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Individual genomic loci, transcript level, and biochemical profile of immune and antioxidant markers associated with genetically identified bacterial mastitis in Shami goats in Egypt

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ABSTRACT

Background: Mastitis in goats is unquestionably a grave concern, with far-reaching implications for both animal well-being and productivity, while also presenting a potential threat to public health.

Aim: The study aimed to compare culture methods and multiplex PCR (m-PCR) in the detection of the most three common mastitis-causing pathogens (*Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus* spp.) and investigate the gene expression, single nucleotide polymorphisms (SNPs), serum concentrations of immunological and antioxidant indicators linked to mastitis in Shami goats.

Methods: One hundred Shami do (50 Shami goats with clinical mastitis and 50 normal goats taken as control group). The culture methods and m-PCR analysis were used to find the bacteria in the milk samples. Blood samples were obtained to assess some hemato-biochemical parameters, detect SNPs, and determine the expression of certain immunological and antioxidant indicators in the genes.

Results: The culture method detected the pathogens causing mastitis in 90% of the milk samples, but m-PCR detected them in 100% of the milk samples. SNPs linked to mastitis resistance/susceptibility in examined does were detected through DNA sequencing of immunological and antioxidant indicators. The magnitude of gene expression varied significantly between the resistant and mastitis-affected groups. Significant ($P < 0.05$) elevations were noticed in WBCs count, mainly neutrophils count, serum levels of BHB, NEFA, triglycerides, LDL-C, AST, ALT, ALP, creatinine, total protein, globulin, Ca, K, GPx, MDA, acute phase proteins, and cytokines in mastitis affected does as compared to control. While RBCs count, PCV%, lymphocytes count, serum concentration of glucose, cholesterol, HDL-C, albumin, Na, Cl, P, GSH, SOD, and catalase significantly ($P < 0.05$) diminished in mastitis affected does compared to healthy ones. APPs and pro-inflammatory cytokines scored high sensitivities and NPVs but TNF- α and serum amyloid A (SAA) had the highest percentages of increase.

Conclusion: The study confirmed that m-PCR is the most sensitive method for bacteria identification (*S. aureus*, *E. coli*, and *Strept. spp.*) while SNPs in antioxidant and immunological genes may be important genetic indicators for mastitis risk or resistance in Shami does. To establish an effective management plan and forecast the most sensitive risk time for illness onset, gene expression profiles of the tested genes may also be employed as proxy biomarkers. TNF- α and SAA may be precious indicators for the detection of caprine mastitis.

Keywords: Shami does, Single nucleotide polymorphisms, Antioxidants, m-PCR, Mastitis.

Introduction

It is impossible to overstate the profitable significance of goats, particularly in underdeveloped nations. Worldwide, goats are an extremely useful source of meat, milk, fur, and skin (Wilson, 1987). Most developing nations' livestock economies and cultures are heavily reliant on goats. The most economically significant part of animal production is goats. They

endure harsh environmental conditions in semi-arid and desert regions. According to Joint (2011), Egypt has a total of 4.2 million goats. The Damascus or Shami goat is well regarded by farmers in the eastern Mediterranean region for its outstanding reproductive and productivity performance (Khazaal, 2009). The capacity of Damascus goats to adapt to high temperatures and their appropriateness for breeding

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in lowlands give them an advantage over sheep in terms of their ability to reproduce while benefiting from arid pastures (Barıtcı and Adıgüzel, 2017). Damascus goats are used extensively to increase the production of other breeds since they are regarded as a dual-purpose animal for the production of meat and milk with a high genetic level (Mavrogenis *et al.*, 2006).

Small ruminants frequently develop mastitis, which is characterized by alterations in the physical, chemical, and microbial characteristics of the milk as well as pathological lesions in the glandular tissue (Mishra *et al.*, 2018). Constructed on etiopathological findings and inspections, mastitis is typically categorized as subclinical, acute, subacute, chronic, and gangrenous (Narenji Sani *et al.*, 2015). Teat injuries, poor management and cleanliness, and malfunctioning milking equipment are examples of predisposing factors that have been shown to speed up the spread of infectious organisms and the progression of disease (Zenebe, 2014). The key factors relating to the economic losses of mastitis are milk production decline, deterioration in product quality, and compromised hygiene standards rendering the final product unsuitable for consumption (Mota, 2008).

In addition, *Staphylococcus species* have emerged as the leading culprits behind subclinical or clinical mastitis in numerous countries (Schukken *et al.*, 2009). Important *Streptococcus species* for bovine mastitis include *Strept. agalactiae*, *Strept. dysgalactiae*, and *Strept. uberis* (Dmitriev *et al.*, 2006). According to Mohamed (2013), they are to blame for over 80% of all mastitis diagnosed in Egypt. It is challenging to distinguish between the many species within this vast group of pathogens (Koskinen *et al.*, 2009). Furthermore, the absence of particular biochemical markers for species identification means that the pathogens causing mastitis cannot be reliably identified simply on phenotypic alterations (Capurro *et al.*, 2009). DNA sequence-based molecular techniques have shown to be an effective means of species identification, rapid, accurate, and trustworthy identification techniques are still needed (Shome *et al.*, 2011). Blood biochemical investigations give much information about animal's nutritional status, health, and wellbeing; therefore, they can be used to assess the health of animals in general (Faraz, 2019).

Based on our present comprehension, the information on immunological, antioxidant states, SNPs, and gene expression alterations associated with caprine mastitis is scarce. In addition, the absence of accurate diagnostic and predictive tools for these conditions hinders our ability to enhance preventative and therapeutic strategies for these disorders. The current aimed to investigate SNPs, the gene expression profile of immune and antioxidant bio-markers, besides identifying the most frequent pathogens causing mastitis in goats using multiplex PCR (m-PCR).

Materials and Methods

Animals

Fifty Shami goats with clinical mastitis and 50 normal goats (the control group), 3–8 years (5.1 ± 1.7) and 27.5–45 kg (33.7 ± 5.8), were raised at Mariut Research Station, El-Amria, Alexandria, Desert Research Center (DRC), Egypt. Shami does do ranged in lactation from the first to the seventh. Water was accessible to the does at all times, and they were kept in semi-open shaded pens with 750 g of concentrate feed mixture (CFM) + 750 g of alfalfa hay/head/day for feeding. Table 1 displays the ingredients of the basal diet. When available, the natural pasture which consisted of grass, berseem, darawa, and green herbage was fed. The investigated were put through clinical examination in accordance with the pre-established standard protocols (Pugh, 2012), and the results were documented at the same time. Clinical mastitis was identified based on physical examination of the glands through inspection and palpation (Jackson and Cockcroft, 2002) and evaluation of produced milk for aberrant color and consistency. The processes were carried out, from September 2022 to June 2023.

Sampling

Milk sample

According to Hogan *et al.* (1999), teats were prepared aseptically before samples were taken manually. 70% ethanol was applied to the mammary teats after cleaning and drying them, and first milk streams were discarded. Then, milk samples (10–15 ml) from both mammary halves of each doe were collected in sterile tubes, labeled, and straight to the laboratory for California mastitis test (CMT) and somatic cell count (SCC) screening.

Using a Bentley Soma Count 150 (Bentley Instrument, Chaska, MN, USA), somatic cells were counted electronically. The CMT supplied by Taba Medical Pharma Company®, was performed using 3–5 ml of goat milk and an equivalent volume of CMT reagent, and they were quickly combined by swirling or rotating. The amount of gel that developed and the degree of color change were used to rate the response (Gebrewahid *et al.*, 2012). The CMT core was given a value of 0, +1, +2, or +3 depending on the reaction's

Table 1. Composition of the concentrate feed mixture (CFM).

Ingredients	Quantity
Corn	530 kg
Wheat bran	240 kg
Soya bean	230 kg
Sodium chloride	5 kg
Calcium carbonate	10 kg
Premix	1 kg
Netro-Nill	0.5 kg
Fylax	0.5 kg

severity, and milk samples with CMT scores of 0 and 1 and SCC levels under 200,000/ml were deemed healthy ($N = 50$), whereas milk samples with CMT scores of 2 or 3 and SCC levels above 200,000/ml were deemed to have clinical mastitis ($N = 50$) (Gebrewahid *et al.*, 2012).

Blood sample

Jugular venipuncture was used to get ten ml of blood from each animal. The drawn blood was added to EDTA-containing tubes, heparin-containing tubes, and plain tubes (i.e., anticoagulant-free) to obtain whole blood, heparinized plasma and serum, respectively. All samples were chilled using crushed ice before being brought right away to the laboratory for additional processing. Whole blood tubes were used for both the PCR assay and complete blood count (CBC).

Microbiological analysis of milk specimens

Samples of milk with a CMT score of +2 or +3 and SCC greater than 200,000/ml were used for the bacteriological test. 10 ml of milk were dispersed using the quadrant streaking method on a 5% sheep blood agar plate. The medium was then examined for typical bacterial colonies after being incubated at 37°C for 24–48 hours. Following their dissemination on brain heart infusion agar, pure colonies on blood agar were incubated for 24 to 48 hours at 37°C in an aerobic environment (Mbindyo *et al.*, 2014). At this stage, the Gram reaction, culture, and shape were used to identify the bacterium (Ewing, 1986). *Staphylococcus aureus* was located using selective media such as Mannitol salt agar and Baird–Parker agar. As representative colonies, black gram-positive cocci colonies with a clear zone surrounding them were chosen and subjected to a catalase test (Staphylex, Oxoid). *Staphylococcus aureus* was discovered in colonies that lacked catalase but had hemolysis.

To isolate *Streptococcus*, all isolate clones that seemed to be gram-positive were dispersed on sheep blood agar slants with 1% esculin (Oxoid, UK). Catalase-negative

Streptococci isolates were then cultured on kanamycin-esculin-azide agar (Oxoid) after drops of hydrogen peroxide were applied to already-growing cultures.

Gram-negative bacilli colonies were then distributed on MacConkey agar plates for isolating *Escherichia coli*, where the pink color of the bacterial colonies indicates that *E. coli* is growing. The most probable number (MPN) method, which uses three tubes, was used to confirm the *E. coli* isolates. The positive MPN tube was disseminated on Eosin methylene blue (EMB) agar and allowed to develop overnight at 35°C. A green metallic sheen dye is produced around the bacterial growth in 24–36 hours.

Bacterial identification

Staphylococcus aureus, *E. coli*, and *Strept. spp.* standard bacterial reference strains ATCC 25922, 25922, and 15913 were received for comparison from the veterinary quality control on poultry production, Animal Health Research Institute, Cairo, Egypt.

The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to extract DNA from milk samples, with certain modifications made in accordance with the manufacturer's instructions. 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Elution buffer (100 µl) was used to elute the nucleic acid.

The primers utilized were given by Metabion (Germany) and are listed in Table 2. Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 5.5 µl of water, and 5 µl of DNA template. An applied biosystem 2,720 heat cycler was used to carry out the process. On a 1.5% agarose gel (Appllichem, Germany, GmbH) in 1× TBE buffer at room temperature, the PCR products were separated

Table 2. Primers sequences, target genes, amplicon sizes, and cycling conditions.

Target	Primers sequences 5'-3'	Amplified product (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
<i>S. aureus</i> 23S rRNA	ACGGAGTTACAAAGGA	1250						Bhati <i>et al.</i> (2016)
	CGAC							
	AGCTCAGCCTTAACG AGTAC							
<i>E. coli</i> phoA	CGATTCTGGAAATGGCA	720	95°C 5 minutes	94°C 30 seconds	55°C 40 seconds	72°C 1 minutes	72°C 10 minutes.	Hu <i>et al.</i> (2011)
	AAAG							
	CGTGATCAGCGGTGACT ATGAC							
<i>Streptococcus</i> spp. 16S rRNA	CGGGGGATAACTATTGGAA	912						Osakabe <i>et al.</i> (2006)
	ACGATA							
	ACCTGTCACCCGATGTACC GAAGTA							

Table 3. Forward and reverse primer sequence, length of PCR product, and annealing temperature for immune and antioxidant genes used in PCR-DNA sequencing.

Primer	Forward	Reverse	Annealing temperature (°C)	Length of PCR product (bp)
<i>CD-14</i>	5'-GTGTGCTTGCCCTGCCTGCTGC -3'	5'-AGTGTGCTTGGGCAATGTTTCAGC-3'	64	538
<i>CCL2</i>	5'-GCCAGTCCAGAGGCCAACAGCTC -3'	5'-AGTCACGGAAGATAAACTGAA-3'	62	534
<i>β defensin</i>	5'-CAGCATGAGGCTCCATCACCTG-3'	5'-CGAAGGCGGCAGTTTCTGACT-3'	62	253
<i>SPP1</i>	5'-ATGAGAATTGCAGTGATTTGCT-3'	5'-CATTCACCAACTAAGCTGAGAA-3'	60	943
<i>BPI</i>	5'-ATGGCCAGCGCCCCGGCAGT-3'	5'-AGGTTGCAGCTTGGAGGACACA-3'	62	645
<i>A2M</i>	5'-AACCGCAATACATGGTGCTGGT-3'	5'-GGTTTGTCTGTCTGGACAAAAGAC -3'	58	325
<i>ATP1A1</i>	5'-ACAAACAAAAGGGTCACAACATG -3'	5'-CTTACCCTAGATCCTGGTCTCAT-3'	60	300
<i>TLR7</i>	5'-ATCTTCTCCCAGCTTACAGCTG-3'	5'-AGAATTATTTTCACAGGGTGTG -3'	60	398
<i>TLR8</i>	5'-GCTTCTGACCTCCCTTTTCTGCTG-3'	5'-GTGTATCTGAATTGAAGCACCT-3'	62	799
<i>SOD1</i>	5'-GTCTGCGTGCTGAAGGGTGACG-3'	5'-CAATTCCAATTACACCACAGGC-3'	60	436
<i>CAT</i>	5'-GAGAGGCAGTTTATTGCAAATC-3'	5'-ATGCTGCACATAGGTGTGAACTG-3'	64	866
<i>Ahp/TSA</i>	5'-CATGTGTATATAGACAGAAATC-3'	5'-AATGTCTTCAATGTCTTAGAAT-3'	58	636
<i>PRDX2</i>	5'-GGCCCTGAGAAATCGAGTCCGT-3'	5'-AGGCAGGCCAAGAGCCCAGGCT-3'	60	706
<i>PRDX4</i>	5'-GCTTGGGCTCGAGCGGGCGTTGA-3'	5'-CTGAATTCGTCAATTCTATCA-3'	60	527
<i>NQO1</i>	5'-AGCACTGGTGACGCCTTCTAG-3'	5'-GCCAGAGAATGATGTTTCATGT-3'	62	635
<i>Nrf2</i>	5'-GAAATGTCAAACAGAAGGGTC-3'	5'-AAGTGATCTAAATCTTGCTCCA-3'	62	442

-CD14= cluster of differentiation 14; CCL2= C–C motif ligand 2; SPP1= Secreted Phosphoprotein 1; BPI= Bactericidal permeability increasing protein; A2M= Alpha-2-Macroglobulin; ATP1A1= ATPase Na⁺/K⁺ Transporting Subunit Alpha 1; TLR7= Toll-like receptor 7; TLR8= Toll-like receptor 8; β defensin= beta defensin; CCL2= Chemokine (C–C motif) ligand 2; SOD1= Superoxide dismutase 1; CAT; Catalase; AhpC/TSA—alkyl hydroperoxide reductase/thiol-specific antioxidant; PRDX2= Peroxiredoxin 2; PRDX4= Peroxiredoxin 4; NQO1= NAD (P) H Quinone Dehydrogenase 1; and Nrf2= nuclear factor erythroid 2–related factor 2.

using 5 V/cm gradient electrophoresis. Each gel slot had 20 µl of the m-PCR products put into it for gel analysis. The sizes of the fragments were measured using a gelpilot 100 bp plus ladder (Qiagen, Gmbh, Germany), a generuler 100 bp ladder (Fermentas, Germany), a 100 bp DNA ladder H3 RTU (Genedirex), and a generuler 100 bp ladder. Computer software was used to analyze the data after the gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

PCR-DNA sequencing of immune and antioxidant genes

Utilizing the Gene JET whole blood genomic DNA extraction kit and adhering to the guidelines provided by the company, the genomic DNA was extracted from whole blood (Thermo Scientific, Lithuania). DNA that was high quality, pure, and concentrated was tested by Nanodrop.

The amplification of coding elements was done via PCR for immune (*CD-14*, *CCL2*, *β defensin*, *SPP1*, *BPI*, *A2M*, *ATP1A1*, *TLR7*, and *TLR8*) and antioxidant (*SOD1*, *CAT*, *AhpC/TSA*, *PRDX2*, *PRDX4*, *NQO1*, and *Nrf2*) genes. The sequence of *Capra hircus* that was published in PubMed was employed in the primer

sequence design. Table 3 presents the primers utilized for the amplification. A 100 µl final volume of the polymerase chain reaction mixture was produced in a thermal cycler. 5 µl DNA, 43 µl H₂O (d.d water), 50 µl PCR master mix (Jena Bioscience, Germany), and 1 µl of each primer were included in each reaction volume. For six minutes, the reaction mixture was exposed to the starting denaturation temperature of 95°C. The cycling was done in 35 cycles: 45 seconds at 95°C for denaturation, 1 minute for annealing temperatures according to Table 3, 45 seconds at 72°C for extension, and 10 minutes at 72°C for the last extension. The exemplary PCR analysis findings were obtained using agarose gel electrophoresis, and the samples were kept at 4°C for storage. The fragment patterns were then observed using an ultraviolet gel documenting technique.

DNA sequencing was preceded by the removal of primer dimmers, nonspecific bands, and other impurities. To purify PCR products with the anticipated size (target bands), a Jena Bioscience #pp-201×s/Germany PCR purification kit was utilized, as stated by (Boom *et al.*, 1990). The manufacturer’s instructions were followed.

Using a Nanodrop (Uv-Vis spectrophotometer Q5000/USA), the PCR product was quantified to guarantee sufficient quantities and purity as well as a high product yield (Boesenberg-Smith *et al.*, 2012). Using an enzymatic chain terminator technique developed by Sanger *et al.* (1977), PCR products with the target band were sent for forward and reverse DNA sequencing using an ABI 3730XL DNA sequencer (Applied Biosystem, USA) to detect SNPs in the genes investigated in the control and mastitis affected does.

Utilizing the programs Blast 2.0 and Chromas 1.45, DNA sequencing data analysis was done (Altschul *et al.*, 1990). Genes under investigation were subjected to PCR product comparison with reference sequences from GenBank, and these differences were categorized as single-nucleotide polymorphisms (SNPs). Tamura *et al.* (2007) evaluated the genes between enrolled does using the MEGA6 software program to do an amino acid sequence variation analysis based on data alignment of DNA sequencing.

Quantitative real time PCR for immune and antioxidant genes

Trizol reagent was used to extract total RNA from doe blood in accordance with the manufacturer's instructions (RNeasy Mini Ki, Catalogue no. 74104). Using a NanoDrop® ND-1000 Spectrophotometer, the amount of isolated RNA was measured and certified. The manufacturing protocol (Thermo Fisher, Catalogue no. EP0441) was followed in the synthesis of the cDNA for every sample. Quantitative RT-PCR was used with SYBR Green PCR Master Mix (2× SensiFast™ SYBR, Bioline, CAT No: Bio-98002) to evaluate the gene expression pattern of genes encoding immunity and antioxidants. SYBR Green PCR Master Mix (Quantitect SYBR green PCR kit, Catalogue no. 204141) was used to perform real-time PCR for the relative measurement of the mRNA level.

The *Capra hircus* published sequence in PubMed was followed in the creation of primer sequences, as indicated in (Table 4). For normalization, the housekeeping gene β . *actin* was employed as a constitutive control. In all, 3 μ l of total RNA, 4 μ l of 5× Trans Amp buffer, 0.25 μ l reverse transcriptase, 0.5 μ l of each primer, 12.5 μ l 2× Quantitect SYBR green PCR master mix, and 8.25 μ l RNase free water made up the 25 μ l reaction mixture. Using a thermal cycler, the final reaction mixture was subjected to the following protocol: reverse transcription for 30 minutes at 50°C, initial denaturation for 10 minutes at 94°C, 40 cycles of 94°C for 15 s, annealing temperatures as indicated in Table 4, and 72°C for 30 s. A melting curve analysis was carried out at the conclusion of the amplification stage to verify the PCR products specificity. Using the $2^{-\Delta\Delta C_t}$ technique, it was established how each gene's relative expression in each sample related to the β . *actin* gene (Pfaffl, 2001).

Hematological and biochemical parameters

Serum biochemical tests were performed using commercial test kits in accordance with the providers' established protocols, and hematological parameters (RBCs, Hb, PCV, MCV, MCH, MCHC, WBCs, neutrophils, lymphocytes, monocytes, and eosinophils counts) was manually calculated according to Feldman *et al.* (2000). GSH, GPx, MDA, SOD, and CAT were detected (Biodiagnostic Egypt, CAT No: MD2529, CA252417, GSH2511, SD 25 21, and GP 2524). Serum haptoglobin (Hp) concentrations were determined by Eagle Biosciences (Columbia) ELISA kits, serum amyloid A (SAA), and plasma fibrinogen (Fb) concentrations were determined by using IBL International Crop (Canada)® ELISA kits. Pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) levels were determined in serum by using BOSTER BIOLOGICAL TECHNOLOGY PicoKine™ ELISA kits (CAT No: EK0412, EK0393, and EK0526, respectively).

Statistical analysis

SPSS (version 20, Inc., Chicago, USA) was used to perform the statistical analyses. All parameters were given descriptive statistics, and the means of the two groups were compared by using the Student's *t*-test. When $p < 0.05$, the results were considered significant. ROC curve analysis for the measured APPs and pro-inflammatory cytokines was done using the Graph Pad Prism 8 program in mastitic goats compared to normal ones.

Ethical approval

The processes were carried out in compliance with the regulations set forth by the Ministry of Agriculture and Land Reclamation in Egypt's Animal and Poultry Health Department, Desert Research Centre, and Ethical Committees.

Results

Clinical findings

Clinically, none of the healthy Shami producing the typical, regular milk had any mammary anomalies. Does with mastitis had swollen, hot, hard, and extremely painful udders. Milk production was also drastically reduced, and the milk's appearance was dense and yellow. The breathing and body heat of this group were increased.

Prevalence of clinical mastitis

Out of 100 goat milk samples analyzed, the results showed that 50 of them tested positive for California mastitis (50%). From 50 samples, 45 had bacteriologically proven bacterial contamination with *S. aureus* ($n = 21$) (42%), *E. coli* ($n = 13$) (26%), and *Strept. spp.* ($n = 11$) (22%), as indicated in (Table 5).

When pathogen occurrence used to be decided by the usage of m-PCR on milk samples that had been infected, the effects were greater favorable than those obtained by way of microbiological subculture with 44% *S. aureus*, 30% *E. coli*, and 26% *Strept. spp.* It found two extra *E. coli* isolates that have been observed in the culture, one greater *S. aureus*, and two *Strept. spp.* (Table 5). The

Table 4. Oligonucleotide primers sequence, accession number, annealing temperature, and PCR product size of immune and antioxidant genes used in real time PCR.

Gene	Primer	Product length (bp)	Annealing Temperature (°C)	Accession number
<i>CD-14</i>	F5'-CAGCTGCAACAAGCTAAGCA-3' R5'-CGGTCTGGAATCATAACGGGA-3'	144	59	DQ457090.1
<i>CCL2</i>	F5'-ACATTCTGCTTCGGCACTCA-3' R5'-AGACCTTCATGTTGCAGCGA-3'	200	60	XM_005693218.3
<i>β defensin</i>	F5'-CTTCCTGGTCTGTCTGTCTG-3' R5'-GTTTCTAGGGCACCTGTCCG-3'	106	62	DQ532360.1
<i>SPP1</i>	F5'-CGCCCTCCAGTTAAACCGA-3' R5'-GCTTCTGAGATGGGTCAGGC-3'	110	62	NM_001285667.1
<i>BPI</i>	F5'-TTCAGCATGGTTATTTCGAGGA-3' R5'-TAGAGATGCCCTCCACACTCA-3'	178	58	NM_001314172.1
<i>A2M</i>	F5'- TGCAGTTTGCCCTCACGAT-3' R5'- CACCGTCTCATTACAGGTGGC- 3'	192	62	XM_018048527.1
<i>ATP1A1</i>	F5'-ACATGACTCCCGAGCAACTG-3' R5'-TGGCAGCCTTCCACAATGAT-3'	103	60	XM_018045956.1
<i>TLR7</i>	F5'-CTTCTCTGTGTCTTCCAGGGTC-3' R5'-CTACCGTTTCTCACCTTGC-3'	194	59	NM_001285543.2
<i>TLR8</i>	F5'-CCCGAAGCTATCCTTGCGAT-3' R5'-CCACTCTGGGACTTGGCATT-3'	217	60	GU936189.1
<i>SOD1</i>	F5'-ATCCACTTCGAGGCAAAGGG-3' R5'-CTGCACTGGTACAGCCTTGT-3'	124	60	NM_001285550.1
<i>CAT</i>	F5'-ACACAGGCACATGAACGGAT-3' R5'-CCGTAGTCAGGGTCTTCGTG-3'	159	58	GQ204786.1
<i>AhpC/TSA</i>	F5'-CCTCAGGAGACCCAGCTCA-3' R5'-AGTCCACATGCTGAACTCCT-3'	144	59	XM_005684152.3
<i>PRDX2</i>	F5'-ACTATAAAAAGGCGCCGCTGG-3' R5'-CAGGCCATGACTGAAAGCTG-3'	111	59	XM_018051260.1
<i>PRDX4</i>	F5'-TCTGAATGACCTTCCCGTGG-3' R5'-TCTTGGCCACCAAGCTTTCA-3'	160	62	XM_013976641.2
<i>NQO1</i>	F5'-TGCACTTCTGTGGCTTCCAA-3' R5'-CCAGGCGTTTCTTCCATCCT-3'	108	60	XM_005692193.3
<i>Nrf2</i>	F5'-CTACGGGCAAAAGCTCTCCA-3' R5'-TCTGCAATTCTGAGCAGCCA-3'	171	60	KM576769.1
<i>β. actin</i>	F5'-CGTGCTGCTGACGGAGGCCCC-3' R5'-GCACAGCCTGGATGGCCACATAC-3'	113	60	AF481159.1

–CD14= cluster of differentiation 14; CCL2= C–C motif ligand 2; SPP1= Secreted Phosphoprotein 1; BPI= Bactericidal permeability increasing protein; A2M= Alpha-2-Macroglobulin; ATP1A1= ATPase Na+/K+ Transporting Subunit Alpha 1; TLR7= Toll-like receptor 7; TLR8= Toll-like receptor 8; β defensin= beta defensin; CCL2= Chemokine (C–C motif) ligand 2; SOD1= Superoxide dismutase 1; CAT; Catalase; AhpC/TSA—alkyl hydroperoxide reductase/thiol-specific antioxidant; PRDX2 = Peroxiredoxin 2; PRDX4 = Peroxiredoxin 4; NQO1 = NAD (P) H Quinone Dehydrogenase 1; and Nrf2= nuclear factor erythroid 2–related factor 2.

Table 5. Distribution of SNPs, type of mutation in immune, and antioxidant genes for tolerant and affected does to mastitis.

Gene	SNPs	Tolerant	Mastitis	Total	Type of mutation	Amino acid number and type
<i>CD-14</i>	G457A	21	-	21/100	Non-synonymous	153 A to T
	A491C	18	-	18/100	Non-synonymous	164 Q to F
<i>CCL2</i>	A262G	-	15	15/100	Non-synonymous	88 R to G
	T479C	31	-	31/100	Non-synonymous	160 L to S
<i>β defensin</i>	C30T	28	-	28/100	Synonymous	10 R
	T105A	35	-	35/100	Non-synonymous	35 D to E
	T197C	24	-	24/100	Non-synonymous	66 V to A
<i>SPP1</i>	C225T	-	19	19/100	Synonymous	75 T
	C857T	-	27	27/100	Non-synonymous	286 T to I
<i>BPI</i>	A75G	-	23	23/100	Synonymous	25 T
	G257A	-	14	14/100	Non-synonymous	86 R to Q
<i>A2M</i>	A456G	38	-	38/100	Synonymous	152 S
	C36G	17	-	17/100	Synonymous	12 S
<i>ATPIA1</i>	C143A	27	-	27/100	Non-synonymous	48 T to N
	T152C	-	23	23/100	Non-synonymous	51 M to T
<i>TLR7</i>	C347T	29	-	29/100	Non-synonymous	116 A to V
<i>TLR8</i>	G709A	-	25	25/100	Non-synonymous	237 G to S
<i>SOD1</i>	C45 G	32	-	32/100	Non-synonymous	15 H to Q
<i>CAT</i>	T728 C	37	-	37/100	Non-synonymous	243 M to T
<i>AhpC/TSA</i>	C126T	16	-	16/100	Synonymous	42 Y
	T418C	-	26	26/100	Non-synonymous	140 C to R
<i>PRDX2</i>	T592C	-	17	17/100	Non-synonymous	198 W to R
	A201G	24	-	24/100	Synonymous	67 L
<i>NQO1</i>	A404G	34	-	34/100	Non-synonymous	135 N to S
	C580T	29	-	29/100	Non-synonymous	194 R to W
<i>Nrf2</i>	G71A	38	-	38/100	Non-synonymous	24 R to H

–CD14 = cluster of differentiation 14; CCL2 = C–C motif ligand 2; SPP1 = Secreted Phosphoprotein 1; BPI = Bactericidal permeability increasing protein; A2M = Alpha-2-Macroglobulin; ATPIA1 = ATPase Na+/K+ Transporting Subunit Alpha 1; TLR7 = Toll-like receptor 7; TLR8 = Toll-like receptor 8; β defensin = beta defensin; CCL2 = Chemokine (C–C motif) ligand 2; SOD1 = Superoxide dismutase 1; CAT; Catalase; AhpC/TSA—alkyl hydroperoxide reductase/thiol-specific antioxidant; PRDX2 = Peroxiredoxin 2; PRDX4 = Peroxiredoxin 4; NQO1 = NAD (P) H Quinone Dehydrogenase 1; and Nrf2 = nuclear factor erythroid 2–related factor 2.

-A = Alanine; C = Cysteine; D = Aspartic acid; E = Glutamic acid; F = Phenylalanine; G = Glycine; H = Histidine; I = Isoleucine; K = Lysine; L = Leucine; M = Methionine; N = Asparagine; P = Proline; Q = Glutamine; R = Arginine; R = Arginine; S = Serine; T = Threonine; V = Valine; W = Tryptophan; and Y = Tyrosine.

presence of combined contamination in 25 animals used to be noted. As indicated in Figure 1, *E. coli* and *S. aureus* had been the maximum common combined infections (8/17, 47%), accompanied with the aid of *E. coli* and *Strept. spp.* (5/9, 55.5%) and *S. aureus*, and *Strept. spp.* (2/14, 14%).

Immunity and antioxidant gene polymorphisms associated with mastitis incidence

PCR-DNA sequencing of *CD-14* (538-bp), *CCL2* (534-bp), *β defensin* (253-bp), *SPP1* (943-bp), *BPI*

(645-bp), *A2M* (325-bp), *ATPIA1* (300-bp), *TLR7* (398-bp), *TLR8* (799-bp), *SOD1* (436-bp), *CAT* (866-bp), *AhpC/TSA* (636-bp), *PRDX2* (706-bp), *PRDX4* (527-bp), *NQO1* (635-bp), and *Nrf2* (442-bp) revealed mastitis resistance/susceptibility in the examined does is correlated with nucleotide sequence changes in the formula of SNPs. Nucleotide sequence variation in the genes under study between the affected and resistant does, and between the examined animals and GenBank reference sequences, validated all of the SNPs that

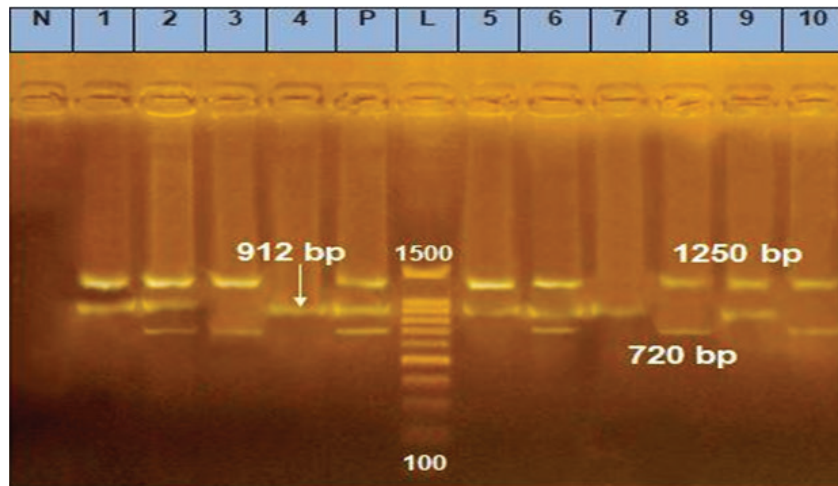


Fig. 1. m-PCR assay, amplifying fragments of 720, 912, and 1250 bp were amplified for *E. coli*, *Strept. spp.*, and *S. aureus*, respectively. Lane1: 100–1500 bp marker, Lanes p: positive control of *E. coli*, *Strept. spp.*, and *S. aureus* standard strains, Lane N: negative control with no DNA, Lanes 2, 3, 6, 8, and 10 positive for *E. coli*, Lanes 1, 2, 4, 5, 6, 7, and 9 positive for *Strept. spp.*, Lanes 1, 2, 5, 6, 8, 9, and 10 positive for *S. aureus*.

Table 6. Pathogen existence in milk.

Organism	The prevalent pathogens by culture (50 sample)	%Relative to the number of infected animal	The prevalent pathogens by multiplex-PCR	% prevalent pathogens by multiplex-PCR
<i>E. coli</i>	13	26%	15	30%
<i>S. aureus</i>	21	42%	22	44%
<i>S. spp.</i>	11	22%	13	26%
Total	45	90%	50	100%

were found. Coding mutations between the mastitic doe and the healthy doe was caused by the exonic changes that are displayed in Table 6 in all immunological and antioxidant indicators that were investigated.

Gene expression configuration of immune and antioxidant markers

The immunological and antioxidant markers' gene expression profiles are displayed in Figures 2 and 3. Mastitis-affected does have significantly greater expression levels of the genes *CD-14*, *CCL2*, β defensin, *SPPI*, *BP1*, *A2M*, *TLR7*, and *TLR8* than resistant does. Mastitis-affected does have significantly lower expression of the genes *ATP1A1*, *SOD1*, *CAT*, *AhpC/TSA*, *PRDX2*, *PRDX4*, *NQO1*, and *Nrf2* than resistant does.

Hemato-biochemical and immunological alterations associated with goat mastitis

There were significant ($p < 0.05$) elevations in WBCs and neutrophils count while significant ($p < 0.05$) decreases in RBCs, PCV, and lymphocytes count in mastitic does as compared with healthy does (Table 7). However, Hb, MCV, MCH, MCHC, and monocyte count were non-significantly altered ($p \geq 0.05$) in mastitic does in relation to the control group (Table 7). There were significant ($p < 0.05$) augments in serum

values of BHB, NEFA, triglycerides, LDL-C, AST, ALT, ALP, creatinine, total protein, globulin, calcium, and potassium with significant ($p < 0.05$) declines in serum concentrations of glucose, cholesterol, HDL-C, albumin, sodium, chloride, and phosphorus in mastitic does compare to healthy ones (Table 8). There were significant ($p < 0.05$) rises in serum levels of GPx and MDA with significant ($p < 0.05$) reductions in serum concentrations of GSH, SOD, and catalase in mastitic does in comparison with healthy ones (Table 9). There was a significant ($p < 0.05$) increase in APPs and cytokines concentrations in mastitic does in relation to healthy ones (Table 9).

ROC curve analysis of the estimated APPs and pro-inflammatory cytokines cleared that they yielded at AUC = 1, sensitivity, and NPV as 100%, with high PPVs and ARs (more than 70%) while their specificities and LR were low. They were almost equal but TNF- α and SAA had unique percentages of increase of 256.37% and 234.78%, respectively (Table 10).

Discussion

Mastitis, especially in the postpartum period, is the term used to describe the inflammation of one or more of the

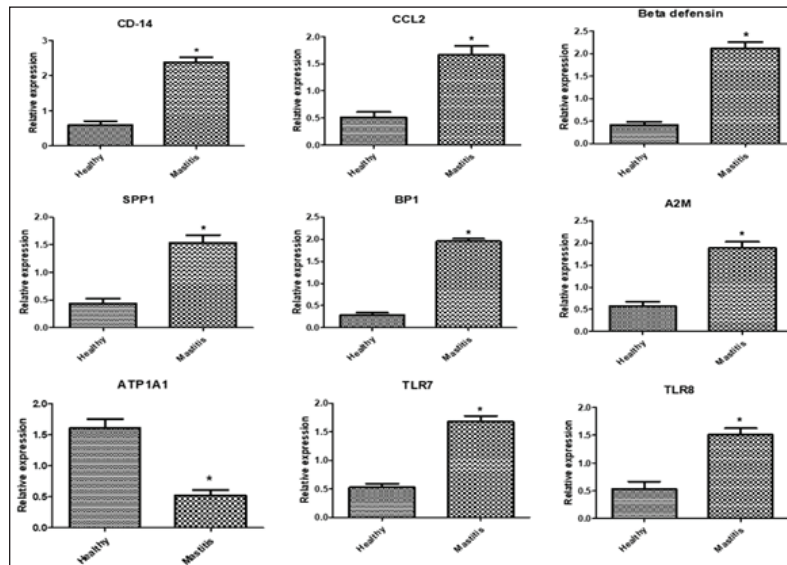


Fig. 2. Relative expression patterns of immune genes in healthy and mastitic Shami goats. Results are expressed as means \pm SEM. * $p < 0.05$.

Table 7. Hematological parameters of normal ($N = 50$) and mastitic ($N = 50$) Shami goats.

Parameters	Normal does	Mastitis does	p values
RBC ($\times 10^{12}/l$)	9 ± 0.9	$5.7 \pm 0.38^*$	0.001
Hb (g/dl)	10.4 ± 0.84	$10.1 \pm 1.$	0.69
PCV%	34.7 ± 2	$27.5 \pm 1.3^*$	0.001
MCV (fl)	38.7 ± 3.2	38.1 ± 3.7	0.82
MCH (pg)	11.7 ± 2.1	11.4 ± 1.4	0.88
MCHC (%)	30.1 ± 3.4	31.4 ± 3.6	0.63
WBC($\times 10^9/l$)	4.9 ± 1.5	$10.8 \pm 4.4^*$	0.04
lymphocyte ($\times 10^9/l$)	7.2 ± 2.9	$2.8 \pm 0.61^*$	0.027
monocyte ($\times 10^9/l$)	0.11 ± 0.05	0.25 ± 0.08	0.22
neutrophil ($\times 10^9/l$)	1.8 ± 0.9	$3.7 \pm 1.1^*$	0.039

*Values with an asterisk within the same row are statistically significant ($p < 0.05$).

mammary gland tissues that customarily occur before lactation (Ondieki, 2018). Infections are typically characterized by local inflammatory symptoms, yet depending on how severe the infection is, systemic symptoms may also be present (Mohammed *et al.*, 2016).

The culture method confirmed the presence of the mastitic pathogens in 95% of milk samples and m-PCR results committed their presence in 100% of milk samples. Whereas, *S. aureus* was identified in 42% of raw milk samples by culture method and in 44% of raw milk samples by m-PCR method. These results were in agreement with those reported in (43.1%) of dairy does in Italy (Cortimiglia *et al.*, 2015) but lower than found in (50%) of buffalo in Bangladesh (Talukder *et al.*, 2013), (76.9%) of goats in Sardinia (Spanu *et al.*,

2013), and higher than those recorded in (30%) of cow in Egypt (Saad *et al.*, 2019), (38.3%) of sheep and goats in Turkey (Abdallah *et al.*, 2018), (35%) of goat in India (Mishra *et al.*, 2018), (4.5%) of camels at Matrouh Governorates, Egypt (Darwish, 2023), and (13.5%) of goat in Brazil (Rupp *et al.*, 2019). According to Le Loir *et al.* (2003), inappropriate hygiene procedures, handling of animals during milking, and milk storage could all be contributing factors to the spread of *Staphylococcus* spp. in milk.

In the present data, the isolation rate of *E. coli* by culture method was 26% and by m-PCR was 30% (focusing on the *phoA* gene). Our findings were consistent with Sameer *et al.* (2013) (26.67%) in Egypt, but higher than those reported by Abd El-Tawab *et al.* (2018) (9.5%) in Sharkia and Dakahlia Governorates, Egypt,

Table 8. Biochemical parameters of healthy ($N = 50$) and mastitic ($N = 50$) Shami goats.

Parameters	Normal does	Mastitis does	<i>p</i> values
Glucose (mg/dl)	104 ± 12.2	70.6 ± 11*	0.02
Cholesterol (mg/dl)	110.6 ± 10.6	80.6 ± 6.6*	0.01
Triglyceride (mg/dl)	25.6 ± 6	50.6 ± 5.1*	0.005
HDL-C (mg/dl)	47 ± 7.2	31 ± 1*	0.01
LDL-C (mg/dl)	36.2 ± 4.7	55.3 ± 5.3*	0.009
Total protien (g/dl)	5.4 ± 0.4	7.3 ± 0.5*	0.008
Albumin (g/dl)	3.4 ± 0.4	2.4 ± 0.2*	0.02
Globulin (g/dl)	3.6 ± 0.2	4.3 ± 0.2*	0.01
Creatinine (mg/dl)	0.42 ± 0.1	0.6 ± 0.05*	0.02
NEFA (mmol/l)	0.6 ± 0.1	1 ± 0.2*	0.03
BHB (mmol/l)	0.7 ± 0.07	1.5 ± 0.2*	0.004
AST (U/l)	48 ± 3.6	72.3 ± 6.4*	0.02
ALT (U/l)	33 ± 4	45.3 ± 3.6*	0.03
ALP (U/l)	96.6 ± 15	218 ± 34.3	0.005
Calcium (mg/dl)	5.2 ± 0.2	8.2 ± 0.2*	0.003
Phosphorus (mg/dl)	4.8 ± 0.1	3 ± 0.1*	0.001
Sodium (mmol/l)	182.6 ± 10.2	160.6 ± 6*	0.03
Potassium (mmol/l)	3 ± 0.2	5 ± 0.7*	0.01
Chloride (mmol/l)	126.1 ± 1	114 ± 1*	0.001

*Values with an asterisk within the same raw are statistically significant ($p < 0.05$).

Table 9. Oxidative stress markers, acute phase proteins and pro-inflammatory cytokines in normal does ($N = 50$) and those with clinical mastitis ($N = 50$).

Parameters	Normal does	Mastitis does	<i>p</i> values
GSH (mg/dl)	37.3 ± 4.5	22.3 ± 4.1*	0.01
GPx (U/gHb)	30.3 ± 2.5	57 ± 6.5 *	0.002
MDA (nmol/ml)	5.5 ± 1.5	17.3 ± 3.5*	0.006
SOD (U/ml)	54.3 ± 7.7	27.6 ± 3*	0.005
Catalase (U/l)	37.6 ± 4.1	17.3 ± 6*	0.009
Hp (ng/ml)	73 ± 22.6	135 ± 19.4*	0.02
SAA (mg/l)	4.6 ± 1.1	15.4 ± 2.5*	0.003
Fb (g/l)	2.2 ± 0.15	3.5 ± 0.1*	0.001
IL-6 (pg/ml)	47.3 ± 8	120.6 ± 36.1*	0.02
IL-1β (pg/ml)	37.3 ± 13.5	106.6 ± 29.2*	0.02
TNF-α (pg/ml)	46.3 ± 6.2	165 ± 42.7*	0.009

*Values with an asterisk within the same raw are statistically significant ($p < 0.05$).

Darwish (2023) (12.7%) at Matrouh Governorate, Egypt, Lan *et al.* (2020) (18.5%) in China, Haftay *et al.* (2016) (21.3%) in Ethiopia and lower than percentages reported by Abdallah *et al.* (2018) and Saad *et al.* (2019) (44% and 36.8%, respectively) in Egypt and Machado *et al.* (2018) (51.9%) in Brazil. The highest prevalence of *E. coli* as an environmental pathogen in

milk samples from infected goats may be attributed to management errors such as overcrowding, insufficient ventilation, inadequate manure removal, subpar hygienic conditions, and an overall deficiency in farm cleanliness and sanitation (Abdallah *et al.*, 2018). According to our investigation, 22% of *Strept. spp.* ($n = 11$) were found in milk samples of mastitic Shami

Table 10. ROC curve analysis of the estimated acute phase proteins and cytokines in mastitic does compare to normal does.

	Hp (ng/ml)	SAA (mg/l)	Fb (g/L)	IL-6 (pg/ml)	IL-1 β (pg/ml)	TNF- α (pg/ml)
Cut-off point	82.50	5.05	2.30	51.50	41.50	49.50
Sensitivity%	100%	100%	100%	100%	100%	100%
Specificity%	66%	68%	68%	68%	68%	66%
PPV%	74.36%	75.76%	75.76%	75.76%	75.76%	74.36%
NPV%	100%	100%	100%	100%	100%	100%
AR%	83%	84%	84%	84%	84%	83%
Percentage of increase	84.93%	234.78%	59.09%	154.96%	185.79%	256.37%

LR = 0.5–5: low; LR = 5–10: moderate; LR > 10: high.

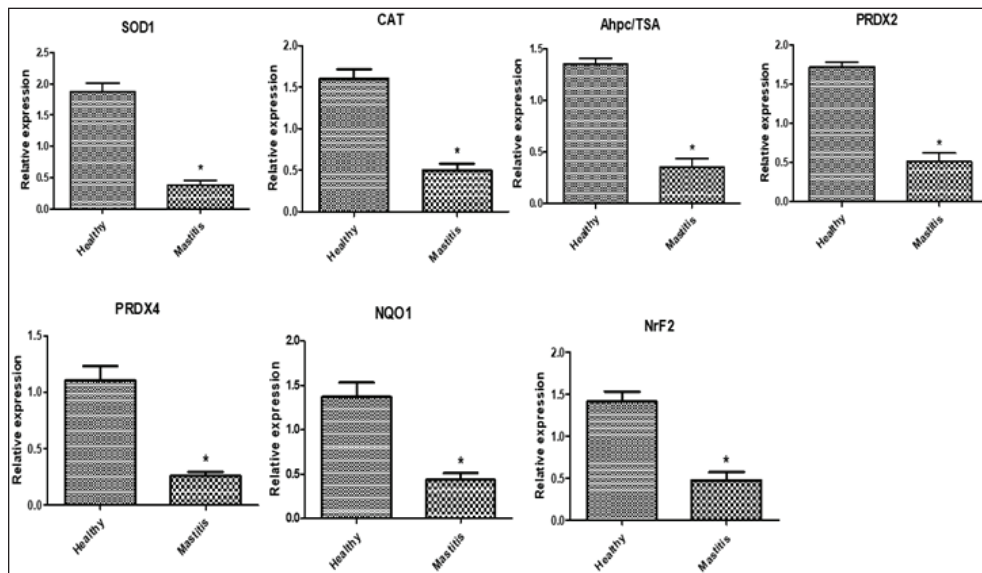


Fig. 3. Relative expression patterns of antioxidant genes in healthy and mastitic Shami goats. Results are expressed as means \pm SEM. * $p < 0.05$.

goats while m-PCR showed the presence of 26% of *Strept. spp.* ($n = 13$) (two more isolates than culture). This was almost the same results reported in 22.5% of dairy cows in China (Ding *et al.*, 2016), and 23% of cows in India (Mishra *et al.*, 2018). 25% of goats in Egypt (Mohamed, 2022), but less than other studies reported (31.2%) of cows in Egypt (Saad *et al.*, 2019), 65% of bovine in Nigeria (Amosun *et al.*, 2010), and higher than 8.7% of cattle in China (Han *et al.*, 2022), 9% of cattle in Pakistan (Ali *et al.*, 2021), 12% of cattle in Egypt (Ahmed *et al.*, 2020), 15.4% of camels in Egypt (Darwish, 2023), 17.4% of small ruminant farms in Denmark (Chatzopoulos *et al.*, 2020). Changes in topographical features and management practices may be to blame for the variation in the percentage of isolated *Strept. spp.*

Mutations in immune function-related genes are partly responsible for the ruminants' sensitivity to harmful microorganisms (Michel *et al.*, 2003). Within this

context, fragments found by PCR-DNA sequencing of *CD-14* (538-bp), *CCL2* (534-bp), β defensin (253-bp), *SPP1* (943-bp), *BPI* (645-bp), *A2M* (325-bp), *ATP1A1* (300-bp), *TLR7* (398-bp), *TLR8* (799-bp), *SOD1* (436-bp), *CAT* (866-bp), *AhpC/TSA* (636-bp), *PRDX2* (706-bp), *PRDX4* (527-bp), *NQO1* (635-bp), and *Nrf2* (442-bp) genes identified SNPs, or nucleotide sequence changes, in mastitis-resistant and afflicted does.

An acceptable explanation for that will be finding SNPs in immune and antioxidant genes is a novel finding in this research that may be relevant to Shami goats' vulnerability or resistance to mastitis. Upon comparing our findings with the relevant GenBank reference sequence, it was intriguing to observe that the variations discovered in the genes under investigation were initially reported. However (Al-Sharif, 2022), identified DNA polymorphisms in the antioxidant and immunological genes as biomarkers for the tolerance and susceptibility to reproductive diseases in Baladi

goats. *TLR7* and *TLR8* single nucleotide polymorphisms (SNPs) and their relationship to the pro-viral load of small ruminant lentiviruses (SRLVs) in goats were found by (Olech *et al.*, 2021).

Relating to mastitis in ruminants and polymorphisms in the immune system and antioxidant genes; *TLR4* and *SOD* SNPs in sheep were linked to postpartum diseases susceptibility in Barki sheep according to Darwish *et al.* (2023), who assessed the connection between *SPP1* and features related to milk yield and composition. A connection between the *SPP1* SNP and somatic cell score was found by statistical analysis. In addition, discovered a link between mastitis resistance and β -defensin gene polymorphisms (Tolone *et al.*, 2016). Investigated SNPs in immunological (*SELL*, *ABCG2*, *SLC11A1*, and *FEZL*) and antioxidant (*SOD1*, *CAT*, *GPX1*, and *AhpC/TSA*) genes linked with mastitis risk in Holstein and Brown Swiss dairy cows were investigated (Ateya *et al.*, 2022). The *CD14* gene polymorphism and its link to mastitis was reported (Kumar *et al.*, 2014).

The osteopontin transcript (*SPP1*) was described by (Alain *et al.*, 2009) as hereditary variations that exist in an early innate immunological candidate of *E. coli* mastitis that may be connected to mastitis resistance. The existence of SNPs in the *CCL2* gene and evaluated how they would affect the variation in somatic cell score (SCS) in Holstein bulls was discovered (Leyva-Baca *et al.*, 2007) Schenkel, Sharma, Jansen, & Karrow, 2007. The regulatory changes in the *A2M* gene contributed to dairy cows' susceptibility to mastitis (Wang *et al.*, 2014). Mastitis and heat tolerance have both been linked to *ATPIA1* gene polymorphism in earlier studies (Elayadeth-Meethal *et al.*, 2021). According to studies on mastitis resistance in buffalo, it was found that variation in the *A2M* gene was connected to SCC, subclinical mastitis, protein production, fat, milk yield, fat and protein percentages, and mastitis resistance (Mingala *et al.*, 2020).

Our findings demonstrated that affected does have a higher pattern of immunological marker expression than resistant does, although antioxidant gene expression showed the reverse tendency. This is the first study to determine the immunological and antioxidant mRNA levels in mastitis-resistant and -susceptible individuals using a real-time PCR approach. We investigated gene polymorphism utilizing SNP genetic markers and gene expression to get beyond the shortcomings of earlier research. We hypothesize that the functional aspects of the genes in caprine mastitis may be impacted by the SNPs found in the examined genes. Consequently, it is well understood how the genes related to immunity and antioxidants that are being studied, both in healthy and mastitis-affected animals, are controlled.

Examining the goat mammary gland's reaction to *S. aureus* infection was done by Cremonesi *et al.*, (2012) using gene expression profiling in milk somatic and white blood cells. The highest increases in expression

were seen in the *NFKB1*, *TNFAIP6*, *BASPI1*, *IRF1*, *PLEK*, and *BATF3* genes after challenge. Goats with *S. aureus* mastitis compared to healthy goats, Pisoni *et al.* (2010) showed that pro-inflammatory cytokines, chemokines, and their receptors—IL-1 α , lymphotoxin alpha, granulocyte chemotactic protein (CXCL6), and IL-2 receptor gamma were all significantly overexpressed.

In previous research examining the gene expression profile of immunological and antioxidant markers linked to ruminant mastitis, Wang *et al.* (2012) showed that mastitis-infected bovine mammary tissues had considerably greater levels of *A2M* mRNA and protein expression than did healthy tissues. According to Ateya *et al.* (2022), compared to tolerant cows, mastitic Holstein and Brown Swiss dairy cows exhibited significantly greater levels of *SELL*, *SLC11A1*, and *FEZL* gene expression. The genes for *GPX1*, *AhpC/TSA*, *SOD1*, *CAT*, *ABCG2*, and *SOD1* were significantly down-regulated. In addition, Kosciuzczuk *et al.* (2014), found that the expression pattern of β -defensin is significantly higher in bovine mammary glands infected with coagulase-positive or coagulase-negative *Staphylococci* than in udders free of bacteria. Asadpour *et al.* (2021) looked into variations in antioxidant gene expression during clinical mastitis in cows brought on by *E. coli* and *S. aureus*. They discovered that, in contrast to *E. coli*, *S. aureus*-induced mastitis milk had far higher levels of *SOD* expression. Furthermore, in terms of mRNA levels, *GPx* was substantially overexpressed in milk from mastitis induced by *E. coli* as opposed to *S. aureus*. Ewes with postpartum issues had significantly greater levels of *IL-5*, *IL-6*, *IL1- β* , *TNF- α* , *TLR4*, and *Tollip* than resistant ewes; conversely, *SOD* and *CAT* gene patterns were down-regulated.

CD14 is a crucial part of innate immunity. One of the most significant molecules that binds and neutralizes bacterial endotoxins is the anti-bacterial peptide *CD14* (Wright *et al.*, 1990). By binding to the *CCR2* receptor that is found on the surface of monocytes and neutrophils, respectively, *CCL2* functions as an effective chemokine for these cells (Proudfoot, 2002). β -defensins were thought to be the only antibacterial substances made by leucocytes and epithelial cells that acted as the initial barrier against pathogens (Caverly *et al.*, 2003).

A cytokine called secreted phosphoprotein 1, also known as *SPP1* or osteopontin, is created by activated T cells and macrophages (Chabas *et al.*, 2001). *BPI* is an endogenous cationic bactericidal/permeability-increasing protein. In addition to eliminating Gram-negative bacteria, endotoxins, and lipopolysaccharide (LPS), which is another name for endotoxin, are neutralized. *BPI* serves a variety of biological purposes, including enhancing opsonization and complement activation for amplified phagocytosis, inhibiting angiogenesis and the release of inflammatory

mediators, and guarding against infection by fungi and protozoan pathogens (Balakrishnan *et al.*, 2013). Along with the complement system's C3, C4, and C5, alpha-2-macroglobulin (A2M) is a member of the alpha-macroglobulin (aM) family of proteins (Levashina *et al.*, 2001). In addition, it promotes the growth of macrophages and T cells (Bonacci *et al.*, 2007).

Genetic variations of TLRs have been discovered to be related to various disease outcomes (Skevaki *et al.*, 2015). Numerous viral infections have been linked to *TLR7* and *TLR8* polymorphisms (Zhang *et al.*, 2020). A transmembrane enzyme called Na⁺, K⁺-ATPase uses ATP to move 3 Na⁺ out of and 2 K⁺ into a mammalian cell via the plasma membrane (Rakowski *et al.*, 1989). To regulate cell volume, osmotic balance, and appropriate resting membrane potentials, ionic homeostasis in the cytoplasm is made easier by this (Pavlov and Sokolov, 2000).

Antioxidants fight free radicals by scavenging them, detoxifying them, preventing their generation, or sequestering the transition metals that produce them (Masella *et al.*, 2005). The primary inducible defense against oxidative stress is the Keap1-Nrf2 stress response system, which controls the production of cytoprotective genes (Yamamoto *et al.*, 2018). According to Bianchet *et al.* (2004), the antioxidant NQO1 protects against harmful oxidative by cleansing radicals and peroxides that include sulfur, thiol-specific peroxidase serves as a sensor of hydrogen peroxide-mediated signaling events and supports cellular defense against oxidative stress (Ateya *et al.*, 2021).

The significant change in the immune (*CD-14*, *CCL2*, β defensin, *SPPI*, *BPI*, *A2M*, *TLR7*, and *TLR8*) and antioxidant (*SOD1*, *CAT*, *AhpC/TSA*, *PRDX2*, *PRDX4*, *NQO1*, and *Nrf2*) expression patterns in mastitic may be ascribed to the severe inflammation that harms the affected tissue and causes the release of cytotoxic radicals and pro-inflammatory cytokines, the excess ROS weakens the immune system in the absence of aentire antioxidant that is appropriate (Sordillo and Aitken, 2009). Our research showed that mastitics do have much lower levels of *ATPIAI* mRNA than healthy ones. Mastitis has been proven to have an impact on milk's potassium and sodium levels (Liu *et al.*, 2012). Milk has a higher concentration of K⁺ than Na⁺ because Na⁺, K⁺-ATPase, which is positioned on the basolateral membrane, actively removes Na⁺ from the secretory cells of the mammary glands (Linzell and Peaker, 1971). However, mastitis causes changes to the K⁺ and Na⁺ contents in milk. K⁺ concentration falls and Na⁺ concentration rises in cows with subclinical mastitis (Auldish and Hubble, 1998). As a result, milk's osmolality changes. As a result, since the Na⁺, K⁺-ATPase system is crucial for the osmoregulation of milk, information on its sensitivity to mastitis would be of relevance. When mastitis affects cows, the osmolality of their milk may alter, indicating a decrease in the activity of the Na⁺, K⁺-ATPase enzyme.

Our study's findings unequivocally show that, in comparison to healthy goats, the hematology of mastitic Shami goats was significantly altered. Of particular concern was the marked increase in WBCs, neutrophils count, and lymphopenia, likely due to differential immunomodulatory effects resulting from increased demand for leukocytes in the infected udder (Carvalho-Sombra *et al.*, 2021). Disease-related stress and neutrophilia (subsequent leukocytosis) as well as lymphopenia may develop as a result of the endogenous production of corticosteroids in the mastitic group (Duncan, 1994; Kaneko, 1997). Our finding was similar to that obtained in mastitis in goats (Abba, 2013), in cattle (Saleh *et al.*, 2022), and in camels (Darwish, 2023).

In the current study, there were noticeable decreases in RBCs and PCV between mastitic and healthy does. These findings agreed with the findings of Hristov (2018) in goats, Saleh *et al.* (2022) in cattle, and Darwish (2023) in camels, but away from that reported by Garba *et al.* (2019) in Red Sokoto goats or Sarvesha *et al.* (2016) in Indigenous cow who did not observe any significance difference of Hb and RBCs level of mastitic animal as opposed to healthy ones. There were not any noteworthy changes in values of Hb, MCV, MCH, MCHC, and monocytes between the mastitic and control groups. These findings were opposite to those found by Saleh *et al.* (2022) in cows who confirmed considerably reduced Hb, MCV, MCH, and MCHC values when in contrast to controls, indicating microcytic hypochromic anemia, which is frequent in inflammatory disorders. The authors delivered that, inflammatory prerequisites and the pro-inflammatory cytokines stimulate the mononuclear phagocytic system to elevate iron trapping and storage, which is notion to be a protecting mechanism with the aid of lowering iron availability to microorganisms, ensuing in a reduction in Hb synthesis and the manufacturing of microcytic hypochromic RBCs (Stockham and Scott, 2013). The considerable increase in the pro-inflammatory cytokines IL-6 in the mastitic group in our study lends weight to this opinion.

The occurrence of hypoglycemia and the observed increase in serum levels of BHB, NEFA, triglycerides, LDL-C, and total lipids in mastitic goats compared to healthy ones may result from reduced appetite in afflicted does, leading to heightened lipolysis, the onset of acidosis, and a ketotic state (Kaneko, 1997; El-Deeb, 2013). On the other hand, the noted reduction in serum cholesterol and HDL-C in diseased does than in healthy does, may be attributed to the hepatic insufficiency usually observed with the disease (Kaneko, 1997). Our results were consistent with those seen in mastitic does (El-Deeb, 2013) and mastitic she-camels (Darwish, 2023).

The current study recorded higher serum total protein levels in mastitic cases compared to healthy ones, which may be explained by the inflammatory process taking

place in the tissue of the mammary glands triggering the body's immunological system. In this study, we also found that mastitic goats' serum globulin levels were higher than non-mastitic does'. This could happen as a result of the gamma-globulin antibody production that is responsible for blocking the effects of the invasive microorganism (Nicholas, 2005). This finding was in part similar to that given by Garba *et al.* (2019) in Red Sokoto goats, and Das *et al.* (2018) in cattle and she-camels Darwish (2023). Contrariwise, the mastitic in our data suffered from obvious hypoalbuminemia, this may be attributed to the albumin nature as a negative APP which decreases in response to inflammation to conserve amino acid for synthesis of positive APPs as haptoglobin (Darwish, 2023).

The present research revealed that does afflicted with mastitis exhibited elevated levels of creatinine. This increase was thought to be the result of severe necrosis in the udder tissue, which has an abundance of smooth muscle. This result shared some similarities with those reported for ewes (Çetin *et al.*, 2005) and camels (Darwish, 2023). Similarly, the serum activity of AST, ALT, and ALP markedly increased in the current study, which is indicative of liver dysfunction. This result shared some similarities with those reported in does (Das *et al.*, 2018), cattle (Garba *et al.*, 2019), and camels (Darwish, 2023). Our study's findings were in contrast to those of Çetin *et al.* (2005) who studied ewes mastitis, and Sarvesha *et al.* (2017) and Sarvesha *et al.* (2016) who studied mastitis in indigenous and crossbred cows, respectively, who found no difference in serum hepatic enzymatic activities between diseased and healthy animals.

Our data about mastitis referred to hypercalcemia in the mastitic does, this may be explained by the fact that infected animals produce less milk, which results in less calcium being excreted from the milk (Wegner and Stull, 1978). These consequences had been similar to those proven in preceding reviews by Das *et al.* (2018) and Sarvesha *et al.* (2017) in cows. The mastitic does (in relation to healthy does) in the present data also showed a pronounced hyperkalemia. This result was attributed to the above-mentioned ketoacidosis and the subsequent H⁺ accumulation. The body tries to get rid of accumulated H⁺ by pumping it intracellularly leading to K⁺ pump extracellularly (Kaneko *et al.*, 1997). Hyperkalemia was noted in mastitis-affected cow and buffaloes (Das *et al.*, 2018; Sarvesha *et al.*, 2017). On the contrary, prominent hyponatremia, hypochloremia, and hypophosphatemia were depicted between the diseased and healthy ones. Anorexia and renal dysfunctions may be acceptable causes for these deficiencies. Similar opinions were mentioned in mastitis in Sahel goats (Abba, 2013) and in she camels (Darwish, 2023).

In the current research, there was a marked higher level of GPx and MDA in mastitic does than healthy ones, along with a significantly lower level of GSH, SOD,

and catalase. These alterations could be attributed to the increased demand for antioxidant activities due to elevated oxidative damage resulting from inflammatory responses in mammary gland tissue. Our results were in line with those reported in ewes (Çetin *et al.*, 2005), does (El-Deeb, 2013), and camels (Darwish, 2023). The current records clear that tissue damage and inflammation may be influenced by the raised serum oxidative stress indicators in clinically mastitic does.

The present data showed that clinically mastitic does had higher APPs (Hp, SAA, and Fb), and pro-inflammatory cytokines (IL-6, IL-1 β , and TNF- α) levels than did healthy does. Gabay and Kushner (1999) mentioned that the release of TNF- α as a result of inflammation may enhance the increased levels of APPs. The elevated APPs and pro-inflammatory cytokines levels are consistent with those found in does with gangrenous mastitis (El-Deeb, 2013), ewes with experimentally induced mastitis with *Staphylococcus* (Winter *et al.*, 2003). In this work, the signals control neutrophil activity and recruitment to infection places are chemotactic agents produced by infecting bacteria as well as different immune system components. However, there are drawbacks to this neutrophil influx. Tissue damage can result from this, which has the potential to lead to fibrosis and a decline in mammary function.

Previous research emphasized the importance of APPs and pro-inflammatory cytokines in diagnosing and predicting various diseases including mastitis (El-Deeb, 2013; Darwish, 2023). The ROC curve analysis conducted in the current study further validated this assertion. Despite their relatively low specificities and LR_s, these biomarkers exhibited high sensitivity, PPV_s, NPV_s, and AR_s with AUC values. Notably, the percentage increase of TNF- α and SAA singled them out as dependable markers for diagnosing goat mastitis.

Conclusion

The current investigation concluded that the m-PCR assay was better than the culture method in the identification of major bacterial species causing mastitis in Shami goats (*S. aureus*, *E. coli*, and *Strept. spp.*). Our results emphasize the importance of SNPs as genetic markers in genes related to immunity and antioxidant defense which would determine the predispose individuals to either resistance or susceptibility to mastitis. Treating caprine mastitis requires a comprehensive approach that considers not only the clinical symptoms but also the associated alterations in immune response and hemato-biochemical parameters. TNF- α and SAA may be reliable biomarkers for the diagnosis of goat mastitis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Authors contributions

Ahmed El-Sayed conceived, and designed the experiment, collected blood samples, performed biochemical analysis, and wrote the manuscript. Ahmed Ateya performed real-time PCR and contributed to writing the manuscript. Amani A. Hafez conducted m-PCR and contributed to writing the manuscript. Eman Ebissy and Asmaa Darwish analyzed the data and contributed to writing the manuscript.

Data availability

On reasonable request, the corresponding author will provide the information supporting the study's conclusions.

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