



Original Article

A study on IL8RB gene polymorphism as a potential immuno-compromised adherent in exaggeration of parenteral and mammo-crine oxidative stress during mastitis in buffalo

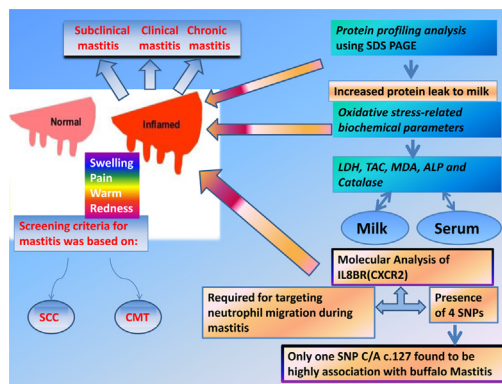


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GRAPHICAL ABSTRACT



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ABSTRACT

The genetic markers in inflammatory responses during mastitis afford a reasonable way for improving milk production in the Egyptian buffalo breed. Among them is the interleukin 8 Receptor Gene (*IL8RB*) (*CXCR2*); a chemokine receptor gene augments the neutrophil migration during infection. To understand its role better during mastitis in Egyptian buffalos, twenty-five dairy animals representing the normal, sub-clinically, clinically and chronically affected buffalos were randomly selected from different districts. Screening criteria for mastitis were based on somatic cell count and California mastitis test assays on their milk samples. Biochemically, mastitis induced an increase in milk lactate dehydrogenase, alkaline phosphatase and catalase activities and serum malanoaldehyde concentration. The total antioxidant concentrations, however, decreased in serum and milk during mammary inflammation. The protein profiling of milk they proved an accelerated mammary inflammatory influx of blood-borne proteins during mastitis. The genomic DNAs were extracted from blood samples and the *CXCR2* sequence of 1246 bp covering a part of intron 1, exon 2 and a part of 3'UTR were submitted to Genbank (accession # KY399457.1). The study clearly defined the presence of four SNPs. Three were detected as synonymous substitutions in coding region and one in the 3'UTR region. Only SNP C/A at c.127 was found to be highly associated with mastitis. In conclusion, the results warrant the potential correlation between the genetic SNP variance for certain genes and the incidence of mastitis in buffalo breed.

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Introduction

The river buffalo is considered as the most effective part of animal production in Egypt with average population around 4 million heads as declared by the study carried out by FAOSTAT (2013) (FAO Statistics Division, FAO, Rome, Italy. www.fao.org). Buffalos play an obvious economical role through their production of milk, meat, and hides besides their animal power in cultivation. Buffalos' milk is the natively-preferred dairy product due to its favored color and taste properties and a valuable fat percentage. The quantitative and qualitative improvements of milk production in Egyptian buffalos, however, are still facing many obstacles with a real need for alternative programs suitable for enhancing their reproductive performance [1]. Mastitis, on the other hand, is a multi-factorial disease that selectively targets certain animals with the same management conditions among the rest of the healthy herd. This may refer to the genetic variance of animals in the same herd [2]. Mastitis stands as the most economically common and damaging threat against milk production in cattle and buffalos. Therefore, selective improvement of production traits responsible for animal resistance against this disease is the utmost option for upgrading overall performance in buffalos [3].

Recently, mastitis is sub-categorized into a clinical (an individual animal health problem) and a sub-clinical mastitis (a herd problem) [4]. The clinical mastitis is characterized by abnormal milk, gland swelling, and systemic illness, whilst subclinical mastitis has apparently normal milk with increase in SCC and reduced milk production [5]. Previous works, however, indicated that heritability experiments for SCC could improve selection criteria in buffalos [6]. The incorporation of major candidate genes in buffalo breeding is currently an important issue in buffalo breeding. This became more obvious since the cattle SNP chip does not offer an optimal coverage of buffalo genome. Thereafter, the construction of novel buffalo-based genetic mapping positively impacts buffalo dairy production [7]. Based on the selection criteria for bovine mastitis two major ways were applied: the traditional approach of udder health of the animal or SCC, and the recent approach of genetic DNA profiling [8]. The resistance against mastitis is a polygenic trait. Thence, there is a need to study the genes related to the resistance against mastitis. The alteration in the genes associated with neutrophil function can be considered as significant marker for mastitis since the migration of circulating neutrocytes to the infection site -as the first line of defense- is crucial for competing most of mastitis pathogens [9]. It was proved that the inflammatory mediators such as neutrophil complement receptors, cytokine, and chemokines potentiate the migration of neutrophils [10]. During this process, interleukin 8 (*IL8*) is considered as the main chemo-attractant binder to the two chemokine receptors surfacing the neutrophils, namely *IL-8 RA (CXCR1)* and *IL-8 RB (CXCR2)* [11]. Moreover, the infection resolution and neutrophil migration to the mammary gland need *IL-8B* receptor gene. It was stated that the locus of *CXCR2* has been genetically mapped close to particular loci as natural resistance associated macrophage protein (NRAMP)-1 locus known to encode disease resistant gene. The *CXCR2* binds to interleukin 8, neutrophil activating peptide-2 (NAP-2) and onco-gene α [11]. The *IL-8B* receptor exhibits importances in immune function during mastitis infection as it belongs to the promising candidate genes contribute in bovine mastitis [12].

Although the dairy animals are subjected to oxidative stress manifested by lipid peroxidation due to the pathogenic invasion of the mammary gland, the study on oxidative stress during buffalo mastitis, however, is not completely recovered [4]. Different enzymes are used as biomarkers in milk samples. In the last few years, measurement of activities of these enzymes is considered as a diagnostic tool for detecting mastitic animals. The identifica-

tion of mastitis can be checked by fluctuation of the activities of milk enzymes e.g. lactate dehydrogenase (LDH) and alkaline phosphatase (AP) during the inflammation of mammary glands [13,14]. During this inflammatory process, the infiltration of defensive macrophages and polymorphonuclear leukocytes into the mammary gland has varied degrees of destructive action resulting in clinical and subclinical mastitis. Consequently, these cells together with other damaged parenchyma cells of the inflamed udder secrete products containing some hydrolytic enzymes (e.g. lysosomal or non-lysosomal LDH) [15] and are considered as the origin of the altered LDH and AP levels in mastitic milk [13]. Cattle milk proteins represent an available source for studying evolution and breeding preservation by reflecting genetic polymorphism. Moreover, previous reports found that milk protein polymorphism has a strong impact on milk quantitative and qualitative traits as well as technological properties [16]. Buffalo milk has gradually replaced cow milk in some regions of the world [17]. This is related to its superior nutritional properties to cow milk due to its high fat and protein contents [18].

This work aims at screening the coding region of *IL-8B* receptor gene (*CXCR2*) to detect any possible SNPs in mastitic and control animals in native buffalo breed. The study also evaluates the oxidative stress parameters (malondialdehyde (MDA), total antioxidant capacity (TAC), and activities of LDH, AP, and catalases) on par-enteral and mammary secretion levels by measuring its parameters in blood and milk of control and mastitic buffalos. Furthermore, the protein profiling of the milk whey during mastitis was performed in comparison with normal ones.

Material and methods

Animals and sampling

Blood and milk samples were randomly collected from twenty-five unrelated (according to the farm records) Egyptian buffalos. The animals were raised either at animal production units, in Mahlet Moussa-Kafr-elshiekh or army forces farm at Fayoum district. *Blood sampling was performed in agreement with the international ethical approval for large animal blood sampling.* All animals were nearly within the same average age (4:6 years) and weight (400:550 kg). The blood and milk samples (10 mL for each animals) were collected during mid lactation period. Based on somatic cell count of milk samples for scaling their degrees of mastitis [19] using NucleoCounter[®] SCC-100™ Somatic Cell Counter (Chemometec, Allerod, Denmark); ten animals were served as controls and fifteen animals were confirmed to have mastitis (5 clinical, 5 subclinical and 5 chronically affected buffalos).

The mastitic buffalos were divided into 3 classes according to their somatic cell counts: subclinical (SCC > 211,000/mL), clinical (SCC > 1,500,000/mL) and chronic mastitis which is detected by case history and records. Normal buffalo's SCC is less than 100,000 cells/mL [20].

Molecular analysis

Genomic DNA extraction

Genomic DNA was extracted from the blood of 25 animals using salting out method [21] with slight modifications. 25 mL of cold 2X sucrose lysis buffer and 15 mL deionized water were added to each sample (10 mL EDTA blood). The mixture was incubated on ice for 30 min and mixed by frequent inversion prior to centrifugation at 5000 rpm for 15 min at 4 °C. The supernatant was discarded and the pellet was washed twice with the lysis buffer and deionized

water then suspended in a 3 mL of nucleic lysis buffer. The suspension was mixed with 108 μ L 20% SDS and 100 μ L Proteinase K then overnight incubated at 37 °C. The incubated content was transferred to a 15 mL polypropylene tube and 1 mL of saturated NaCl was added with vigorous shaking for 15 s. The mixture was centrifuged at 3500 rpm for 15 min at 4 °C and the supernatant was collected with its double volume of ice-cold absolute ethanol. The contents were mixed gently by inversion until cotton-like threads of DNA were seen. A heat-sealed Pasteur pipette was used to collect the formed DNA, which was then twice-washed in 70% ethanol. After air-drying, the recovered DNA was dissolved in 200 μ L TE buffer and then incubated at 37 °C for 2 h in a water bath. The concentration of DNA samples was measured using Nanodrop 1000 (Thermo-Scientific, Waltham, USA).

Primers design for IL-8B receptor gene PCR

The two primer pairs (below) were designed using buffalo accession # XM_006046377.1 to flank CXCR2 Exon2. Primers were designed using Primer3 software and their specificity was tested (Oligo Analyzer program version 1.0.3) and manufactured by Eurofins, Luxembourg, Germany.

Primer 1

F: 5'-GGCTAGAATCTGGGGAGGTT-3' R: 5'-GCACGACAGCAAA GATGA-3'

Primer 2

F: 5'-GAGGACATGGGTGCCAATAC-3 R: 5'-ATGGCCTCAG CAACTTC-3'

Polymerase chain reaction and sequencing

The reaction mixture for PCR was prepared by adding 25.5 μ L of nuclease free water, 5 μ L of 10X DreamTaq™ DNA polymerase buffer, 5 μ L (100 μ M) dNTPs, 5 μ L of each primers (20 μ M), 0.5 μ L (5 U/ μ L) of DreamTaq™ DNA polymerase (Fermentas, Waltham, USA) and 4 μ L genomic DNA (50 ng/ μ L) in all the tubes for each primers sets. The reaction mixture was run for 35 cycles in a Q-Cycler, (HVD Lifesciences, Wien, Austria) proceeded by initial denaturation at 95 °C for 5 min followed by 1 min denaturation at 95 °C; 2 min annealing at 66 °C and 67 °C for primers 1 and 2; respectively, and 2 min extension at 72 °C. The run was then terminated in both sets by a final extension at 72 °C for 10 min.

The amplification products were separated by electrophoresis using 1.5% agarose gel at 100 V for 1 h, stained with ethidium bromide (Applichem, Darmstadt, Germany), and photographed using InGenius Gel documentation system (Syngene bioimaging, Cambridge, UK). The target PCR products were purified using MEGA quick-spin™ total fragment DNA purification Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea).

The amplicons of twenty-five buffalo samples were two ways sequenced. The sequencing was performed after Sanger method using reverse and forward primers (Macrogen, Seoul, Republic of Korea).

Sequence and protein analyses

The first primer amplifies 832 bp covering part of intron1 and part of exon2. The second primer amplifies 613 bp covering exon 2 (part of which overlaps with the first segment) and 3' UTR.

The complete CXCR2 sequence was deduced for each buffalo from the resulted amplicons of the two primer pairs. Multiple sequence alignment of buffalo's CXCR2 gene was performed using Clustal Omega program [22]. The polymorphic sites were detected.

The protein sequence was predicted using Open Reading Frame (ORF) (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and the possible SNPs-based amino acids substitutions were evaluated.

Characterization of the protein architecture domains

The protein domains were investigated using Signal P 4.1 software (<http://www.cbs.dtu.dk/services/SignalP/>) to predict the cleavage sites and signal peptide of CXCR2 gene. SMART analysis (<http://smart.embl-heidelberg.de/>) was used to detect the protein domains of genes and the Phobius software (<http://phobius.binf.ku.dk/>) was used to predict the transmembrane topology from the amino acid sequence of a protein.

Biochemical analysis

Protein profiling by SDS-PAGE electrophoresis

Since milk protein content mirrors the actual performance of mammary gland, therefore, milk whey protein profiling affords a direct and easy way to evaluate the mammary homeostasis. To study this hypothesis during buffalo mastitis, the milk whey protein profiling was compared in normal and mastitic buffalos' milk.

Milk samples were skimmed after standing in cold room for 2 h by separating the fat from whole milk by centrifugation (Sorvall, Model RC 2-B, Thermo-Scientific, Waltham, USA) at 3000g for 10 min at 4 °C and protein concentration was estimated [23]. Protein separation by electrophoresis was performed after previously described [24]. Samples were mixed with the sample buffer (1:4) and 5 min denatured at 95 °C. The denatured samples were loaded onto vertical slab gel and subjected to run through 4% stacking gel and 15% separating gel. A wide range protein molecular weight marker (10–245 kDa) was used to determine the molecular weights of separated proteins. Separation was performed in mini-gel (Bio-Rad, California, USA) at 80 V for 4 h. After separation, protein bands were visualized by Coomassie blue staining (50% dH₂O, 40% methanol, 10% glacial acetic acid and 0.1% Coomassie brilliant blue) for 2 h and de-stained (50% dH₂O, 40% methanol and 10% glacial acetic acid) for 40 min and photographed.

Oxidative stress-related biochemical parameters

The oxidative stress parameters in blood and milk were later followed. The LDH [25], catalase [26]; AP [27] activities were measured. The MDA level in serum [28] and the activity of total antioxidant capacity [28] were calculated.

Statistical analysis

Association of SNPs and mastitis

All statistical analyses were performed using R statistical program (<http://www.r-project.org/>) and *P* value was corrected using Bonferroni method [29]. Here, the Fisher's exact test was applied for the analysis of contingency Tables since the sample size is small. Bonferroni correction was then used as an adjustment made to *P* values resulting from the Fischer exact test. In order to apply the Bonferroni correction the number of normal and mastitic samples should be equal. *P* values of less than or equal to 0.05 were considered statistically significant.

Biochemical association

A randomize complete design with one factor was used to analyze all obtained serum data with five replications for each param-

eter. The treatment means were then compared by the least significant difference (L.S.D.) test as given by Snedecor and Cochran [30]. To study any possible correlation among measured parameters, the Pearson simple correlation coefficient for each pair was calculated. The matrix of these correlations was initiated. The statistical significance of correlations was preceded according to [31]. A “*P*” value of ≤ 0.01 was considered to evaluate the statistical results.

Results

In the present study Egyptian buffalo *CXCR2* gene sequence was investigated for the first time using 2 overlapping primers pairs. The deduced sequence was 1246 bp and was submitted to GenBank (accession # KY399457). It included intron 1 (1–46 bp) and the 3' UTR (1137–1246 bp) of buffalo *CXCR2* gene and exon 2 (47–1136 bp). The latter constitute the full coding region of *CXCR2* gene except the first codon present in exon 1.

Sequence analysis of Egyptian buffalo *CXCR2* coding and non-coding region was depicted for nucleotide polymorphic sites (SNPs) in normal and in different groups of mastitic animals. Four nucleotide polymorphic sites were detected (Table 1). Three SNPs were in the coding region; C/A at c.127, C/T at c.546 and C/A at c.562 positions. They were all synonymous with no variations in amino acids. The 4th SNP was detected in 3'UTR at position c.1092 + g.62. The chromatograms and the calculated genotype frequencies of the four investigated groups were presented in Table 1. As seen from the results, the most interesting SNP is C/A c.127. Normal and subclinical buffalo samples were 100% CC homozygous at c.127, whereas in both clinical and chronic samples 32% were CA heterozygous 4% were AA homozygous.

In order to find out the correlation between SNPs and mastitis, we used the Fisher exact test to calculate the *P* value for allele and genotype frequencies followed by correcting the obtained *P* value using Bonferroni correction. It is worth mention that Bonferroni correction analysis uses equal the number of normal and mastitic

samples, thus in the analysis 2 mastitic groups were put together (Tables 2 and 3). The analysis showed that mastitis is highly correlated with SNP c.127, whereas the other SNPs were not significant ($P > 0.05$). Allele A at c.127 was only present in animals with mastitis.

Analysis of the coding region using Smart program revealed the presence of seven overlapping transmembrane receptors from codon 68 to 317. However, using Phobius program, the seven transmembrane detected receptors ranged from codons 55 to 320. They were present between codons 55–77, 89–108, 133–152, 164–186, 221–242, 254–270, and 304–320. Only the c.546 SNP, at codon 182, was located in the 4th transmembrane receptor. This SNP was synonymous leading to the same amino acid.

Protein profiling analysis

The control samples show bands representing lactoferrin, buffalo serum albumin (BSA), glycomacropeptide, and B-lactoglobuline and α -lactalbumine. In mastitic animal, however, there are increase in the bands related to milk proteins as BSA, immunoglobulins, and lactoperoxidase (Fig. 1).

Biochemical analysis

Blood LDH shows significant elevation in subclinical, clinical, and chronic mastitis (5 samples for each group) when compared to the control group of buffalos (1858.8 ± 71.04 , 2265.25 ± 129.77 , and 1848.44 ± 73.086 vs. 1578.5 ± 28.04 U/L, respectively) as in Fig. 2a.

It was observed that clinical mastitic animal in LDH serum more significant than subclinical and chronic when compared to control one ($P \leq 0.01$).

The serum TAC levels (mM) displayed a significant decrease in mastitis especially clinical, chronic and subclinical; respectively (Fig. 2b).

Table 1
Single nucleotide polymorphism and genotypic frequencies detected in *CXCR2* gene of Egyptian buffalo.

SNPs position and genotypes	Genotypic frequencies of control and mastitic animals				Chromatogram
	Control animals	Subclinical animals	Clinical animals	Chronic animals	
c.127 C/A					
CC	100%	100%	64%	64%	
CA	0%	0%	32%	32%	
AA	0%	0%	4%	4%	
c.546 C/T					
CC	83%	100%	64%	36%	
CT	16%	0%	32%	48%	
TT	1%	0%	4%	16%	
c.562 C/A					
CC	69%	16%	4%	16%	
CA	28%	48%	32%	48%	
AA	3%	36%	64%	36%	
c.1092 + g.62					
A/G					
AA	36%	20%	20%	16%	
AG	48%	60%	60%	48%	
GG	16%	20%	20%	36%	

Table 2

Genotypic and allelic association between CXCR2 SNPs and subclinical and clinical mastitis in buffalo.

SNP position	Genotype			Fisher exact test <i>P</i>	Bonferroni correction <i>P</i>	Allele		Fisher exact test <i>P</i>	Bonferroni correction <i>P</i>
	Genotype frequency					Allelic frequency			
c.127C/A	CC	AC	AA	0.00000363	Highly significant	C	A	0.0016	Highly significant
Healthy	100	0	0			1.00	0.00		
Diseased (subclinical and clinical)	83	16	1			0.91	0.09		
c.546C/T	CC	CT	TT	1.0000	1.000	C	T	1.0000	1.000
Healthy	83	16	1			0.91	0.09		
Diseased (subclinical and clinical)	83	16	1	0.91	0.09				
c.562A/C	CC	AC	AA	0.3253	0.487	C	A	0.3769	0.653
Healthy	69	28	3			0.83	0.17		
Diseased (subclinical and clinical)	59	36	5	0.77	0.23				
c.1092+g.62A/G	AA	AG	GG	0.1428	0.214	A	G	0.2007	0.301
Control	36	48	16			0.60	0.40		
Diseased (subclinical and clinical)	25	50	25	0.50	0.50				

Table 3

Genotypic and allelic association between CXCR2 SNPs and chronic and clinical mastitis in buffalo.

SNP position	Genotype			Fisher exact test <i>P</i>	Bonferroni correction <i>P</i>	Allele		Fisher exact test	Bonferroni correction <i>P</i>
	Genotype frequency					Allelic frequency			
c.127C/A	CC	AC	AA	0.00000	Highly significant	C	A	0.00006	Highly significant
Healthy	100	0	0			1.00	0.00		
Diseased (chronic and clinical)	69	28	3	0.83	0.17				
c.546 C/T	CC	CT	TT	0.00000672	0.06	C	T	0.0011	0.09
Healthy	81	18	1			0.90	0.10		
Diseased (chronic and clinical)	50	41	9	0.71	0.29				
c.562 A/C	CC	AC	AA	0.32530650	0.325	C	A	0.3769	0.377
Healthy	69	28	3			0.83	0.17		
Diseased (chronic and clinical)	59	36	5	0.77	0.23				
c.1092+g.62 A/G	AA	AG	GG	0.1428	0.214	A	G	0.2007	0.301
Control	36	48	16			0.60	0.40		
Diseased (chronic and clinical)	25	50	25	0.50	0.50				

Both of control and subclinical mastitic groups were significant when compared to chronic and clinical group, respectively ($P \leq 0.01$).

The results recorded a significant increase in serum MDA level (nmol/mL) during mastitis. The dramatic order of increase was in clinical case then chronic and subclinical groups (Fig. 2c).

The clinical mastitis group showed higher fluctuation away the control group regarding serum MDA than subclinical and chronic groups ($P \leq 0.01$).

Catalase activity shows significant decrease in subclinical, chronic and clinical mastitis in buffalos (Fig. 2d). In mastitis infection, serum catalase is less significant than healthy one. It was found that clinical and chronic groups are considered to be more significant than subclinical one ($P \leq 0.01$).

The serum AP levels, however, displayed a significant increase in mastitis especially subclinical, chronic and clinical respectively (Fig. 2e). Unlike the previous parameters, serum AP is the most significant in all cases of mastitis infection (subclinical, clinical and chronic) when compared to control one ($P \leq 0.01$).

It was observed that the level of MDA and the activities of LDH, ALP, and catalase were significantly higher in mastitic milk than in normal milk ($P \leq 0.01$), while, the activity of TAC was significantly lower in mastitic milk than in normal milk ($P \leq 0.01$) as mentioned in Figs. 3a–3e.

The correlations between the levels of the measured oxidative stress parameters are presented in Table 4 for serum and Table 5 for milk.

Discussion

Mastitis is a major source of economic loss in dairy buffalos. The genetic makeup could play a role in the development of mastitis in buffalos. The milk protein profiling and biochemical parameters related to oxidative stress in blood and milk mirror the degree of mastitis. This is the first work investigating the genetic polymorphism of the interleukin receptor and its potential correlation to mastitis based on biochemical parameters in Egyptian buffalos.

The molecular investigation in this study covering CXCR2 gene full coding region except for the first codon (present in Exon1) revealed that only one SNP C/A at c.127 is associated with mastitis. The presence of allele A only in mastitic animals is of significance. This calls for analysis of large numbers of samples to confirm this finding. Controversial results on CXCR2 gene association with mastitis have been reported. A significant association between CXCR2 SNP (C/G) + 777 and percentages of cases with subclinical mastitis has been reported in cattle [10]. However, non-significant association was previously reported in cattle by Shivanand et al. [32] and in buffalo by Wani et al. [33]. The presence of a SNP c.546 in the 4th transmembrane receptor could be of significant value. A Transmembrane polymorphism of Fcγ receptor IIb was reported to be associated with kidney deficiency syndrome in rheumatoid arthritis [34].

Measuring the activities of different milk enzymes, on the other hand, has diagnostic value as a basic biomarker for discrimination between normal, subclinical and clinical mastitis.

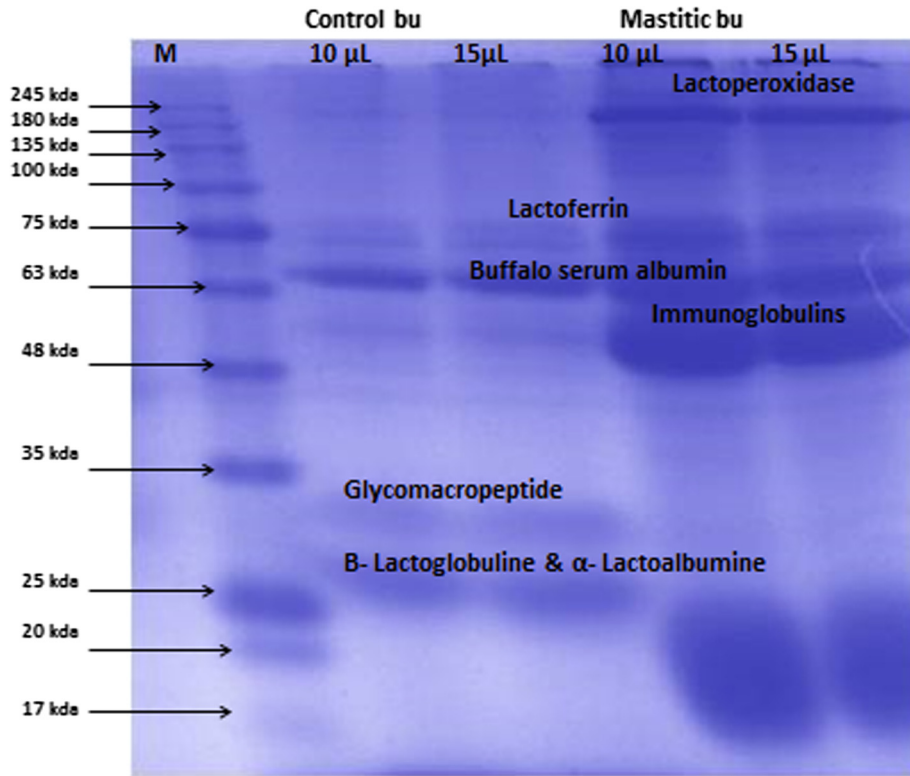


Fig. 1. The protein separation pattern of milk whey using SDS PAGE electrophoresis in normal during mastitis. Lanes 1 is whey milk from control group (10 µL) loading volume. Lane 2 is whey milk from control group (15 µL). Lane 3 is whey milk from mastitic group (10 µL). Lane 4 is whey milk from mastitic group (15 µL). The concentration of loaded protein was 1 µg/µL. Lane M is a wide range protein molecular weight marker.

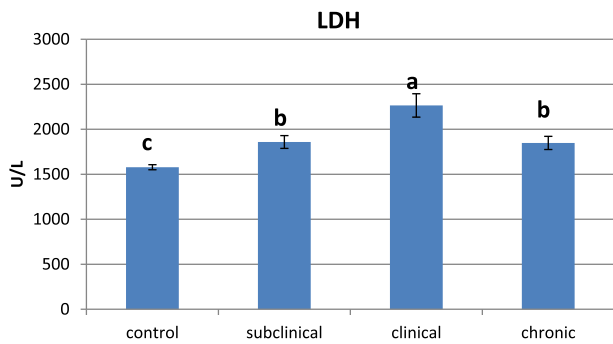


Fig. 2a. Serum activities of lactate dehydrogenase enzyme (LDH) in normal and mastitis samples.

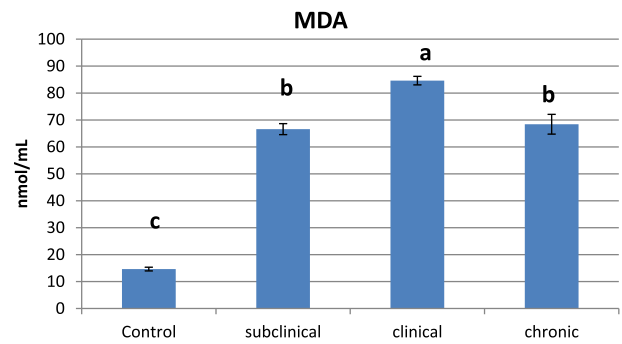


Fig. 2c. The serum malondialdehyde (MDA) levels in normal and mastitis samples.

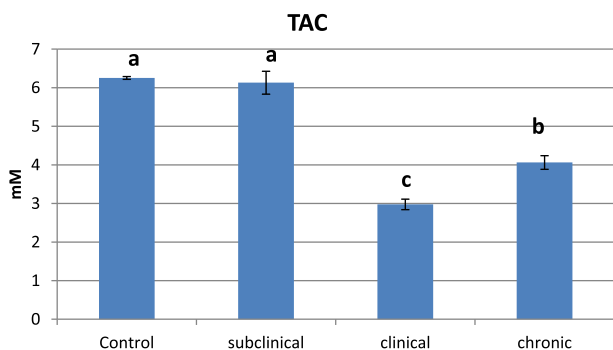


Fig. 2b. Determination of total antioxidant capacity (TAC) in serum in normal and mastitis samples.

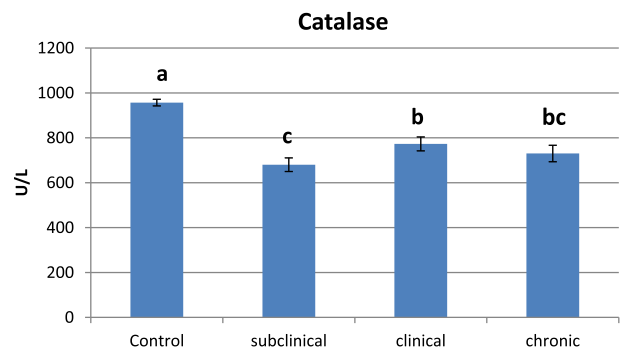


Fig. 2d. Determination of catalase enzyme activity in serum in normal and mastitis samples showing significant elevation in its activity in control samples when compared to other groups.

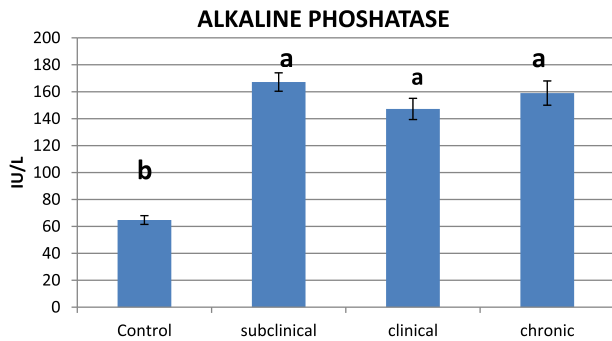


Fig. 2e. Serum activities of alkaline phosphatase enzyme (AP) in normal and mastitis samples showing significant decrease in its activity in control samples when compared to other groups. The columns with the different letter are significantly differed at $P \leq 0.01$. Two columns with the same letter are not significantly differed at $P \leq 0.01$.

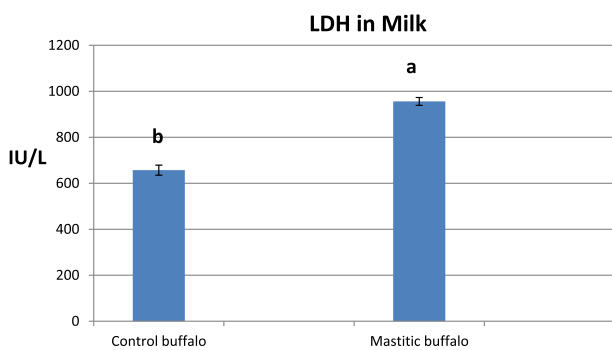


Fig. 3a. LDH activities in control milk and during mastitis show a significant increase in milk LDH activity during mastitis.

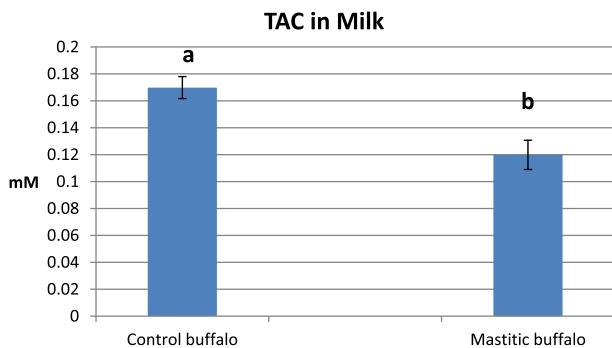


Fig. 3b. Determination of TAC in normal milk samples and during mastitis with significant elevation in its value in control milk samples.

Inflammation of mammary gland can affect the milk composition in several ways. Because of the increased permeability of blood-milk barrier, the serum proteins can leak to the milk. Also the damaged epithelial cells make intracellular components release into milk and finally synthesis of milk-specific components produced in the mammary epithelium is reduced. Intra-mammary infection can increase its micro-vascular permeability through secretion of the chemical mediators such as histamine, prostaglandins, and oxygen free radicals from inflammatory cells. This can explain the recognized increase in soluble protein reported by SDS PAGE protein foot printing.

The results of the present study show that the average LDH activities in milk from buffalos affected by mastitis (956.01 ± 17.02 U/L) were significantly higher than those from

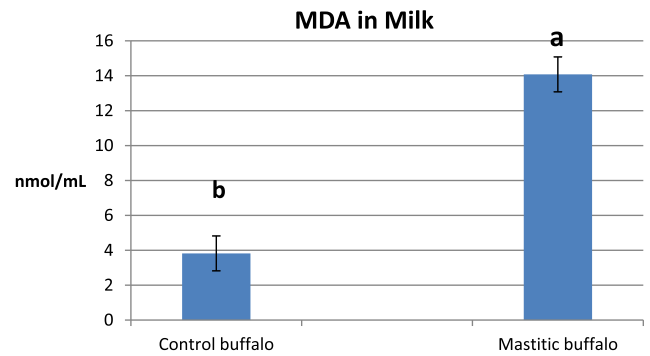


Fig. 3c. The levels of MDA in milk in normal samples and during mastitis with observed significant elevation in its level during mastitis.

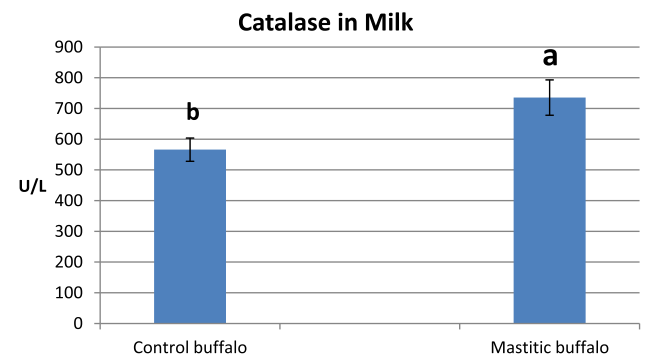


Fig. 3d. Determination of catalase activities in normal milk samples and during mastitis showing significant elevation in its activity during mastitis.

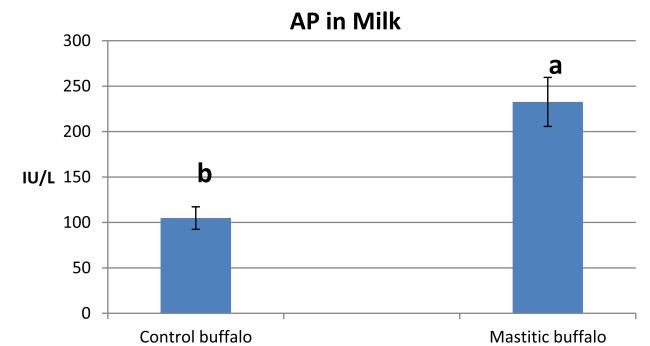


Fig. 3e. The alkaline phosphatase activities in normal milk samples and during mastitis with doubling of its activity during mastitis. The columns with the different letter are significantly differed at $P \leq 0.01$. Two columns with the same letter are not significantly differed at $P \leq 0.01$.

healthy ones (657.2 ± 21.84 U/L). In addition, it is proven that mean AP activities in buffalo's milk during mastitis (232.6 ± 26.9 IU/L) were also higher than those from healthy ones (104.84 ± 12.37 IU/L). Our finding is consistent with the previous studies on cattle mastitis [13,35].

Several early works evaluated the milk LDH and AP activities as diagnostic tools for udder infection in cattle breeds. The fluctuation in their activities served as a sensitive marker for inflammatory changes of mammary glands during subclinical mastitis [13]. In addition, it was postulated in several studies that the activity of alkaline phosphatase enzyme in mastitic milk increases significantly over its level in normal milk [14].

Our investigation reported a significant elevation in the blood MDA levels during sub-clinical (66.6 ± 2.04 nm/mL), clinical

Table 4
Correlation between oxidative stress parameters in serum.

	MDA in serum	TAC in serum	Catalase in serum	LDH in serum	ALP in serum
MDA in serum	1.00				
TAC in serum	-0.68**	1.00			
Catalase in serum	-0.58*	0.16	1.00		
LDH in serum	0.68***	-0.74***	-0.30	1.00	
ALP in serum	0.72***	-0.24	-0.71***	0.50*	1.00

* Significant at $P \leq 0.05$ level of probability.

** Significant at $P \leq 0.01$ level of probability.

*** Significant at $P \leq 0.001$ level of probability.

Table 5
The oxidative stress parameters correlations in milk.

	MDA in Milk	TAC in Milk	Catalase in Milk	LDH in Milk	ALP in Milk
MDA in Milk	1.00				
TAC in Milk	-0.81**	1.00			
Catalase in Milk	0.96***	-0.85**	1.00		
LDH in Milk	0.98***	-0.84**	0.99***	1.00	
ALP in Milk	0.99***	-0.86**	0.98***	0.99***	1.00

** Significant at $P \leq 0.01$ level of probability.

*** Significant at $P \leq 0.001$ level of probability.

(84.6 ± 1.58 nm/mL) and chronic mastitis (68.4 ± 3.6 nm/mL) when compared with its level in healthy controls (14.6 ± 0.69 nm/mL). Further, milk samples analysis revealed appreciable increase of MDA levels in mastitis cases (14.07 ± 1.59 nm/mL) in comparison with healthy ones (3.82 ± 0.404 nm/mL). The findings revealed a 4-fold elevation in MDA levels in milk of sub-clinically, clinically and chronically mastitic buffalos. This reported higher MDA levels in milk during mastitis referred to the increase in the auto-oxidative activity in milk during mastitis. This fact affords a new reason for the poor quality of this type of milk, which also suffers from a relatively high somatic cell count [36]. In fact, malondialdehyde –the main defined product of accelerated lipid peroxidation– is known to be a mutagen and a suspected carcinogen reacting with DNA to generate mutations [35]. Moreover, the reported significant increase of lipid peroxidation in blood and milk MDA levels might reflect the uncompromised oxidative damage in buffalos during sub-clinical, clinical and chronic mastitis [37].

The mechanisms by which inflammation causes damage to mammary gland tissue during mastitis are still not fully understood. It is well known that inflammatory reactions, in which vascular permeability increases and leukocyte migration occurs, involve several mediators including neutrophil-derived proteinases and free radicals, such as superoxide, hydrogen peroxide and hydroxyl radical [38].

Neutrophil-induced mammary cell damage and LDH release were scavenged by catalase enzyme. Therefore, the catalase activity is probably the best known and most widely used enzymatic test for detecting mastitis in milk samples. Observations suggested that morphological changes might be induced by hydrogen peroxide and its derived oxidants since the addition of catalase increased cell survival in activated neutrophil-induced cell damage model [38].

The findings revealed that there was an elevation of catalase enzyme levels in milk of mastitic buffalos (735.4 ± 57.43 U/L) when compared with those of healthy controls (565.9 ± 37.87 U/L), which agrees with the previous work of Fox and Kelly [39].

In contrast, the measurement of catalase activity in serum showed a decline of its level during subclinical (680.38 ± 30.2 U/L), clinical (772.95 ± 30.95 U/L) and chronic (730.33 ± 36.77 U/L) mastitis in buffalos when compared with those of healthy ones (957.08 ± 14.8 U/L).

The TAC was proved to be lower in milk from affected mammary glands with mastitis (0.11 ± 0.010 mM) when compared to normal mammary gland-voided milk (0.16 ± 0.008 mM). Concomitantly, the TAC in serum records lower levels in subclinical (6.13 ± 0.29 mM), clinical (2.9 ± 0.13 mM) and chronic (4.06 ± 0.17 mM) mastitis compared to the normal serum (6.25 ± 0.03 mM). These results could imply that mastitis alters the antioxidant homeostasis leading to a decrease in antioxidant levels of milk. Therefore, any alterations in TAC in milk could be used to monitor the degree of mastitis [40].

There was a positive correlation between serum MDA level and LDH ($P \leq 0.01$) and AP ($P \leq 0.01$). Conversely, the serum MDA was not correlated with TAC level ($P \leq 0.01$) and catalase activity ($P \leq 0.01$). In addition, serum TAC has a positive correlation with catalase activity. Similarly, LDH correlated positively with AP ($P \leq 0.05$). Our study also found that the MDA level in milk is positively correlated to both LDH and AP activities. This is consistent with the previous finding [35]. On the contrary, the catalase activity in milk has significantly strong positive correlation with MDA ($P \leq 0.01$) unlike in serum, in addition to the negative correlation with TAC ($P \leq 0.01$).

Since mastitis remains one of the most important diseases of dairy cattle in the world [41], milk protein can be a useful marker for monitoring its progression in dairy animals [42]. As proved by protein foot printing in our results, it is generally accepted that during mastitis, there is an increased leak of cellular proteins into milk. This is attributed to the influx of blood-borne proteins (possibly serum albumin, immunoglobulins, and the minor serum proteins, transferrin, α -macroglobulin) into the voided milk. This increase in proteins of blood serum origin during mastitis is possibly due to a disruption to the integrity of the mammary epithelia by microbial toxins and opening of the tight junctions.

The broadening of protein bands at 65 and 75 kda might explain the possible increase in their corresponding soluble proteins e.g. serum albumin or lactoferrin proteins during mastitis when compared with healthy animal-derived whey. Although these speculations need further immune-blot assessment, our finding is consistent with the previously reported results regarding lactoferrin [43]. In addition, there is a potential increase in immunoglobulin level at the range of 60 kda and peroxidase at 200 kda in mastitic whey.

Conclusions

This is the first research to screen the *IL-8B* receptor gene in Egyptian buffalos. The results reveal a significant association between the SNP C/A c.127 in *CXCR2* and the incidence of mastitis in Egyptian buffalo. In addition to the blood and milk biochemical parameters that indicate an increased oxidative stress during mastitis, there is a dramatic change in protein profiling in the whey of the affected milk. This novel approach warrants the remote clinical relevance of the genetic makeup of buffalo as a putative element in selection of mastitis-resistant breed in this economically recognized animal.

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Conflict of interest

The authors have declared no conflict of interest.

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