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PCR detection of *Staphylococcus aureus* isolated from sub-clinical mastitis of goat with expression immune responses genes.

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Abstract

Objective: Goat sub-clinical mastitis is a multi-bacterial infection. The proper selection of a medicine for therapy depends on correctly identifying the bacterium. Via use of the 16S ribosomal RNA (16S rRNA) gene, the goal of this work is to quantify the gene expression of IL2 and TNFα while also identifying the phenotypic and genotypic traits of Staphylococcus aureus that contribute to illness. Materials and Methods: Utilizing 16S rRNA gene-specific universal primers for the bacterial polymerase chain reaction (PCR), 100 samples of goats with healthy and subclinical mastitis were analyzed. The expression levels of the staphylococcus aurus and the inflammatory cytokines interleukin (IL-2) and (TNFα) in sub-clinical mastitis and healthy animals were evaluated and compared using the primers.Results: According to the PCR, 70 (64.3%) samples had favorable outcomes. Sub-clinical mastitis goats had considerably higher TNF gene expression than healthy goats ($p< 0.001$). The relative gene expression in subclinical mastitis-affected animals of TNFα and IL2 is 6.2±3.23 and 1.02±0.311, respectively, and the relative gene expression of these cytokines is 3.012±2.12 and 3.33±0.821, respectively.

Keywords

goat Mastitis, Staphylococcus aureus, Cytokines and pro-inflammatory

Mastitis, a swelling of the gland that produces milk, typically results from an intra-mammary bacterial infection. .(Kibebew 2017, Pal, Regasa et al. 2019). Depending on the severity of the inflammation, phases of clinical mastitis can also be divided into subacute, acute, and acute phases. (Ibrahim 2017). When opposed to clinical mastitis, the incidence of subclinical mastitis (SCM), which doesn't cause obvious changes to the milk's or udder's appearance, is substantially higher in a dairy herd (Shaheen, Tantary et al. 2016, Cheng and Han 2020). This illness is characterized by inflammation of the mammary gland, which can be triggered by germs, most frequently bacteria that cause tissue loss, as well as by physical, chemical, or traumatic stress(Bae, Jeong et al. 2017, Shoaib, Aqib et al. 2021).Along with these impacts, producers will experience economic loss as a result of inadequate milk quality and reduced milk output. In most cases, bacterial infections are what cause mastitis(Pleguezuelos, Fern et al. 2015, Gelasakis, Angelidis et al. 2016).The most prevalent microbe among the many varieties recovered from instances of animal mastitis are Staphylococci (Staphylococcus aureus), Streptococci (Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis), in addition to relatives Enterobacteriaceae (Escherichia coli, Klebsiella pneumoniae) also other. Least prevalent causal factors include Pseudomonas aeruginosa,Mycobacterium species, Nocardia asteroides, Candida species, Cryptococcus species, and Aspergillus species. Occasionally are viruses thought to be responsible for mastitis in cattle.

Increased milk leucocyte count indicates pathogen entry into the breast gland through the teat canal (Freitas, Mendes et al. 2018, Tomazi, de Souza Filho et al. 2018). Staphylococcus (S.) aureus is a facultative, gram-positive, incapable of producing spores, catalase-positive, coagulase-positive, and typically organized in irregular clusters that resemble grapes. S. aureus is among the most significant emerging zoonotic pathogens, causing human food poisoning and mastitis in ruminants(Sheet, Hussein et al. 2021, Sheet, Jwher et al. 2021). Bacterial compounds known as virulence factors increase the ability of the bacteria to colonize and live in the host and, as a result, help the host sustain harm. Numerous virulence factors are present in staphylococci(Madigan, Stahl et al. 2018). The nuc gene generates a thermostable nuclease named staphylococcal nuclease. Staphylococci spread and tissue degradation result from the hydrolysis of DNA and RNA in host cells.When held by neutrophil extracellular traps (NETs), it also promotes microbial egress, enabling the germs to circumvent this host protection(McClure, Zaal DeLongchamp et al. 2017, Torres, Vargas et al. 2019). Cytokines are immunological-regulatory molecules that are essential for controlling immunological reactions to various illnesses.(Vlasova and Saif 2021). Interleukins (IL) IL1,IL2,IL3,IL4,IL5, IL6,IL8,IL10 and IL12) in milk (Vitenberga-Verza, Pilmane et al. 2022).

Tumor necrosis factor alpha (TNF-α) is a type of mediator that causes inflammation when it is locally produced during the acute stage of mastitis. It has been studied how immunity to TNF- affects mastitis; TNF- is assumed to be crucial for the development of phagocytic leucocytes in the udder(El-Deeb, Fayez et al. 2021). Tumor necrosis factor alpha is assumed to be essential for neutrophil leucocytes in the udder to activate their antibacterial activities (Bassel and Caswell 2018). Mastitis-causing bacteria cause the release of IL-2 into milk, which is crucial for battling infection in the breast(Fang, Cui et al. 2019). One of the most useful techniques currently used in biological research, diagnostics, and investigative research is PCR (polymerase chain reaction). Testing is a fast, incredibly accurate approach to spot particular viral diseases and genetic anomalies(Tahamtan and Ardebili 2020). Reverse transcription-polymerase reactions that occur in real time pcr (RT-PCR) assay Real-time RT-PCR, which was created to quickly identify the cause of diseases, offers obvious improvements over more traditional RT-PCR formats, but assay results must still be read carefully (Ruiz-Romero, Martнnez-Gymez et al. 2020).Aims of this study is detection of s.aureus bacteria phenotyping and genotyping from sub clinical mastitis in goat and study of gene expression of IL2 and TNFα as mechanical responses to disease.

Material and Method

Samples collection

For the study, one hundred goats with healthy and subclinical mastitis were gathered. The samples ranged in size from 5 to 10 mL and came from various places in Basra province. The first streams of milk were removed before to sampling, and teat ends were swabbed with 70% alcohol for hygiene(Asmelash, Mesfin et al. 2016). The samples obtained in Sterilized tubes were immediately submitted to the laboratory for further processing.

The California mastitis test (CMT)

was employed to gather samples. The following are categories to look for for the outcomes: The possible results are negative (score 0), trace (score 1), mildly positive (score 2), positive (score 3), and extremely positive (score 4)(Rossi, Amarante et al. 2018).

Isolation, identification, and Phenotypic detection of S. aureus

The species have been isolated and recognized as mentioned by (Bautista-Trujillo, Solorio-Rivera et al. 2013).On nutrient agar medium, the samples were swabbed and then incubated at 37 °C overnight. Due to their morphology, color and consistency, various bacterial colonies were closely examined the next day. As a primary recognition check, to check the purity of the cultures, the colonies were streaked on mannitol salt agar and incubated for 24 hours at 37 ° C. The colonies were moved to coupled nutrient slants and kept at 4° C under refrigeration. Phenotypic identification was carried out by demonstrating the typical cellular morphology in Gram's-stained smears. The bacterial isolates were correspondingly assigned code numbers after determining the pure growth.

Catalase test

A loop of the young culture of bacterial isolates received a drop of 3% hydrogen peroxide. before being spread over a spotless glass slide. Or A few drops of a solution containing a pure culture of a bacterium cultured on a NA plate were added, and the production of bubbles within a few seconds or not was monitored(MacFaddin 2000, Javid, Taku et al. 2018).

Oxidase test

Tetra methyl-p-phenylenediaminedihydrochloride is dissolved in an aqueous solution and used as a test

reagent. Three drops of a freshly made 1% aqueous solution of the color indicator tetra methyl-p-phenylene-diamine-dihydrochloride (Whatman filter paper No. 2) were added to a strip of the paper. A platinum loop was used to smear a loopful of each isolate's young (24-hour) bacterial culture on the surface of the filter paper. (MacFaddin 2000, Hassain and Abbood 2019).

Genotypic confirmation of S. aureus

Amplification of 16S rRNA gene

The genotypic acknowledgement via 16S rRNA was performed as shown in the methods mentioned (Miyoshi, Iwatsuki et al. 2005) where the primers were used . (700-750 bp).

F- 5′-AGAGTTTGATCATGGCTCAG

R-5′-GGTTACCTTGTTACGACTT-3′

Bacterial genomic DNA was isolated using the Geneaid DNA extraction program in Korea. Nano drop Quawell (USA) was used to calculate DNA concentration. The DNA polymerase, dNTPs, and reaction buffer in the(Accu Power® PCR Premix)were previously combined and vacuum-dried into a single packet. A total of 50 µl of distilled water, template DNA (15 µl), primers (6 µl), and AccuPower®PCR Premix tubes were used.The PCR combination was assigned through using Thermal (Techne; UK) to the corresponding thermal cycle conditions: 5 min of 95 ° C until 2-31 thermal cycles at Thermal (Techne; UK),ninety four °C for About a minute and a half , fifty five °C in a minute and a half and seventy seven °C for A minute and a quarter, then a final extension lasting 10 minutes at 72 ° C. The PCR results were processed in 0.8 percent agarose gels made in 1x TBE buffer after being stained with a safety dye (Green-DNA DYE; Biotech, USA). Under a UV transiluminator (Vilber Lourmal- CE; Taiwan), the PCR result was seen.

Extraction of RNA and reverse transcription

A TRIzolTM Plus RNA Purification Kit (USA) was used as directed by the manufacturer to extract nucleic acid RNA. A milk somatic cell pellet was lysed in one milliliter of 1.0 mM TRIzol reagent. Phase separation was performed by centrifuging the mixture after adding 0.2 ml of chloroform. By combining it with an equal amount of isopropyl alcohol, washing it many times with 75% alcohol, and then adding a sufficient amount of diethylpyrocarbonate-treated water, the RNA was precipitated in the aqueous phase. A BioPhoto meter plus (NanoVue, USA) was used to spectrophotometrically measure the extracted RNA. The ratio of OD260/OD280 was utilized to calculate efficiency. 1 g of total RNA and 1 g of (dT12-18) oligo were mixed together and heated to 70 °C (232.2 °F) for 10 minutes to start the reverse transcription procedure. After cooling on ice, we added 200 U of SuperScript-II RNase-H Reverse Transcriptase, 0.5 mM of each dNTP, and 5 l of first-strand buffer. The mixture was prepared in advance by stabilizing it at 25°C for 10 minutes.

PCR quantification

The manufacturer's instructions were implemented for the quantitative PCR procedure. The Promega GoTaqTM qPCR Master Mix Kit with SYBR green dye was used. According to published guidelines, the primers used to assess gene expression were created (Table1). The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous guide. With a reaction volume of 20µ l each sample, 5 µl of sample-complemented deoxyribonucleic acid (cDNA), 1 µl each of forward and reverse primers, 1µl each of GAPDH, and 13 µl of sterile deionized water, the reaction was carried out in triplicate. The reaction was heated to 95°C for 2 minutes, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 55°C, and 1 minute at 72°C to produce the appropriate level of amplification. The 2 Ct method was used to quantitate each sample, which was done in triplicate.

Table (1): Primer pairs were used to measure the expression of the bovine cytokine gene using quantitative real-time PCR (Leutenegger, Alluwaimi et al. 2000).

Gene	5'-3' Oligonucleotides R: reverse; F: forward
IL-2	F: GGATTTACAGTTGCTTTTGGAGAAA R: GCACTTCCTCTAGAAGTTTGAGTTCTT
TNF-α	F: TCTTCTCAAGCCTCAAGTAACAAGT R: CCATGAGGGCATTGGCATAC
GAPDH	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT

Statistic evaluation

To determine whether there was any link between the results, A 5% significance threshold was used when running the t-test. Version 22 of SPSS software is used for statistical analysis.

Results and Discussion

Bacterial isolation and identification

One of the most prevalent issues in dairy animals, mastitis led to significant financial losses

because it reduced the quality and quantity of milk. Identification of the causes of mastitis. In order to prevent and manage dairy farms, it was crucial for the veterinary profession and veterinary medicine(Pumipuntu, Kulpeanprasit et al. 2017) .One hundred milk samples from goats with subclinical mastitis were collected for this study. Bacterial isolates were included in both Table (2) and Figure (1). Growth on mannitol salt agar was one of the essential techniques for identifying S. Aureus in food. In the subclinical goat milk experiment, approximately (70%) of the bacterial isolates developed rounded and yellowish colonies on MSA, allowing for the initial identification of these isolates as S. aureus infections (Zhao, Liu et al. 2015) He said more than 95% of the dairy goat milk samples had bacteria in them. However, several investigators only obtained a low proportion of positive samples from SCM goats (38%, 29.4%) ((Zhao, Liu et al. 2015, Dore, Liciardi et al. 2016, Pirzada, Malhi et al. 2016) .

Table (2): The number and percentage of S. aureus isolated bacteria. Using various methods, bacteria were identified from normal and subclinical mastitis in goats.

Type of bacteria	No. of positive bacteria isolates	Percentage positive bacteria isolates
S. aureus	70	70%
Other bacteria	10	10%
No growth	20	20%
Total	100	

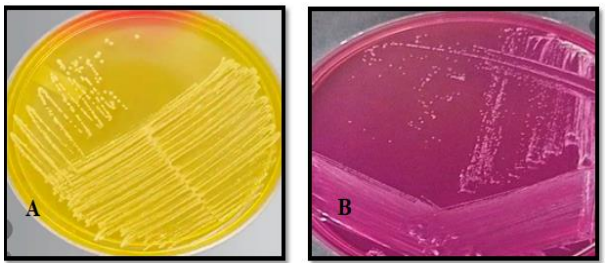

Figure (1): Bacterial staph. aureus isolates grow on mannitol salt agar
staph.aureus growth on mannitol salt agar
Other bacteria

Positive with yellow colonies isolates were subjected to several tests (Gram stain, oxidase, catalase and coagulase). And the results appear positive cocci in Gram stain , Catalase and Coagulase, with Oxidase negative , Through the results, they agree with the researchers (Khaleel, Othman et al. 2016).figure (2)

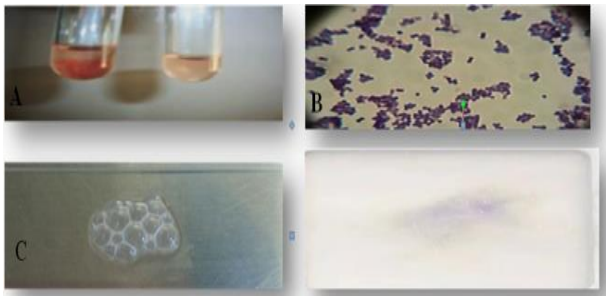

Figure (2):
Coagulase test B. Grram stain
Catalase test D. Oxidase test

Detection of Staphylococcus aureus 16SrRNA

The molecular detection of bacterial isolates by using PCR with specific primers 16SrRNA, S. aureus were positive in pcr with a percent of (64.3%) at molecular size (700-750 bp), In the examined milk samples, the most common bacterial causes of gaot sub clinical mastitis was S. aureus were detected by using single PCR assays, The PCR technique has various advantages, including being a faster, simpler, less expensive, and more accurate method of detecting several forms of harmful bacteria, this result agreement with authors (Hameed, Xie et al. 2018). Fig. (3) and table (3).

Table (3): Identification S.aureus by PCR and 16SrRNA

Samples No.	MSA+No.	PCR +No.
100	70	45
(%)	70	64.3

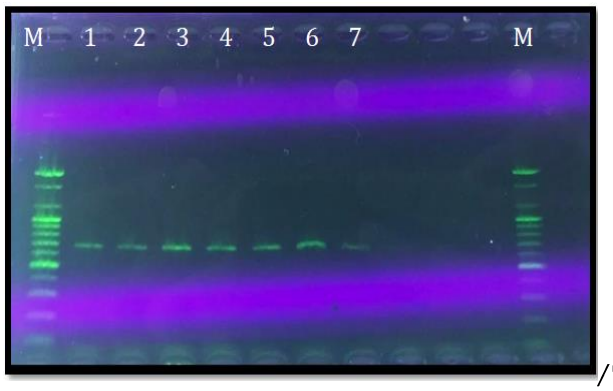

Figure (3): PCR Amplification of staph.aureus , Primer 16SrRNA at molecular size (700-750bp). The product was electrophoresis on 1.5% agarose. Lane M: DNA ladder, Lane (1-7) positive PCR amplification results.

Real-time PCR

Animals with subclinical mastitis have their gene expressions for (IL-2 and TNFα) compared. TNFα

gene expression was increased in mastitis-affected goats compared to animals without the condition ($p < 0.001$). Animal mammary gland inflammation is crucial to the pathogen's ability to infect host tissue. An important part of the body's natural defense system that affects how the infection develops is the transportation of neutrophils to the infection site. Inflammatory mediators, which the body produces in reaction to bacterial toxins or metabolites, cause neutrophil migration(Shaheen, Ahmad et al. 2020).Several cytokines, including interleukin-1b (IL-1b), interleukin-2 (IL-2), interleukin-10 (IL-10), interleukin-6 (IL-6), tumor necrosis factor (TNF-a), transforming growth factor (TGF-b), lactoferrin, interferon gamma, and granulocyte-macrophage colony-stimulating factor, facilitate leukocyte accumulation at the site of inflammatory conditions. (Katsanos, Kyriakidi et al. 2017). This investigation looked at IL2 and TNF expression. The results showed that TNF was elevated, which was consistent with the findings of past research, and we assessed the relative expression of genes connected to immune response mechanisms to mastitis to investigate their expression.(Akhtar, Guo et al. 2020) as shown in figure (4).

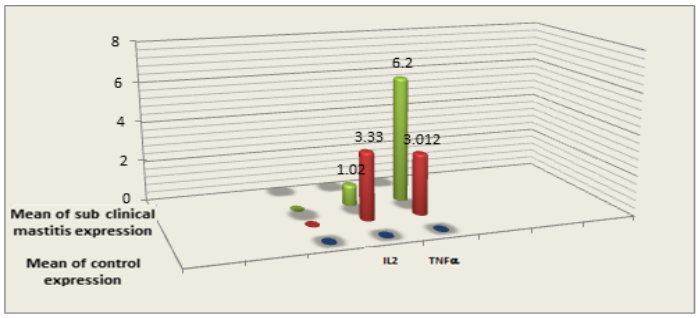

Figure (4): Real-time PCR analysis of the relative expression of the inflammatory cytokine genes (IL2 and TNFα) in goat milk with subclinical mastitis. The data are shown using the 2−ΔΔCt method as the fold change in gene expression compared to the control samples and normalized to an endogenous reference gene (GAPDH). The results are expressed as Mean ± SD as well as $P < 0.05$.

TNFα level of significance is $P < 0.001$ and Significance Level with IL2 are $P < 0.0001$ and the Relative gene expression of TNFα and IL 2 in Animal with sub clinical mastitis 6.2±3.23 and 1.02 ±0.311 respectively and Relative gene expression of these cytokines are 3.012±2.12 and 3.33±0821 respectively And if this indicates something, it indicates with the results of (Vitenberga-Verza, Pilmane et al. 2022) table (4).

Table (4) Relative gene expression of TNFα and IL2 in animals with sub clinical mastitis and healthy animals, significance level of the means

Variables	Relative gene expression Mean±SD	
	TNFα	IL 2
Animal with sub clinical mastitis	6.2±3.23	1.02 ±0.311
Healthy animal	3.012±2.12	3.33±0821
Significance level	$P < 0.001$	$P < 0.0001$

Ratio for expression IL2, Healthy animal expression = 3.33/3.33=1

And Animal with Disease or with sub clinical mastitis expression = 1.02/ 3.33= 0.3063 according to this the Samples expression at 0.3063 and the fold less than Healthy animal expression table (5).

Ratio for expression TNFα Healthy animal expression = 3.012/ 3.012=1 Animal with Disease or with sub clinical mastitis expression = 6.2/ 3.012= 2.06 So the Samples expression at 2.058 and fold higher than Healthy animal expression table (6).

Table (5): Ratio for IL2 gene expression in goat sub-clinical mastitis-Healthy goat

Variables	Ratio of gene expression of IL2	Fold change
Healthy goat	3.033/3.33	1
Sub-clinical mastitis goat	1.02/3.33	0.3063

Table (6): Ratio for IL2 gene expression in goat sub-clinical mastitis-healthy goat

Variables	Ratio of gene expression of TNFα	Fold change
Healthy goat	3.012/ 3.012	1
Sub-clinical mastitis goat	6.2/ 3.012	2.06

Finally, The acquisition and characterization of microorganisms in milk caused by SCM in dairy goats was completed. The SCM bacteria in this experiment could only be identified down to the genus level. One of the most used technologies in bioscience, diagnostics, and forensic research is now PCR. A more precise identification technique, such as a molecular method using multiplex polymerase chain reaction to identify the actual species of bacteria, would be required to avoid drawing any incorrect conclusions from the data. The research contributes to our understanding of the immunological reactions that take place in goat subclinical mastitis, a condition that has a negative influence on milk output and quality. It is crucial to establish their pathogenic relevance, the virulence components required to induce poisoning, and penetration into the mammary gland in order to assist veterinarians in administering the right antibiotic treatment.

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