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Original Article

Molecular characterization of lactoferrin gene as genetic marker to subclinical mastitis in water buffaloes (*Bubalus bubalis*)

Yancy Ogdamin Waminal[1,2], Gabriel Alexis San Perdo Tubalinal[3,4], Claro Niegos Mingala[2,3,4*]

[1]Department of Animal Science, College of Agriculture, Tarlac Agricultural University, Camiling 2306, Tarlac, Philippines

[2]Department of Animal Science, College of Agriculture, Central Luzon State University, Science City of Munoz 3120, Nueva Ecija, Philippines

[3]College of Veterinary Science and Medicine, Central Luzon State University, Science City of Munoz 3120, Nueva Ecija, Philippines

[4]Biosafety and Environment Section, Philippine Carabao Center, Science City of Munoz 3120, Nueva Ecija, Philippines

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**Corresponding author email:*
cnmingala@hotmail.com

Abstract

The study characterized the lactoferrin (Lf) gene in different water buffalo breeds and determined its association with subclinical mastitis (SCM). A total of seventy five (75) and five (5) milk and blood samples, respectively were collected for the conduct of this study. Amplified Lf gene demonstrated a 2224 base pairs (bp) molecular weight. Nucleotide and amino acid sequence of Lf gene of riverine- and swamp-type water buffaloes revealed 98.83% and 98.29% identity, respectively with that of *Bubalus bubalis* Lf gene in the GenBank. Phylogenetic studies showed that Lf genes of both types of water buffaloes grouped with Lf gene of water buffalo sequences registered in the GenBank. Three water buffalo genotypes were documented using the restriction enzymes *AluI* and *HaeIII*. The *AluI* can produce three genotypes (AA, AB, BB) resulting in three cuts of 561, 217 and 123 bp. Using *HaeIII*, three restriction patterns were observed producing three genotypes as well with four fragment sizes of 561, 318, 112, and 70 bp. Based on the statistical analysis, no direct association has been established between the Lf genes of water buffalo with SCM using *AluI* and *HaeIII* restriction enzymes although a higher number of animals with genotype BB belongs to those with SCM group. The results of the study merit for further studies regarding the BB genotype of Lf gene as a possible resistance gene marker for SCM in water buffaloes.

Keywords: *Bubalus bubalis*, Lactoferrin, Subclinical mastitis

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Introduction

Domesticated water buffaloes (*Bubalus bubalis*) are major milk-producing animals in several countries and contribute significantly to global milk production (FAO, 2016). Unlike dairy cows, water buffaloes are resistant to most dairy diseases even with stressful environments and settings of low feeding (Sahin et al., 2016a; Sahin et al., 2016b; Sahin et al., 2017). Distinct anatomical features, such as long narrow teat canal,

teat skin less prone to chapping and sores, thicker epithelium and keratin layer and tighter sphincter of streak canal, and absent milk cisterns may be the reason for this relative resistance (Thomas et al., 2004). However, buffaloes that are managed intensively, mastitis begins to occur even with these distinct anatomical features (Preethirani et al., 2015). The impact of mastitis is comparable to that of dairy cows involving production losses, culling and treatment costs, and decreasing animal health and welfare (Hogeveen and Van der Voort, 2017; Fagiolo and Lai, 2006; Halasa et al., 2007).

Mastitis is an economically significant infectious disease of dairy animals. Losses are due to decrease quantity and quality of milk, heightened by medicine and labor cost. A retrospective study on the prevalence of subclinical mastitis (SCM) conducted in the Philippines from September 2006 to June 2009 showed 42.76% with recurrence of 75% in water buffaloes (Salvador et al., 2012). In reducing economic losses, lowering the cases of SCM in farms is a vital strategy. To understand the mechanism of the susceptibility of animals to mastitis, genetic traits for milk production should be considered. Thus, genetic markers in the selection of animals for breeding has also been considered (Gholizadeh et al., 2008).

Lactoferrin (Lf), also known as "red protein from milk", is an iron-binding protein that can be found in most bodily fluids. Lf is released by neutrophils and inflamed tissues and has relationship with innate immunity due to its direct antimicrobial property as it limits bacterial proliferation and adhesion in the process of killing the microbes (Walker, 2010; Van Der Strate et al., 2001; Valenti and Antonini, 2005; Legrand et al., 2005; Legrand et al., 2008; Siqueiros-Cendon et al., 2014).

Information about Lf gene in water buffaloes (*Bubalus bubalis*) is limited. Genetic characterization of the gene would augment the ability to understand its role in the occurrence and onset of mastitis (Pawlik et al., 2009). The potential of Lf gene as a genetic marker for mastitis resistance in dairy animals needs to be elucidated to validate its role (Shimazaki and Kawai, 2017).

Identification of nucleotide polymorphism as a marker-associated resistance or tolerance to mastitis may be used as a tool in improving the performance of water buffaloes. Molecular characterization and genetic diversity of Lf gene of cattle have been associated with different production trait and diseases, thus, have been incorporated for selection criteria and production (Sharma et al., 2015). With this, it is rational to study the relationship of Lf gene with resistance to mastitis for water buffaloes.

The objective of the study is to characterize and identify Lf gene as a genetic marker for SCM resistance in water buffaloes by molecular analysis and association of nucleotide polymorphisms with the presence of SCM.

Material and Methods

Sample collection

A total of seventy-five (75) milk samples from riverine type water buffaloes on the 2nd month of lactation and five (5) blood samples from swamp-type water buffaloes were collected for the study. Fifty (50) and twenty-five (25) samples were non-mastitic and mastitic, respectively. There were no alterations made on the overall management of the water buffaloes included in the study.

The test animals were manually milked from the udder and pooled to collect 30 mL of milk sample for RNA extraction. The milk was collected in a 50 mL conical tube and was placed in a cooler with ice transported to the laboratory and processed on the same day of collection. For samples that were not processed on the same day were stored in a freezer (-20 °C) until used.

For the five swamp-type water buffaloes, RNA was extracted from blood as these animals were not lactating. Five mL of blood was collected from the jugular veins of the animals using EDTA tube. Samples were processed on the same day of collection.

RNA extraction

RNA extraction from milk and blood samples from the water buffaloes was done following the Promega protocol with modifications. For milk, 30 mL of raw milk was centrifuged at 4,000 rpm for 20 mins. After centrifugation, the fat layer and the supernatant were discarded, and afterward, the pellet was suspended in 1 mL PBS (phosphate-buffer-saline, a pH of 7.4). One mL of PBS was mixed again with the pellet and was centrifuged for 14,000 rpm for 1 min. This process was repeated twice and the supernatant was removed to collect the pellet.

For blood, 500 µL of the buffy coat was aspired after centrifugation at 4,000 rpm for 5 min and transferred into the 2 mL microtube, and then added with 2-3 volumes of 1 ml of 0.14 NH_4Cl. The mixture was mixed by vortex and centrifuged at 14,000 rpm for 1 min. The supernatant was removed and the process

was repeated twice to collect the pellet.
The pellet from the milk and the blood was added with 1 mL cell lysis solution and was subjected to vortex to mix the pellet and cell lysis. After this, it was centrifuged to 14,000 rpm in 1 min. The supernatant was discarded and 500 µL of nuclei lysis and 300 µL protein precipitation solution was added.
After discarding the supernatant, isolated cells were dissolved with 1 mL Trizol reagent and incubated for 10 min at room temperature. The solution was decanted and pellets were washed by 1 ml 75% ethanol and centrifuged for 5 min at 8,000 rpm at 4°C. Pellets were dried and reconstituted with 30 µL diethyl pyrocarbonate (DEPC) treated water.

Reverse transcription PCR (RT- PCR)
Complementary DNA (cDNA) was done using the cDNA Synthesis Kit (Takara Bio, Inc, Japan). One µL of oligo dT random primers were mixed with 1 µL dNTP, 5 µL of RNAse free water and 3 µL of RNA template. The solution was incubated for 5 min at 65°C before running for PCR. The prepared RNA primer mix was mixed with 4 µL 5X buffer, 0.5 µL RNAse inhibitor, 1 µL reverse transcriptase, and 4.3 µL RNAse free water. This was subjected to PCR run for segment 1, 10 min at 30°C; segment 2, 45 min at 50°C; and segment 3, 5 min at 95°C. The synthesized cDNA was tested for *β-actin* gene amplification to ensure cDNA synthesis protocol.

PCR primers
Three primer sets were used to amplify the Lf gene (Table 1). These primers were designed using the Primer3 server (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the bubaline sequence (Accession number: JF825526) from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).
The designed primers were analyzed for self-annealing and loop properties using Oligo analyzer software (https://sg.idtdna.com/calc/analyzer). Primers forming hairpin loops, self-annealing, dimers, cross dimer, and repeats were avoided as much as possible. Suitable primers were checked using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that it amplifies the Lf gene.

Table-1: Nucleotide and amino acid sequence percentage similarity of Lf gene in ruminant species regarding swamp-type and riverine-type water buffaloes.

Primers	Nucleotide Sequence	Expected Product Size
Primer 1 - F	TAGCCATGAAGCTCTTC	835 bp
Primer 1- R	GAGTACGGACAACACCGGGC	
Primer 2 - F	GCTTCTCTGCCTAAACAACAC	1008 bp
Primer 2 - R	GTCTCAGCACACAATCTAGG	
Primer 3 - F	AACATCCCCCATGGGCCTG	694 bp
Primer 3 - R	TTTCTTCGATCGGACGGA	

Gene amplification
All PCR assays were performed in 20 µL reaction volume containing 2 µL of genomic DNA template, 10 pmol of each primer and PCR master mix. The amplification of the Lf gene was carried out in a thermocycler (SimpliAmp, Thermofisher) under optimized conditions. Briefly, initial denaturation at 94˚C for 4 min, followed by 40 cycles consisting of denaturation at 94˚C for 30 sec, annealing at 57˚C for 1 min and extension at 72˚C for 45 sec, with a final extension at 72˚C for 4 min.
After amplification, 1 µL of the PCR product was electrophoresed on 1.5 % agarose gel containing 1X TAE buffer at 70 volts for 30 min and visualized under ultraviolet light using UV transillumination advanced imaging system. A 1kb plus DNA ladder (Invitrogen, CA, USA) was used as a ladder to determine the sizes of the PCR products.

Nucleotide sequencing and phylogenetic analysis
Lf products were submitted for sequencing at the Philippine Genome Center. DNA sequences were assembled using Mega 7 software. The forward and reverse sequences for each primer were assembled to form contigs of the respective regions. The gene sequences were compared with the Lf mRNA sequences to annotate different exonic regions putatively to identify SNPs in respective regions. The contiguous Lf gene nucleotide sequence was subjected to Basic Local Alignment Search Tool (BLAST) at the NCBI database to determine the sequence similarity with the corresponding regions of other species.
Nucleotide sequences were aligned and translated to the amino acid sequence using the MEGA 7 software.

The phylogenetic tree was constructed using the neighbor-joining method. Confidence in the groups was estimated by a bootstrap of data using 1000 replications. Phylogenetic trees were constructed using MEGA 7 software (Tamura et al., 2011).

RFLP analysis

The Lf PCR new set of primers sequence in water buffaloes (F-5' ATCCTTCGCCCGTTCTTGAG 3' and R-3' CGCCGAATCTACTTTTGAGG 5') was designed to amplify the ligand-binding region in exon 2, 3 and 4 of Lf gene in water buffaloes in which the putative area is located with an amplicon size of 561 bp.

Restriction enzymes *AluI and HaeIII* were used based on the suggested enzymes by Sequence Manipulation Suite: Restriction map (http://www.bioinformatics.org/sms2/rest_map.html) (Stothard, 2000).

Restriction fragment length polymorphism (RFLP) was conducted by preparing the reaction mixture composed of 7 µl of PCR product, 5.15 µl of sddH_2O, 0.35 RFLP buffer and 0.075 µL for each enzyme. Samples were incubated at 37°C for 4 hr. Restriction fragments were resolved on 2% agarose gel in horizontal electrophoresis. The restriction-digested gene fragments were visualized on a UV transilluminator (FlourChem E by ProteinSimple ™) and photographed.

Association of Lf gene to SCM

The amplified fragment of the Lf gene from the milk of water buffaloes showed different genotypes based on the exhibited banding patterns from *AluI* and *HaeIII* restriction enzymes. The different genotypes were associated based on the result of the CMT test of each of the milk samples. A total of 62 samples from water buffaloes were used in studies that associated expression of Lf gene and the occurrence of SCM.

CMT analysis

CMT was used as an indirect method of measuring Somatic Cell Count (SCC) as the indicator of intramammary infection as it has the advantage of being quick, cheap, and simple "animal side" test.

All milk samples collected were subjected to CMT. A small amount of milk (approximately 5 mL) was put into the paddle. The paddle was tilted almost vertically so that only 2 to 1 teaspoon of milk remains in each cup. An equal amount of CMT reagent was added to the milk and swirled for about 15 seconds. Observation of the reaction was done immediately to see if there is any thickening of the milk. The thicker the mixture, the higher the SCC. In goats and water buffaloes, the reaction scores are: N (negative) with no reaction and with estimated SCC of below 200,000 cells/mL; T (trace) with slight slime, tends to disappear with continued swirling with 150,000 to 500,000 cells/mL; 1 when there is distinct slime but without gel with 400,000 to 1,500,000 cells/mL; 2 when there are immediate gel formation and moves as a mass during swirling with 800,000 to 5,000,000 cells/mL; and 3 when gel develops a convex surface and adhere to the bottom of the cup with cell count estimated above 5,000,000 cells/mL (Rahman et al., 2010).

CMT scores for all animal subjects were classified as non-mastitic if the CMT score result is 1 or lower and mastitic if the CMT score result is 2 or higher.

Statistical analysis

Univariate analysis on the possible association between the genotypic frequency and the occurrence of SCM was examined using Chi-square (X^2) test analysis for the goodness of fit (Petrie and Watson, 2006).

$X^2 = E\,(O-E)^{2E}$

Where,

O= Observed Frequencies

E= Expected Frequencies

Results and Discussion

A study on the detection and molecular characterization of Lf gene in blood and milk samples of water buffaloes (riverine- and swamp-types) was undertaken. Out of all samples collected, one sample of each type of buffalo was sent for sequencing.

Figure 1 shows that the target primers 1, 2, and 3 were amplified and generated an amplicon size of 835 bp, 1008 bp, and 694 bp, respectively.

Sequence analysis of Lf Genes

In water buffaloes, swamp-type Lf mRNA sequence resulted in an average of 2224 bp (LC425118.1) while 2226 bp (LC425117.1) in riverine-type water buffalo.

Table-2: Nucleotide and amino acid sequence percentage similarity of Lf gene in ruminant species regarding swamp-type and riverine-type water buffaloes.

SPECIES (Accession Number)	NUCLEOTIDE SEQUENCE		AMINO ACID SEQUENCE	
	Swamp (LC425118.1)	Riverine (LC425117.1)	Swamp	Riverine
B. bubalis (JF825526.1)	98.29%	98.83%	97%	98%
B. Taurus (FJ589071.1)	95.42%	95.98%	96%	96%
B. indicus (GU059864.1)	95.28%	95.84%	95%	96%
B. grunniens (DQ387455.1)	95.35%	95.91%	95%	96%
C. hircus (U53857.1)	90.69%	91.19%	91%	92%
O. aries (NM_001024862.1)	92.66%	93.23%	93%	94%
E. caballus (NM_001163974.1)	74.06%	74.37%	75%	75%
S. scrofa (M81327.1)	73.69%	74.01%	74%	73%
H. sapiens (U076343)	70.76 %	71.98 %	69%	71 %

The riverine Lf gene nucleotide sequence was aligned with other ruminants' sequence of Lf gene using the database from GenBank. The 14 aligned sequences showed an average of 2127 identical pairs, 31 transitional pairs, and 18 tranversional pairs. Between riverine-type, swamp-type water buffalo, and *B. bubalis* (JF825526.1) nucleotide sequence, the average identical pairs were 2221 bp with 3 transversional pairs. This showed that there is a high similarity of sequence between the two types of water buffaloes

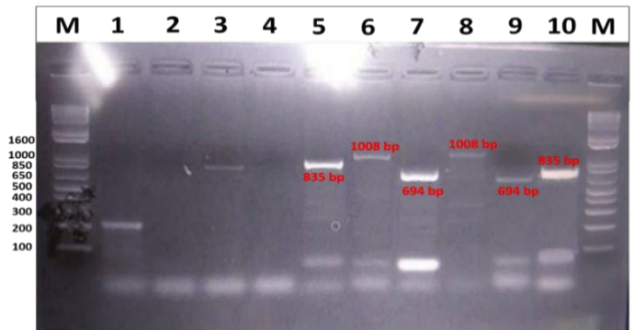

Figure-1: Amplification of Lf gene of swamp-type and riverine-type water buffaloes using primers 1, 2, and 3. Lane M (1kb plus ladder); Lanes 1-4 unamplified PCR products. Lane 5 and 10 (Primer 1); Lane 6 and 8 (Primer 2); Lane 7 and 9 (Primer 3)

The nucleotide BLAST of Lf coding sequence of the riverine and swamp samples revealed a high percentage of similarity between the query sequence and other species of Lf sequence available in the NCBI database (Table 2). Riverine-type Lf gene nucleotide sequence was 98.83% similarity with that of *B. bubalis* (JF825526.1), 95.98% similarity with *B. taurus* (FJ589071.1), 95.91% with *B. grunniens* (DQ387455.1) and 95.84% *B. indicus* (GU059864.1). The lower similarity was observed with small ruminant species such as *O. aries* (NM_001024862.), *C. hircus* (U53857.1) and other species such as *E. caballus* ((NM_001163974.1), *S. scrofa* (M81327.1), and *H. sapiens* (U076343).

Similarly, the protein BLAST result presented a high similarity of translated amino acid sequences of riverine-type Lf to *B. bubalis* (JF825526.1). A lower similarity in water buffaloes Lf amino acid sequence was observed with *B. taurus*, *B. grunniens*, *C. hircus* and *O. aries*, respectively.

Phylogenetic analysis

The maximum likelihood NJ algorithm with 1000 bootstrap resampling revealed the clustering of water buffaloes. Swamp-type water buffalo Lf gene clustered together under a single clade with *B. bubalis* sequence from GenBank being related to swamp-type water buffaloes but more descendants of riverine-type water buffaloes. *B. taurus* and *B. grunniens* can also be descendants of the bubaline Lf while *C. hircus* and *O. aries* are distant relatives.

However, the phylogenetic tree analysis (Figure 2) showed a similar evolutionary origin of riverine-type and swamp-type water buffaloes in consonance with *B. bubalis* Lf generated from the Genbank. This, therefore, could indicate minimal variation on the susceptibility or resistance to diseases.

Polymorphism analysis

The coding sequences of Lf gene in water buffaloes were analyzed for differences in nucleotides and functional amino acid substitution. Furthermore,

restriction enzyme capable of digesting the sequence to identify the nucleotide polymorphism and distinguish the genotype has been identified and results are herein presented.

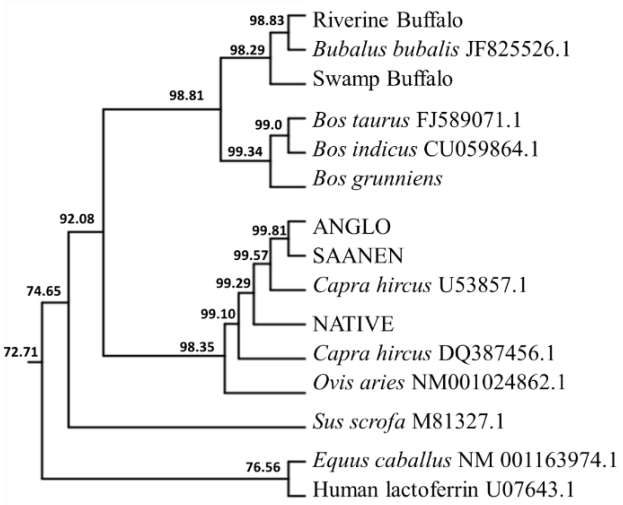

Figure-2: Phylogenetic tree showing the relationship of Lf gene between water buffaloes, goats, other ruminants' and human

To develop rapid recognition of polymorphic site of Lf gene, restriction enzymes *AluI* and *HaeIII* were used to digest 561 bp that was amplified by the primer 2. This 561 bp segment covered exons 2 and 3 of the coding region. SMS Restriction Digest (http://www.bioinformatics.org) application was used for the four samples with a complete Lf gene nucleotide sequence.

The availability of these restriction patterns on extracted Lf gene was examined by using a PCR product that was subjected to *AluI* and *HaeIII* digestion and electrophoresed. Results showed a restriction pattern that was almost the same base on the conceptualized expected pattern. From the 2224 bp nucleotide sequence, it revealed three-nucleotide polymorphism in riverine-type at locus 514, 857, and 1039 (Table 3) with two amino acid substitutions and in swamp-type water buffalo there are four nucleotide polymorphism in the Lf coding sequence at locus 315, 817, 1039 (Table 4), and 1488 with two amino acid substitutions.

The nucleotide polymorphism at locus 857of two types of water buffalo Lf gene sequences resulted in a functional amino acid substitution from threonine to isoleucine. However, there is no amino acid substitution at locus 1039, although there are changes in their nucleotides.

Lee et al. (1997), Martin-Burriel et al. (1997), Li and Chen (1999) and Kaminski et al. (2006) reported that polymorphisms in Lf gene reportedly occur in the coding and regulatory regions and polymorphisms are encoded in exons and introns.

Table-3: Nucleotide polymorphism in Lf gene of riverine-type water buffalo and corresponding sites of amino acid substitution

Base location		Nucleotide polymorphism		Amino acid location	Amino acid substitution	
1	514	C	T	202	L (Leucine)	P (Phenylalanine)
2	857	C	T	286	T (Threonine)	I (Isoleucine)
3	1039	C	T	347	Same	

Table-4: Nucleotide polymorphism in Lf gene of swamp-type water buffaloes and corresponding sites of amino acid substitution

Base location		Nucleotide polymorphism		Amino acid location	Amino acid substitution	
1	315	G	C	105	E (Glutamic acid)	D (Aspartic acid)
2	857	C	T	286	T (Threonine)	I (Isoleucine)
3	1039	C	T	347	Same	
4	1488	C	T	496	Same	

The results of this study corroborated with the result of previous Lf gene studies. In addition, Seyfert et al. (1996) mentioned that the number of exons in the gene and the number of amino acids encoded by 15 of the 17 exons are identical among them. The major differences lie in exons 2, where bovine and other animals' Lf contains one or two amino acids less than human Lf. However, Rupp and Boichard (2003) reported that there is still no strict association found between known Lf gene polymorphisms and mastitis susceptibility.

Restriction enzyme *AluI* cuts AG/CT nucleotide sequence was used to examine polymorphisms in the nucleotide sequence of Lf gene via banding patterns and were compared to the Lf gene sequences of water buffaloes in the GenBank. It was revealed that *AluI* can produce three genotypes at the nucleotide sequence of the 561 bp amplicon. Genotype AA produced two bands of 561 and 123 bp size; one cut was also produced in genotype AB of 217 and 123 bp, and genotype BB that produced two cuts resulting in 561, 123, and 217 bp amplicon sizes (Figure 3).

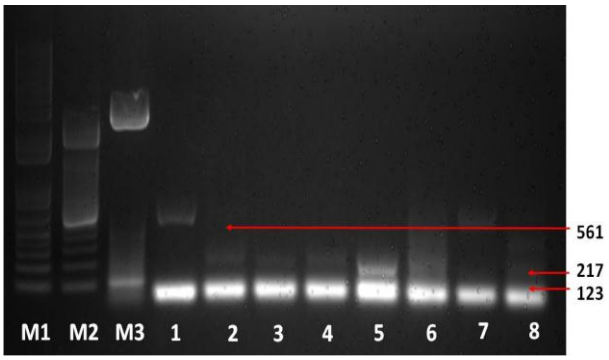

Figure-3: Restriction patterns of Bubaline Lf gene PCR product using *AluI* in 2% agarose gel. M1. 1000 bp ladder, M2. 100 bp ladder. M3. 25 bp ladder, Lanes 1 and 7 genotype AA (fragment size: 561, 123 bp), Lanes 2, 3, 4 and 5-genotype AB (217, 123 bp), Lanes 6 and 8-genotype BB (size: 561, 217, 123, bp).

Figure 4 shows the result of the digestion of water buffalo Lf gene PCR products using *HaeIII* enzyme. From the conceptualization four restriction patterns from nucleotide sequence were observed, genotype AA had one cut with a fragments sizes of 561 and 70 bp; genotype AB with three cuts of 561, 318, 112, and 70 bp, and genotype BB with two cuts of 318, 112, and 70 bp.

No recent studies supported in the three different genotypes found in the riverine water buffalo Lf gene using *HaeIII* and *AluI* restriction enzyme, hence this study will be the baseline for other researchers in identifying mastitic resistance genotypes.

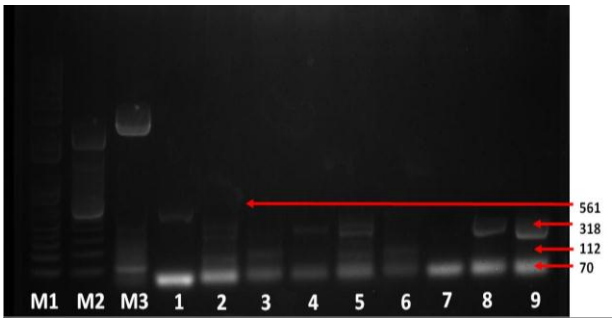

Figure-4: Restriction patterns of Bubaline Lf gene PCR products using *HaeIII* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder. M3 25 bp ladder, Lane 1 and 7, genotype AA (fragment size: 561, 70 bp), Lanes 2, 3, 5 and 6, genotype AB (fragment size: 561, 318, 112 and 70 bp), Lanes 4, 8 and 9, genotype BB (fragment size: 318, 112 and 70 bp)

Association of Lf Gene to SCM

From the total of 75 milk samples, only 62 milk samples were used in for RFLP analysis.

The 561 bp putative ligand-binding domain was targeted in this segment of the study. Table 5 shows the percentage frequency of Lf *AluI*-based genotype in non-mastitic and sub-clinically mastitic riverine water buffalo.

Table-5: Percentage frequency of Lf *AluI*-based genotypes in non-mastitic and SMC riverine-type water buffaloes

Genotype	Category of animal				Total	
	Non- mastitic		SMC			
	%	(n)	%	(n)	%	(n)
AA	66.67	14	33.33	7	34.4	21
AB	57.14	12	42.86	9	34.4	21
BB	52.63	10	47.37	9	32.2	19
Total	59.02	(36)	40.98	(25)	100	61

* bp = base pair

From a total of 61 milk samples tested, 40.98% (n=25) were considered sub-clinically mastitic, while 59.02 % (n=36) were non-mastitic. Genotype AA was found to be more frequent in non-mastitic animals, although Chi-square analysis did not show any significant difference with that of genotype. Furthermore, Statistical analysis of the result could not provide concrete evidence that resistance to SCM was due to the specific genotype.

The Lf gene in water buffaloes has a bactericidal and bacteriostatic activity that could lessen the bacterial population in the milk through phagocytic killing (Valenti and Antonini, 2005; Legrand et al., 2008). This may not be enough to sequester the microbial flora in the mammary gland of the animals. Underlying factors such as climate, housing system, type of bedding, and rainfall and wetness in the vicinity of dairy premises interact to influence the degree of exposure of teat and tend to increase mastitis pathogens that cause SCM (McEwen and Cooper, 1947).

Table 6 shows the percentage frequency of Lf *HaeIII*-based genotype in non-mastitic and SCM riverine water buffalo. From a total of 62 milk samples tested, 35.48% (n=22) were considered SCM, while 64.52% (n=40) were not mastitic. Genotype AA and AB were found to be more frequent in non-mastitic animals, however, Chi-square analysis did not show any significant difference with these genotypes.

The inflammation of the mammary gland induces epithelial cells to secrete Lf. The regulatory region of this gene is useful in the expression of the other antimicrobial proteins with the more potent anti-inflammatory action than Lf itself. Furthermore, Lf cannot work alone thus, animal genetics must coincide with good management practices to prevent or minimize the possible occurrence of SCM in a dairy herd.

Both digestion enzyme used may not have shown a significant association of the investigated genotype, but the presence of extensive polymorphic nucleotide in Lf gene can still be investigated using other restriction enzymes that could target the said polymorphic loci.

Table-6: Percentage frequency of Lf *HaeIII*-based genotype in non- mastitic and sub-clinically mastitic riverine-type water buffaloes

Genotype (fragment sizes)	Category of animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA	28.57	15	71.43	6	33.87	21
AB	70.00	14	30.00	6	32.26	20
BB	47.62	10	52.38	11	33.87	21
Total	35.48	(22)	64.52	(40)	100	62

* bp = base pair

Conclusion

The whole mRNA coding sequence of water buffaloes had an average molecular weight of 2224 bp. Both nucleotide and translated amino acid sequences of Lf in water buffaloes were highly similar (99%) and phylogenetic analysis found an evolutionary relationship with water buffaloes from NCBI GenBank. The Lf gene of riverine water buffaloes established three different genotypes (AA, AB, and BB) using restriction enzymes *AluI* and *HaeIII*. No direct relationship existed between Lf gene and SCM. These initial findings are applicable in the field of immunity and disease resistance. The incidence of association between restriction sites and clinical parameter converts PCR-RFLP into a powerful tool in relating specific amino acid substitution at a critical position to possible disease resistance. Moreover, the selection of disease resistance genes will provide potential avenues for improving the health status of the animal and increasing productivity.

Lactoferrin is a very polymorphic gene that merits further investigation and its significance on disease resistance and susceptibility. Polymorphism of this gene may be useful as a selection marker for resistance against SCM. The result of this study may not statistically prove that lactoferrin can be a marker for SCM. However, the study also points out that most of the water buffaloes with SCM fall under the BB genotype.

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Contribution of Authors

Waminal YO: Designed and conducted the study and prepared the manuscript of the draft

Tubalinal GASP: Conducted the study and prepared the manuscript draft

Mingala CN: Designed and supervised the study and prepared and approved the final version of the manuscript draft