

Article

DNA Polymorphisms and mRNA Levels of Immune Biomarkers as Candidates for Inflammatory Postpartum Disorders Susceptibility in Italian Buffaloes

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Simple Summary: Future reproduction methods for boosting crucial traits are likely to be influenced by the proportion of genetic and phenotypic variances. Using PCR-DNA sequencing, the variations in the nucleotide sequences of the immunological genes between healthy and buffaloes suffering from inflammatory reproductive disorders were discovered. When buffaloes experienced inflammatory reproductive disorders, they were noticeably more likely to express the examined genes. The molecular alterations may therefore provide crucial information about the relationships between the physiologies of the various reproductive pathways and help diagnose inflammatory postpartum disorders.

Abstract: The immunological genes that may interact with inflammatory postpartum diseases in Italian buffaloes were examined in this study. A total number of 120 female Italian buffaloes (60 normal and 60 with inflammatory reproductive diseases) were employed. Each buffalo's jugular vein was pierced to get five millilitres of blood. To obtain whole blood and extract DNA and RNA, the blood was placed within tubes containing sodium fluoride or EDTA anticoagulants. The immunological (*IKBKG*, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ*) genes' nucleotide sequence differences between healthy and buffaloes affected by inflammatory reproductive diseases were found by employing PCR-DNA sequencing. According to Fisher's exact test ($p < 0.01$), there were noticeably different probabilities of all major nucleotide changes spreading among buffalo groups with and without reproductive problems. Buffaloes were significantly more likely to express the examined genes when they had inflammatory reproductive diseases. The outcomes might sup-

port the significance of these markers' nucleotide variations and gene expression patterns as indicators of the prevalence of inflammatory reproductive disorders and provide a workable buffalo management policy.

Keywords: immune biomarkers; transcript levels; reproductive disorders; Italian buffaloes

1. Introduction

The postpartum period, or the first 45 days following delivery, is the most stressful time due to decreased feed intake, endocrine and metabolic changes during parturition and lactation, and it is typically accompanied by a number of physiological stresses with immunological roots [1]. If these changes are not effectively treated, many inflammatory and non-inflammatory pathological illnesses will emerge during this time, including mastitis, endometritis, and hypocalcemia [2–4]. By lowering buffalo conception rates and ages and raising newborn mortalities, these illnesses have a deleterious impact on the flock breeding economic yield. Oxidative stress is the primary factor in immunosuppression and increased disease susceptibility during transition [5]. Due to the increased metabolic demands brought on by late pregnancy, delivery, and the beginning of nursing, reactive oxygen species (ROS) and lipid peroxidation are known to harm tissues, particularly immune cells [6]. The fight against oxidative stress brought on by free radicals involves preventive, repair, physical, and antioxidant defenses [7].

Bos taurus is predisposed to inflammatory reproductive disorders after delivery since about majority of diseases in adult dairy cows often appear in the first month [8,9]. Inflammation is important for repeating tissue receptiveness and founding conception in postpartum dairy cows [11], which is characterized by the exclusion of bacterial infection and renewal of the inflamed tissue. Due to a poor immunological response, cows may struggle to quickly clear infections [12]. Inflammatory reproductive problems are a result of a persistent inflammatory response in tissue [13], which is related to this. Dairy cows are susceptible to bacterial reproductive infections during the time following parturition [14]. It is believed that bacterial infections are the primary cause of the majority of inflammatory reproductive illnesses since a range of bacteria may be easily isolated from the reproductive lumen [15]. Inflammatory reproductive problems have also been connected to increased amounts of pro-inflammatory cytokine transcripts because of poor inflammation activation and bacterial clearance [16].

In mammals, there are two types of immunity: innate immunity and adaptive immunity. Both vertebrates and invertebrates have innate immunity, whereas only vertebrates have adaptive immunity [17]. Examples of various cascades that significantly affect the immune system include the nutritional stress of cattle production methods. It was previously believed that problems with general health at this time had a secondary impact on immunological response. However, as more and more cellular and molecular immunological pathways were clarified, these problems and their effects on the immune system began to take on a stronger molecular reality [18]. Innate immunity supports host genetic resistance primarily, providing defence against pathogens without vaccination or exposure to illnesses [19]. Indeed, some genetic variants that may be responsible for variance in reproductive features have been highlighted by association studies [20]. We will probably be better able to identify the genes and variants that are responsible for these traits as we continue to study the function and expression of genes in relation to reproductive disorders in cattle. This could eventually result in more precise methods of genomic selection that are more effective across generations and breeds [21].

Since genetic variations are the source of all evolution, describing the genetic makeup of a population necessitates an understanding of that group's evolutionary history [22, 23]. Additionally, the proportion of genetic and phenotypic differences is anticipated to

have an impact on future reproduction techniques for enhancing critical features [24]. Because of this, the development in molecular genetics has given us the chance to identify genes and the linked pathways. The latter is more insightful into the physiological mechanisms behind these candidate genes and has the potential to be used as genetic markers in MAS programs [25]. Notably, large numbers of important genes have been found in populations of cattle. These important genes have been linked to various reproductive, illness, or production traits in the general population [26]. Additionally, resistant animals expressed hundreds of genes implicated in immunological function, according to gene expression studies [27,28]. Association analysis performed on a variety of animals typically identifies genetic variants that have an impact on phenotypic variation [29].

According to our current understanding, there is little data on the immunological changes and genetic variations linked to inflammatory postpartum disorders in cattle, and there are also few reliable diagnostic and prognostic tools to help us improve our preventative and therapeutic measures. Furthermore, it is significant to note that additional study is required to track potential immunological markers linked to reproductive abnormalities that determine whether buffalo are resistant to or susceptible to these diseases. In this study, real-time PCR and PCR-DNA sequencing techniques were used to assess the efficiency of putative immune genes as candidates for inflammatory reproductive diseases incidence prediction and tracking in Italian buffaloes.

2. Material and Methods

2.1. Dairy Buffaloes and Research Samples

The 120 pure Italian buffaloes used in this study were grown on a private farm in the Delta region of northern Egypt, 60 of which had inflammatory reproductive problems and the remaining 60 appeared to be healthy. The investigated buffaloes were split into two groups of similar size throughout the postpartum period according to their level of health. The first group was designated as the healthy group and consisted of buffaloes that were clinically healthy and had a history of regular reproductive performance (i.e., normal feed intake, body temperature, no uterine discharge, and normal udder). The buffaloes in the second group exhibit mastitis (high body temperature, low appetite, swollen and tender udder, reddish and yellowish milk colour and bad odour, clotted milk, and teat cracks) and endometritis (pyrexia, persistent coloured uterine discharge with offensive odour, anorexia, and depression). Each buffalo's jugular vein was pierced to get five milliliters of blood. The samples were placed into tubes containing anticoagulants (EDTA or sodium fluoride) in order to collect whole blood and recover DNA and RNA. All animal management techniques, test experimental collection, and sample eradication were carried out under the direction of the Veterinary Medical School of the University of Sadat City (Code VUSC-019-1-23) in accordance with IACUC guidelines.

2.2. DNA Extraction and Amplification

The genetic material JET complete blood genomic DNA isolation kit and the manufacturer's instructions (Thermo Scientific, Vilnius, Lithuania) were used to extract DNA from the genome using total blood. DNA with good purity and concentration was taken into consideration using Nanodrop. Immunity-related genes (*IKBKG*, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ*) were amplified. The *Bubalus bubalis* genome, which was available in PubMed, was used to create the oligonucleotide sequences for amplification. The primers used during the PCR are listed in Table 1.

A heat cycler with a final volume of 150 µL had been used to process the polymerase chain cloning mixture. Every reaction container contained the following components: 66 µL d.d. water, 6 µL genetic material, 1.5 µL of each primer pair, and 75 µL of the master combination (Jena Bioscience, Jena, Germany). At a starting temperature of 94 °C for unwinding, the PCR combinations were used for five minutes. The 35 cycles consisted of 30-

second rounds 30-second rounds of denaturation at 94 °C for 1 minute, one-minute annealing cycles depending on the temperature range shown in Table 1, elongation at 72 °C for 1 minute and final elongation at 72 °C for 8 minutes. The materials were kept at 4 °C. A gel certification method was applied, which involved evaluating PCR segment configurations under UV light and using agarose gel electrophoresis to produce demonstrable results.

Table 1. Immune gene oligonucleotide primers for the investigation of genetic polymorphisms.

Investigated marker	Sense	Antisense	Annealing Temperature (°C)	Size of PCR product (bp)
<i>IKBK</i>	5'- CCGGCTTGGACAGCCTCT CTTG-3'	5'- TGCCCTGCTCTGAAGGCA CATG-3'	58	412
<i>LGALS</i>	5'- CTCATTCTCCTGCAGAG TCT-3'	5'- AACTTGAATTCGTATCCA TCAG-3'	58	395
<i>IL1B</i>	5'- CCATGGCAACCGTACCT GAAC-3'	5'- ACTGACTGCACGGGTGCG TCAC-3'	58	359
<i>CCL2</i>	5'- CTTGAATCCTCTCGCTGC AAC-3'	5'- AGATTCTTGGGTTGTGGA GTGA-3'	60	347
<i>RANTES</i>	5'- CAGCCGGAGCTGCAGAG GATCA-3'	5'- CAAGCTGCTTAGGACAA GAGCG-3'	58	399
<i>MASP2</i>	5'- AGAGCACAGACAGAGAGATGCGGCCATTAGGTG CGGGC-3'	5'- GCGC-3'	60	513
<i>HMGB1</i>	5'- CGCTGGCTGGAGAGTAA TGTTA-3'	5'- CACCAATAGACAGGCCA GGATGT-3'	60	540
<i>S-LZ</i>	5'- TCTGGACATTGACTTCT CAG-3'	5'- CTCAATGTAAGTCTGAC GTC-3'	58	455

IKBK = Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma; *LGALS* = Galectin; *IL1B* = Interleukin 1 beta; *CCL2* = C-C Motif Chemokine Ligand 2; *RANTES* = Regulated on activation, normal T cell expressed and secreted; *MASP2* = Mannan-binding lectin serine protease-2; *HMGB1* = High mobility group box 1; and *S-LZ* = serum lysozyme.

2.3. Discovering Polymorphism

Prior to DNA sequencing, Jena Bioscience # pp-201s/Munich of Hamburg, Germany offered techniques to purify PCR in order to remove primer dimers, non-specific bands, and other contaminants and provide the desired amplified result for the projected scope [30]. When assessing PCR output, a Nanodrop (Waltham, Massachusetts, USA, UV-Vis spectrometer Q5000) was used because it provided adequate quality and good concentrations [31]. Using normal and inflammatory reproductive disorders affected buffaloes, sequence analysis of the PCR-produced amplification results was used to identify SNPs. The PCR results were sequenced on an ABI 3730XL DNA sequencer (United States: Applied

Biosystems, Waltham) using the enzyme chain terminator approach described by Sanger et al. [32].

Chromas 1.45 and BLAST 2.0, the tools for examining DNA analysis findings [33]. Comparing the immune gene PCR results to the GenBank provided reference gene sequences has revealed polymorphisms. The MEGA6 tool can detect differences in the amino acid categorizations among the examined genes according to sequence matching among the buffaloes under investigation [34].

2.4. Levels of Immune Gene Transcripts

According to the manufacturer's recommendations, the RNA was entirely extracted from the blood samples taken from the investigated buffaloes using the Trizol solution (RNeasy Mini Ki, 74104, Product No.). Using a NanoDrop® ND-1000 spectrophotometer, we measured and verified the quantity of RNA that was separated. The producer's method was used to create the whole nucleic acid in each sample (Waltham, Massachusetts, USA: Thermo Fisher, Product No. EP0441). SYBR Green PCR Master Mix and quantitative RT-PCR (2x SensiFast™ SYBR, Bio-line, CAT No. Bio-98002) were used to assess the immune genes' expression profiles. SYBR Green PCR Master Mix (Toronto, Ontario, Canada: Quantitect SYBR green PCR reagent, Catalogue No. 204141) has been used to determine the relative amount of mRNA that each sample possesses.

The sense and anti-sense primer oligonucleotides were made using the *Bubalus bubalis* genome from PubMed (Table 2). The β . *actin* gene served as the constitutive normalization reference. The reaction mixture was given up in over-all volume of 25 microliter consisted of entire RNA 3 microliter, 4 microliter 5xTrans Amp buffer, 0.25 microliter reverse transcriptase, 0.5 microliter of each primer, 12.5 microliter 2x Quantitect SYBR green PCR master mix and 8.25 microliter RNase-free water. The finished reaction mixture was then put through the following steps inside a heater cycler: reverse transcription for 30 min at 55 °C, preparatory denaturation for 8 min at 95 °C, 40 cycles of 15 s at 95 °C, and primer binding temperatures as listed in Table 2 with a 1-min extension at 72 °C. Following the amplifying process, a melting curve analysis was used to show the specificity of the amplified product. By comparing each gene's expression in the examined sample to that of the β . *actin* gene, the $2^{-\Delta\Delta C_t}$ approach has been utilized to examine the variations in each gene's expression [35,36].

2.5. Statistical analysis

H₀: The resistance/susceptibility of inflammatory reproductive diseases in Italian buffaloes could not be explained by polymorphisms and transcript levels of immunological biomarkers.

H_A: The resistance/susceptibility of inflammatory reproductive diseases in Italian buffaloes could be explained by polymorphisms and transcript levels of immunological biomarkers.

Using Fisher's exact test analysis ($p < 0.01$), the significant distribution of SNPs for the identified genes was observed between the buffaloes under study. The Statistical Package for Social Science (SPSS) version 17 computer program and the t-test (SPSS Inc, Chicago, IL, USA) were used to determine the statistical significance of the differences between normal and inflammatory reproductive illnesses affected buffaloes. To present the findings, mean and standard error (Mean $\pm$ SE) were employed. Differences were considered significant at $p < 0.05$.

Table 2. Oligonucleotide-based real-time PCR primers for the under investigation immunological genes.

Investigat ed marker	Primer	Prod uct	Annealin g	GenBank isolate	Origin
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		size (bp)	Temperat ure (°C)		
<i>IKBK</i>	F5'- CTGGCAGGAGAA- GCCATCAA -3 R5'- CAGCAGACAGGACAC- TAGCC -3'	101	58	XM_044936 650.2	Current study
<i>LGALS</i>	F5'- GGCCCAAAGCTCATTCTCC -3 R5'- GCACTCCCCAGGTTTGA- GAT -3'	129	58	XM_025282 898.3	Current study
<i>IL1B</i>	F5'- AGGTGGTGTCTCGTTCATTGTG -3' R5'- AACTCGTCGGAGGAC- GTTTC - 3'	142	60	NM_001290 898.1	Current study
<i>CCL2</i>	F5'- AAGCCTTGAGCACTCAC- TCC -3' R5'- GCAGTTAGGGAAGCCG- GAA - 3'	72	56	HQ889748. 1	Current study
<i>RANTES</i>	F5'- CCCATATGCCTCAGACAC- CAC -3' R5'- GGCGGTTCTTCCTGGTGATA - 3'	140	60	XM_006056 212.4	Current study
<i>MASP2</i>	F5'- ACAGACAAGGCGGAA- TACGG -3' R5'- GTGGTCTACAG- CAGGCAAGT- 3'	162	58	XR_0065511 48.1	Current study
<i>HMGB1</i>	F5'- TGCCTCGCGGAG- GAAAAATA -3' R5'- GCAGA- CATGGTCTTCCACCT - 3'	188	60	XM_006078 587.2	Current study
<i>S-LZ</i>	F5'- CTGTAGCCTGTGCAAA- GCAG -3' R5'- CAGGGTG- CAACCCTCAATGT - 3'	113	60	EF535848.1	Current study
<i>β. actin</i>	F5'- GATGATGA- TATTGCCGCGCTC-3' R5'- AGGGTCAG- GATGCCTCTCTT-3'	197	58	NM_001290 932.1	Current study

IKBK = Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma; *LGALS*= Galectin; *IL1B* = Interleukin 1 beta; *CCL2*= C-C Motif Chemokine Ligand 2; *RANTES*= Regulated on activation, normal T cell expressed and secreted; *MASP2*= Mannan-binding lectin serine protease-2; *HMGB1*= High mobility group box 1; and *S-LZ*= serum lysozyme.

3. Results

3.1. Genetic Polymorphisms of Immune Genes

SNP variations in amplified DNA nucleotides associated with inflammatory reproductive disorders were found in the results of PCR-DNA sequencing on both normal and affected buffaloes for the *IKBK* (412-bp), *LGALS* (395-bp), *IL1B* (358-bp), *CCL2* (347-bp), *RANTES* (399-bp), *MASP2* (513-bp), *HMGB1* (540-bp), *S-LZ* (455-bp) genes. The DNA sequence variations between the GenBank-sourced reference gene sequences and the re-

productive indicators examined in the studied buffaloes were used to validate all the detected SNPs (Figures S1–S8). Table 3 shows the dissemination of a single base variation as well as a type of inherited change for immune indicators in normal and inflammatory reproductive disorders affected buffaloes. The SNPs' Fisher's exact test analysis revealed considerably different occurrences of the investigated markers in the normal and inflammatory reproductive disorders affected buffaloes ($p < 0.01$). All of the reproductive markers under research had the exonic region mutations, which resulted in different coding DNA sequences in normal buffaloes versus inflammatory reproductive disorders affected ones. Eighteen SNPs were discovered using DNA sequencing of immune genes; 10 of them are non-synonymous and 8 is synonymous. The amino acid variations between the immune genes in all the research buffaloes and the reference sequences retrieved in GenBank were used to validate all observed SNPs and the corresponding amino acids (Figures S1–S16).

3.2. Patterns for transcript levels of immune indicators

In inflammatory reproductive disorders affected buffaloes, expression levels of the genes *IKBK*G, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ* were noticeably up-regulated (Figure 1). The greatest potential level of mRNA for each gene assessed in the inflammatory reproductive disorders affected buffaloes was found for *CCL2* (2.21 ± 0.18) and the lowest level was found for *LGALS* (1.47 ± 0.15). Among all the genes analyzed in the normal buffaloes, the gene with the highest potential level of mRNA was found for *S-LZ* (0.65 ± 0.06), while the lowest level was found for *IKBK*G (0.46 ± 0.15).

Table 3. Single base difference dispersal as well as sort of inherited change for immune markers in healthy and inflammatory reproductive disorders affected buffaloes.

Gene	SNPs	Healthy <i>n</i> = 60	Reproductive disorders <i>n</i> = 60	Total <i>n</i> = 120	kind of inherited change	Amino acid order and sort
<i>IKBK</i> G	C78G	-	31	31/120	Non-synonymous	26 I to M
	G328A	27	-	27/120	Non-synonymous	110 G to S
<i>LGALS</i>	T192C	42	-	42/120	Synonymous	64 N
<i>IL1B</i>	C53T	19	-	19/120	Non-synonymous	18 T to M
	T125C	47	-	47/120	Non-synonymous	42 V to A
	T205C	-	23	23/120	Non-synonymous	69 L to S
	C311T	49	-	49/120	Non-synonymous	104 S to L
<i>CCL2</i>	C201T	-	53	53/120	Synonymous	67 P
<i>RANTES</i>	G96C	-	29	29/120	Synonymous	32 T
	A141G	-	43	43/120	Synonymous	47 S
	C370T	54	-	54/120	Synonymous	124 L
<i>MASP2</i>	T465A	22	-	22/120	Synonymous	155 A
<i>HMGB1</i>	C227T	34	-	34/120	Non-synonymous	76 S to L
	C275T	-	29	29/120	Non-synonymous	92 P to L

S-LZ	T407C	56	-	56/120	Non-synonymous	136 F to S
	G33A	39	-	39/120	Synonymous	11 R
	A222G	-	52	52/120	Synonymous	74 L
	G254A	-	37	37/120	Non-synonymous	85 R to K

Single base difference dispersal for immune markers in healthy and reproductive disorders buffaloes showed a highly significant variation ($p < 0.01$) according to Fisher's exact analysis.

IKBKG = Inhibitor of nuclear factor kappa B kinase regulatory subunit gamma; *LGALS* = Galectin; *IL1B* = Interleukin 1 beta; *CCL2* = C-C Motif Chemokine Ligand 2; *RANTES* = Regulated on activation, normal T cell expressed and secreted; *MASP2* = Mannan-binding lectin serine protease-2; *HMGB1* = High mobility group box 1; and *S-LZ* = serum lysozyme. A = Alanine; F = Phenylalanine; G = Glycine; I = Isoleucine; K = Lysine; L = Leucine; M = Methionine; N = Asparagine; P = Proline; R = Arginine; S = Serine; T = Threonine; and V = Valine.

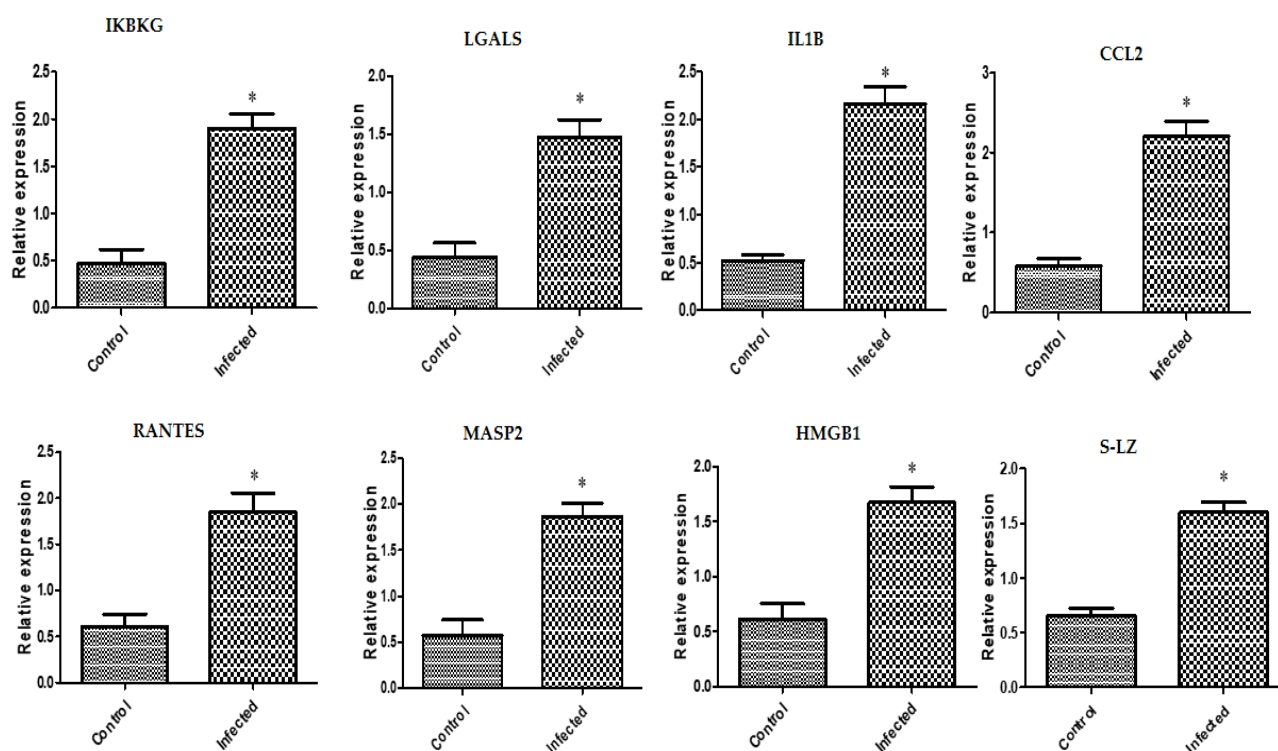


Figure 1. Differential transcript levels of immune genes between normal and inflammatory reproductive disorders affected buffaloes. The symbol * denotes significance when $P < 0.05$.

4. Discussion

4.1. Association of Immune Genes Polymorphisms with Inflammatory Reproductive Disorders

Knowing the genes, underlying mutations, and interactions with other variables that confer resistance is crucial for the efficient exploitation of disease-resistant cattle or the complete elimination of diseased animals [37]. The immune (*IKBKG*, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ*) genes in inflammatory reproductive disorders affected and healthy Italian buffaloes were identified in this study utilizing sequenced amplified PCR products. The findings show that there are differences between the SNPs involving the two categories. The assessed buffaloes had a considerable nucleotide poly-

morphism dispersion ($p < 0.01$), according to the Fisher's exact test. It is crucial to emphasize that, when compared to the relevant datasets obtained from GenBank, the polymorphisms discovered and made available in this context provide new data for the examined indicators.

There is little information on polymorphisms and expression profile of immune biomarkers in inflammatory postparturient disorders in buffaloes. The initial evidence for this connection can be found in the gene sequences from the *Bubalus bubalis* that were used in our work and were published in PubMed. The variance of the immune (*IKBKG*, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ*) markers and how they relate to inflammatory reproductive disorders in Italian buffaloes have not, to our knowledge, been the subject of any prior research. The candidate gene method has, however, been utilized to assess the veracity of postpartum problems in buffalo. For instance, sequence analysis variations of the *TLR2* and *TLR4* genes were linked to endometritis and mastitis in river buffalo [38–40].

In order to better understand the resistance and vulnerability of inflammatory reproductive diseases in livestock, genetic polymorphisms of immunological markers for postpartum endometritis were examined in Holstein dairy cows [41]. Using PCR-DNA sequencing for the immunological (*TLR4*, *TLR7*, *TNF- α* , *IL10*, *NCF4*, and *LITAF*) genes, nucleotide sequence differences between healthy and endometritis-affected cows were discovered. In addition, nucleotide sequence changes in the form of SNPs linked with mastitis tolerance/susceptibility were discovered in the examined Holstein and Brown Swiss dairy cows using PCR-DNA sequencing of immunological (*SELL*, *ABCG2*, *SLC11A1*, and *FEZL*) markers [42]. In cows, the *LTF* gene was linked to mastitis resistance [43–45]. *TLR4* gene polymorphisms and mastitis susceptibility were found to significantly correlate in sheep [46] and cattle [47,48]. According to Ruiz-Rodriguez et al. [49], there is a correlation between goats' susceptibility to or resistance to mastitis and variations in the *TLR2* gene. In Barki sheep, *TLR4* gene SNPs were likewise linked to postpartum disorder risk [27].

4.2. Immune Markers' Gene Expression Profile

In the current work, we proposed that the course of inflammatory reproductive disorders may be influenced by genetic variation in an individual's transcriptional response to those conditions. *IKBKG*, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ* genes were quantified using real-time PCR in both normal and inflammatory reproductive disorders affected buffaloes. Our results demonstrated that buffaloes with inflammatory reproductive diseases expressed immune genes at higher levels than healthy ones. In order to ascertain the mRNA levels of these immunological markers linked to the resistance/susceptibility to inflammatory reproductive diseases in Italian buffaloes, real-time PCR was used in our experiment for the first time. Our investigation of gene polymorphism utilizing SNP genetic markers and gene expression overcame the drawbacks of past studies. As a result, in both healthy and affected by inflammatory reproductive illnesses buffaloes, the mechanisms of the examined gene regulation are well understood.

Buffaloes suffering by reproductive problems have a sparsely developed gene expression profile of immunological markers. Results of an investigation into the expression of cytokine genes in the uteri of *Bubalus bubalis* associated with endometritis infection showed that *IL-1*, and *IL-6* gene expression in endometritis-infected buffaloes increased by 1.3, 1.7, and 5 folds, respectively, in comparison to that in healthy animals. In contrast, when compared to uninfected animals, the expressions of two additional genes, *IL-10* and *TNF- α* , revealed down-regulations of 0.2 and 0.4 folds, respectively [50].

In dairy cows, the bovine endometrium of cows with inflammation had greater transcript levels of immunological (*CCL5*, *CXCL8*, *IL6*, and *IL1B*) genes than cows without inflammation [51]. In Chinese Holstein cattle with mastitis, transcript level expression alterations showed that *TLR4*, *MyD88*, *IL-6*, and *IL-10* were up-regulated while *CD14*, *TNF- α* , *MD-2*, *NF-B*, and *IL-12* were significantly down-regulated [52]. The expression levels of

the genes *TLR4*, *TLR7*, *TNF- α* , *NCF4*, and *LITAF* were significantly greater in cows with endometritis than in resistant cows. Meanwhile the expression of the *IL10* gene was much decreased [41]. Mastitic Holstein and Brown Swiss dairy cows had considerably higher levels of *SELL*, *SLC11A1*, and *FEZL* gene expression than tolerant ones [44]. Dairy cattle with subclinical endometritis exhibit specific mRNA expression patterns of *C3*, *C2*, *LTF*, *PF4*, and *TRAPPC13* in the blood and endometrium. Additionally, the gene expression profiles of *C3*, *CXCL8*, *LTF*, *TLR2*, and *TRAPPC13* were temporally altered in healthy cows' circulating WBC during the postpartum period as opposed to cows with sub-clinical endometritis [53]. The three separate time points of the transition phase did not significantly differ in *CCL5* gene expression [18].

In goats, Damascus goats were found to have an up-regulated expression profile of the *LTF* gene for mastitis resistance [54]. Goats with *Staphylococcus aureus* mastitis were found to have a considerable increase in pro-inflammatory cytokines, chemokines, and their receptors compared to tolerant goats [55]. In the goat model of subclinical endometritis created by Shao et al. [56], the mRNA expression levels of *TLR4*, cytokines, and beta-defensin significantly increased. In terms of the gene expression profile of immune markers in sheep, According to Darwish et al. [27], sheep with postpartum problems had considerably higher levels of *IL5*, *IL6*, *IL1- β* , *TNF alpha*, *TLR4*, and *Tollip* than tolerant ewes.

The inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (*IKBK γ*) gene encodes the regulatory subunit of the inhibitor of kappaB kinase (IKK) complex. When NF-kappaB is activated, genes implicated in immunity, cell survival, and other pathways are also activated [57]. Animal lectins known as galectins have an affinity for β -galactosides [58]. Through lectin-carbohydrate interactions, they can interact with glycoproteins on the surface of cells and in the extracellular matrix [58]. Galectins influence cell survival, alter cell adhesion, and trigger cell migration through this process. Numerous cell and tissue types contain them, and a variety of their functions, including encouraging macrophage migration and playing a part in both acute and chronic inflammation, have been documented [59]. In inflammatory circumstances, cytokines such *IL-6*, *TNF-*, *IFN-*, *IL1B*, and *NFKB* serve as indirect indicators [60].

C-C Motif Chemokine Ligand 2 (*CCL2*), which encodes two tiny proteins that bind to the *CCR2* receptors present on the surface of monocytes and neutrophils, respectively, acts as a potent chemokine for these cells [61]. These molecules interact with receptors on neutrophils and monocytes to cause conformational changes that allow cytosolic free Ca^{2+} to promote chemotaxis towards inflammatory stimuli [62]. Canadian Holsteins were used to study the association between single nucleotide polymorphisms in the bovine *CCL2* gene and productivity and health [63]. The chemokine regulated on activation normal T-cell expressed and secreted (RANTES), also known as *CCL5*, is expressed by a wide variety of cells, including blood lymphocytes, in response to inflammatory signals [64]. It regulates the migration and activation of inflammatory and non-inflammatory cells [65]. Additionally, it affects the acute phase response [66].

Mannose-binding lectin-associated serine protease 2 (*MASP2*) serves as the primary protease in the complement system [67]. Autoimmune disorders have a substantial association with polymorphisms in the *MASP2* gene [68]. Numerous inflammatory illnesses and infections have been associated with *MASP2* serum levels and gene variants [69]. It was shown how the polymorphisms of the *MASP2* gene relate to mastitis and milk output in Chinese Holstein cattle [70]. High-mobility group box 1 (*HMGB1*) is a protein with important biological roles that is both abundant and conserved. It can stimulate the *CXCR4* to increase chemotaxis by binding to the chemokine *CXCL12* or activate the toll-like receptor 4 (*TLR4*) to generate cytokines [71]. In addition to its intrinsic action, *HMGB1* interacts with PAMPs, cytokines, and chemokines to extend its extracellular effects [72]. An anti-bacterial peptide called lysozyme is essential for giving animals immunity [73]. Lysozyme plays a substantial role in treating cow mastitis, according to earlier investigations [73,74].

Italian buffaloes with inflammatory reproductive disorders exhibit a marked up-regulation in the expression pattern of immune (*IKBK*G, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ*) markers. This may be attributed to severe inflammation that damages the affected tissue as well as cytotoxic radicals and proinflammatory cytokines released by the phagocytic cells [75,76]. Additionally, the immune system is compromised by the preponderance of ROS caused by the excess ROS in the absence of an optimal total antioxidant [76]. Inflammation of the reproductive tract is frequently brought on by bacterial infections [77]. The buffaloes' immune systems are triggered as a result of being exposed to more infections. The neutrophil recruitment cascade is initiated when macrophages and epithelial cells are exposed to either lipoteichoic acid (LTA, from Gram-positive bacteria), or LPS, from Gram-negative bacteria, which both stimulate the release of TNF and IL1B [78]. When TNF, IL1, C5a, and histamine interact with their unique receptors, vascular endothelial cells react [79]. Therefore, we assume that an infectious agent was to blame for the vast majority of the inflammatory reproductive diseases cases in this study that affected dairy cows. Furthermore, our Real Time PCR results provide compelling evidence that the affected buffaloes were experiencing a significant inflammatory response due to the inflammatory reproductive diseases. Our idea is that variations in the essential genes associated to these defense systems can lead to variations in disease resistance. Buffaloes have essential, shared innate immune defense mechanisms against multiple reproductive tract infection sources.

5. Conclusions

Single nucleotide variations (SNPs) in the genes for immunological (*IKBK*G, *LGALS*, *IL1B*, *CCL2*, *RANTES*, *MASP2*, *HMGB1*, and *S-LZ*) indicators were discovered using PCR-DNA sequencing in healthy and sick Italian buffaloes with inflammatory reproductive diseases. Furthermore, these indicators' mRNA levels varied between healthy and sick buffaloes with inflammatory reproductive diseases. By employing genetic markers along with normal welfare during buffalo selection, these unique functional variants offer a promising chance to reduce the prevalence of inflammatory reproductive illnesses. The gene domains identified here may facilitate future methods to treating inflammatory reproductive diseases.

Supplementary Materials: Figure S1. Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|XM_044936650.2| and *IKBK*G marker (412-bp) sequences. Figure S2. Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|XM_025282898.3| and *LGALS* marker (395-bp) sequences. Figure S3. Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|NM_001290898.1| and *IL1B* marker (358-bp) sequences. Figure S4. Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|HQ889748.1| and *CCL2* marker (347-bp) sequences. Figure S5. Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|XM_006056212.4| and *RANTES* marker (399-bp) sequences. Figure S6 Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|XR_006551148.1| and *MASP2* marker (513-bp) sequences. Figure S7 Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|XM_006078587.2| and *HMGB1* marker (540-bp) sequences. Figure S8 Analysis of nitrogenous bases matching for DNA in healthy and reproductive disorders affected buffaloes using GenBank gb|EF535848.1| and *S-LZ* marker (455-bp) sequences. Figure S9. Analysis of amino acid matching in healthy and reproductive disorders affected buffaloes using GenBank gb|XM_044936650.2| and *IKBK*G marker (412-bp). Figure S10. Analysis of amino acid matching in healthy and reproductive disorders affected buffaloes using GenBank gb|XM_025282898.3| and *LGALS* marker (395-bp). Figure S11. Analysis of amino acid matching in healthy and reproductive disorders affected buffaloes using GenBank gb|NM_001290898.1| and *IL1B* marker (358-bp). Figure S12. Analysis of amino acid matching in healthy and reproductive disorders affected buffaloes using GenBank gb|HQ889748.1| and *CCL2* marker (347-bp). Figure S13. Analysis of amino acid matching in healthy and reproductive disorders

affected buffaloes using GenBank gb|XM_006056212.4| and *RANTES* marker (399-bp). Figure S14. Analysis of amino acid matching in healthy and reproductive disorders affected buffaloes using GenBank gb|XR_006551148.1| and *MASP2* marker (513-bp). Figure S15. Analysis of amino acid matching in healthy and reproductive disorders affected buffaloes using GenBank gb|XM_006078587.2| and *HMBG1* marker (540-bp). Figure S16. Analysis of amino acid matching in healthy and reproductive disorders affected buffaloes using GenBank gb|EF535848.1| and *S-LZ* marker (455-bp).

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