencing of LTF promotor to explore variations and discuss possible relationships with other candidate genes with potential association to mastitis resistance in dairy cows from Honduras.

Methods

A dataset of 126 Holstein and Holstein crossbred cows from the dairy farm of Escuela Agrícola Panamericana Zamorano were used to select animals for mastitis vulnerability. The mastitis incidence was evaluated by confirmation of clinical diagnostics by a California Mastitis Test (CMT). Positive cows were separated from the herd and treated with immunological stabilizers. A sample of the secreted milk was taken and an antibiogram was performed to detect the proper antibiotics treatment.

Mastitis incidence data were used to select cows without mastitis history (n = 10), and cows with at least 1 event of mastitis (n = 26). Two groups were classified as resistant (RG) and susceptible (SG). From these selected cows, hair follicles were collected and stored until DNA extraction using the Genelute genomic DNA kit CAT G1N350 (Sigma Aldrich, SL Missouri, USA). Mastitis incidence data was used to select cows without mastitis history (n = 10), and cows with 1 event of mastitis (n = 26). Two groups were classified as resistant (RG) and susceptible (SG). From these selected cows, hair follicles were sampled and stored until DNA extraction using the Genelute genomic DNA kit CAT G1N350 (Sigma Aldrich, SL Missouri, USA).

Three primer pairs were designed to sequence the Lactoferrin promoter region including an Exon I, (Table 1) based on Primer3 [Ye et al., 2012], considering the reported sequence ENSBTAG00000001292 from Ensembl database [Cunningham et al., 2022].

A touchdown PCR was performed using 20 ng of DNA, 1.5mM $MgCl_2$, 0.2 mM of dNTPs, 1U of Taq-DNA polymerase and 0.1µM of each primer: Initial step of 95°C for 5 min; 30 cycles at 95°C for 45 s; 65°C for 45 s/- 2°C each cycle and 72°C for 1:30 m; a second 30-fold phase included a denaturing at 95°C for 45 s with an annealing temperature of 60°C for 45 s, and 72°C for 1:30 m; followed by a last step of 72°C for 5min. Each amplicon was sequenced using the SimpleSeq™ service (Eurofins MWG Operon LLC technologies, USA). Amplicons were edited in Unipro UGENE (Okonechnikov et al., 2012) and ensembled using CAP3 software [Huang and Madam, 1999]. Complete sequences were aligned in MAFFT [Katoh and

Standley 2013] and analyzed with Unipro UGENE [Okonechnikov et al., 2012] using the reference sequence GenBank: AY319306.2 [Zheng et al., 2005] to identify polymorphisms.

To examine the putative association amongst the mastitis susceptibility and found polymorphisms, a Fisher exact test was performed comparing the RG and SG groups observed polymorphism frequencies using the FREQ procedure in SAS OD for Academic software (SAS Institute Inc., Cary, NC, USA). An interaction network was also created to elucidate and discuss the possible relationship with other candidate genes and ontology using STRING v. 11.5 [Szklarczyk, et al., 2018].

Table 1
Details of primers designed for resequencing of Lactoferrin promotor region and exon I.

Primer name	*Sequence*	*Product size (bp)*
LTF1-F	5'TGCATATCCACCCCCAACAGG3'	750
LTF1-R	5'CGCAATACAGCTCCCAGAAAACAG3'	
LTF2-F	5'GTTCCTGTCTCCCACCTCATA3'	656
LTF2-R	5'GCGCCCCAGCCTCCCTCCTC3'	
LTF3-F	5'GGTTCCCAAGCACTTTAGATAC3'	624
LTF3-R	5'ACTGAGGGGTGAGGGGACTGGATG3'	

Results And Discussion

Eight polymorphisms were found within the promotor LTF region (Table 2). Four previously reported variations and four novel polymorphisms were identified. An insertion in -/G in the − 478 position has been previously reported by Li et al. [2004] and Daly et al. [2006]. Presumably, this insertion is the same reported by O'halloran [2009] in the − 479 position. A transition found in the − 271 position was reported by Daly et al. [2006], in Normande, Holstein Friesian, and New Zealand Holstein breeds, and might be the same reported in -270 by O'halloran et al. [2009]. Interestingly, a transition G/A polymorphism found in the − 190 position has been reported by Daly et al. [2006] and O'halloran et al. [2009]. The former study suggests the transcription importance of this variation by assessing its adjacent position to a potential SP1 transcription factor binding site, introducing an adenine nucleotide into this GC-rich region. Another previously reported polymorphism found, was a G/A transition in the − 156 position [Li et al., 2004, Daly et al., 2006, O'halloran et al., 2009].

Table 2
Confirmed and discovered polymorphisms in Lactoferrin promotor region in Holstein cows.

*Position in AY319306.2**	*Position from exon I*	*Nucleotide change*	*Variation*	*Allele frequency*
3878	-478[1,2]	-/G	Insertion	G = 0.05
3935	-421	A/T	Transversion	T = 0.15
4083	-274	G/A	Transition	A = 0.35
4086	-271[2]	C/T	Transition	T= 0.09
4094	-265	C/A	Transversion	A = 0.25
4100	-256	A/C	Transversion	C = 0.27
4166	-190[2,3]	G/A	Transition	A = 0.27
4200	-156[1,2,3]	G/A	Transition	A = 0.54

*AY319306.2 Bos taurus lactoferrin (Lf) gene, 5' flanking region exons 1, 2 and partial cds (Zheng et al., 2005). [1]Li et al. (2004), [2] Daly et al. (2006). [3]O'halloran (2009)

Two novel variations showed a significant difference between the cow's resistant and susceptible extreme groups. The variation in position − 421 A/T was significant (P = 0.0188) between groups, suggesting that the A allele carriers are susceptible to clinical mastitis. The variation in position − 256 A/C also showed a relevant difference (P = 0.0700) between groups. Li et al., (2004) reported three nucleotide variations in the 5' region of the LTF gene of Holstein cows but not a significant relationship with somatic cell count. In another study, O'Halloran et al., [2009] found in a multi-breed dairy cattle sample twenty-nine polymorphisms within the regulatory region of the bovine LTF, from which 19 were novel variants; some associated with putative transcription factor binding sites, suggesting a potential effect on LTF expression levels. In a posterior analysis, Huang et al [2010], found three SNPs in the 5' flanking region of the LTF gene in a Chinese Holstein population: these variations showed no individual association with somatic cell score but had a significant association in a combined haplotype. The present work outcomes significant association of at least one polymorphic variation in promoter region to clinical mastitis susceptibility by probability differences amongst extreme groups.

The interaction network assessment showed meaningful relationships among LTF gene and Cationic trypsin precursor mRNA, protein s100-a9, Lysozyme Z (LYZ), monocyte differentiation antigen cd14 precursor (CD14) and neutrophil gelatinase-associated lipocalin (LCN2). On the other hand, reactome analysis revealed a relationship with some antimicrobial peptides, Clusterin preproprotein (CLU), LYZ, and elastase, neutrophil expressed mRNA, but also specific granule lumen proteins, including the previously described proteins and LCN2 (Fig. 1). Direct association between these protein genes and mastitis incidence, somatic cell count, or related traits is not widely documented for all genes or proteins. Chen et al., (2010), found in a Chinese Holstein population polymorphism in the coding region of the LYZ gene

associated with SCS, proposing its use as a candidate marker for mastitis. Ibeagha-Awemu et al. [1998] reported a significant association of genotype in 1908 non-synonym locus to a higher percentage of neutrophils expressing CD14 molecules on their surfaces and suggesting an important role of CD14 in mediating bacterial infections. On this basis, Kumar et al. (2014) demonstrated a significant association between polymorphisms in CL14 and mastitis frequency in Sahiwal cows. Similarly, Gupta et al. [2018], assessed the coding region of the CD14 gene and found a polymorphic pattern associated with SCS and differential expression between extreme genotypes indicating this gene is a candidate for mastitis resistance/susceptibility.

Enrichment analysis revealed nine related biological processes related to LTF, with a false discovery rate (FDR) < 0.001 and three ≤ 0.04. Most GO-terms were related to some degree of defense response (e.g bacterium, external stimulus, stress, and fungus). One of the most important GO terms (GO 0002523 FDR = 0.0226), was related to leukocyte migration involved in inflammatory response according to the AmiGO2 database. LTF is a protein present in milk, and interaction with other molecules and proteins is highly likely these evidence association outcomes indicate a gene concurrence on the potential typical role of these genes on the assistance of mastitis incidence in dairy cows, supporting further investigation for effective implementation of marker-assisted strategies.

Conclusions

Four novel polymorphisms were found in the promotor region of the LTF gene in a Holstein crossbred population. The − 421 A/T variation is associated with mastitis susceptibility supporting the purported key role of the regulatory region. Seven proteins are proposed with concurrence with LTF with potential mastitis effects from which, at least, three have documented association with mastitis traits. Further investigation on LTF and related genes might provide insight into concurrent effects for the improvement of this relevant health problem.

Statements And Declarations

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Contributions

Conceived and designed the experiments: Parra-Bracamonte G.M., Moncada-Lainez M., Castillo R.; Performed the experiments: Valladares-Medina P.A., Sifuentes-Rincón A.M., Parra-Bracamonte G.M.;

Statistical analysis: Parra-Bracamonte G.M.; Wrote the paper: Valladares-Medina P.A., Parra-Bracamonte G.M., and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to disclose.

Ethical approval

Animals were managed under all ethical considerations applicable during the hair-follicle sampling. Sampling was performed during regular milking management.

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Figures

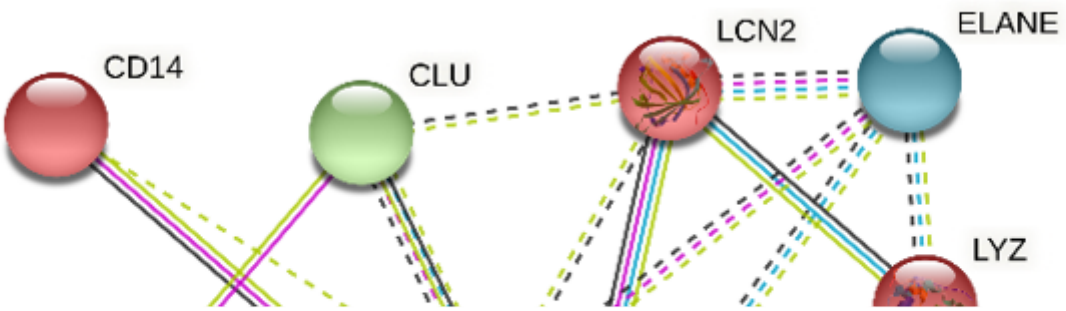

Figure 1

Interaction network of Lactoferrin and functional cluster partners predicted by STRING (Szklarczyk, et al., 2018).