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Original Article

Non-Tuberculous Mycobacteria *hsp65* in Relation to Bovine Subclinical Mastitis and IL6 Concentration in Cow's Milk

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Abstract

Mastitis is a condition in which the mammary tissue becomes inflamed. Changes in color and the appearance of clots, as well as increases in cell counts in the milk, are all indicators of inflammation. Mastitis is a common occurrence in cows as a result of inframammary infections. The present study aimed to find out how often nontuberculous mycobacteria (NTM) mastitis occurs and how *hp65* affects Interleukin (IL) 6 concentrations. The findings of the Modified Whiteside Test (MWT) on the milk samples from 70 cows, 50 sheep, and 30 goats revealed that 82.9%, 76.7%, and 46.7% of milk samples from cows, sheep, and goats were positive, respectively. This test demonstrated a range of positive milk sample MWT reactions, and the difference among the current positivity score results was statistically significant ($P<0.05$). The presence of NTM in analyzed milk samples from cows and sheep was confirmed by *hsp65*-based polymerase chain reaction (PCR) and gene sequencing, with significant differences ($P<0.05$) in 71.4% and 20% of milk samples from cows and sheep, respectively. The PCR detection of the NTM *hsp65* gene in fecal samples from cows, sheep, and goats indicated that cows (80%) had the highest proportion of NTM *hsp65* gene amplification, followed by goats (70%), while sheep fecal samples had the lowest amount (22%). The difference among the positive NTM *hsp65*-based PCR was statistically significant ($P<0.05$). The phylogenetic tree and sequence analysis of the *hsp65* gene revealed two novel variant NTM *hsp65* genes that were deposited in Gene Bank (GenBank acc. LC636294 and LC636295). The current examined NTM *Hsp65* Mycobacterium sequences which were included in the Mycobacterium avium clade in the currently produced tree ELISA detection of IL6 concentration in cow's milk revealed that IL-6 concentration in mastitis milk was varied. The mean of IL-6 concentration in cow's mastitis milk with MWT scores (+++ve) and mean of IL6 concentration in each MWT scores (++ve), MWT scores (+ve), and -ve MWT cow's milk had a highly significant difference ($P<0.001$).

Keywords: hsp65, IL6, Non-tuberculous mycobacteria, Polymerase chain reaction

1. Introduction

Non-tuberculous mycobacteria (NTM), also known as "mycobacteria other than tuberculosis" (MOTT) or "environmental mycobacteria" (EM), can be observed in water, soil, domestic and wild animals, milk, and food. The NTM infections are opportunistic infections that can harm both humans and animals, including fish and poultry (1). Mastitis is a condition in which the mammary tissue becomes inflamed. Changes in color and the appearance of clots, as well as increases in cell counts in the milk, are all indicators of inflammation (2). Mastitis is a common occurrence in cows as a result of inframammary infections (IMIs) (3). Mastitis affects animals all around the world, although the prevalence and incidence rates vary widely (2). It is the most costly disease in the dairy sector. Reduced milk yield and quality, as well as the cost of medications, veterinary services, diagnostics, and culling of

incurable cases, all result in huge losses (4).

Mastitis is diagnosed by looking for elevated somatic cell counts (SCCs) in the milk, which is usually performed in conjunction with bacteriological testing using either phenotypic (milk cultures) or genotypic polymerase chain reaction approaches (5). Several prior investigations assessed the immune mediators that play a role in the inflammatory response to an IMI. Cytokines, acute phase proteins, and eicosanoids were found in milk from mastitic udders (6). The bulk of previous studies contained laboratory data on NTM isolation and speciation; nonetheless, they lacked clinical, microbiological, and radiological correlations, as well as treatment outcome information. Recent studies on NTM diseases considered these issues (7). However, reports of animal diseases caused by NTM are uncommon in Iraq, except for a few investigations in Basrah, Iraq, on *Mycobacteriumavium* sub sp. paratuberculosis, the etiological agent of Johne's disease (8). Other studies conducted in Iraq have detected NTM obtained from clinical samples suspected of Mycobacterium tuberculosis complex (MTBC) (9, 10) and MTBC from patients suspected of tuberculosis (9, 10).

For the identification and differentiation of NTM, molecular techniques, such as PCR, real-time PCR, multiplex real-time PCR, and restriction fragment length polymorphism (RFLP) have been used (8, 11, 12). Genes encoding the conserved and non-conserved portions of mycobacterial 65 kDa heat shock protein have been discovered to be useful for the identification of NTM (13). The 441bp amplicon of the *hsp65* gene, which is found in all mycobacterial species, has more diversity than the 16S rRNA gene sequence, making it useful for distinguishing genetically related species. A variation in the *hsp65* gene sequence can be used to distinguish between slow-growing and fast-growing mycobacteria at the species level.

In veterinary medicine, some NTM species may colonize the host without causing disease; however, they incorrectly stimulate the host's immune system, preventing tuberculosis immuno-diagnosis with tuberculin-based assays (14). Furthermore, NTM may have a negative impact on animal immunization with Mycobacterium Bovis Bacillus Calmette-Guérin (15). In light of the aforementioned issues, the present study aimed to find out how often NTM mastitis occurs and how *hp65* affects IL6 concentrations.

2. Materials and Methods

2.1. Sample and Sampling

The present study was conducted in different areas of Basrah and Thi-Qar provinces, south of Iraq, from October 2019 to December 2020. The presence of visibly abnormal milk (flakes, clots, or serous milk) with systemic disease or symptoms of mammary gland inflammation was described as clinical mastitis. Clinical samples included milk from 70 cows, 50 sheep, and 30 goats of different breeds and ages through cooperation with veterinary hospital staff and owners. Fresh fecal samples (0.2-10 g) were collected from 60 cows, 50 sheep, and 30 goats and placed in cleaned screwed tubes. After cleaning the udder, 10 ml of milk was collected from the 4 quarters into a sterile plane tube by hand milking, and the first three jets were discarded.

2.2. Screening for Mastitis by Modified Whiteside Test

The modified white side test was carried out according to a procedure suggested by Kahir, Islam (16). On a slide with a black background, 100 µl of 4% sodium hydroxide solution was poured into 250 µl of cool milk, and the mixture was violently agitated for 20 sec. After the addition of sodium hydroxide solution to regular milk, there was no reaction and the milk remained uniformly opaque. While the mastitis milk exhibited a reaction, it was graded as follows: Negative (N): a milky, opaque combination with no precipitant: (+), There is a small amount of clumping: (++), and There are coagulated components in the mixture: (+++). A large number of precipitants are present.

2.3. Non-tuberculous Mycobacteria Detection in Milk and Faecal Samples Using Molecular Methods

The NTM *hsp65* genes were identified in milk and fecal samples using PCR and sequence analysis of the

NTM hsp65 genes. Telenti, Marchesi (17) recommended primer sets for amplifying the NTM *hsp65* gene gSYNCTM DNA Extraction Kit, Genaid, Korea and PrestoTM Stool DNA Extraction Kit, Genaid, Korea were used to extract genomic DNA from milk and fecal samples, following the manufacturer's instructions. The isolated DNA was validated on a 1% agarose gel stained with GreenStarTM Nucleic Acid Staining BioNeer (Korea). The concentration and DNA purity were then determined using a Nanodrop spectrophotometer (Quawell, USA) set to 260/280 nm.

The PCR reaction premix was AccuPower PCR premix (Cat # K-2012, Bioneer, Korea), which comprised 1 U of Top DNA polymerase, 250 µM of dNTPs, and 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, and 1.5 mM of MgCl2.Taq PCR Premix (5µ l), Tb11 (1 µl), Tb12 (5 µl), DNA (3 µl). Distill water (10 µl) made up the reaction mixture (20µ l). The hsp65 gene was amplified using a PCR thermocycler (Bioneer, Korea). Tb11 (5-ACCAACGATGGTGTGTCCAT-3) and Tb12 (5 CTTGTCGAACCGCATACCCT-3) were used in the PCR, targeting a 441-bp fragment in the Mycobacterium species' hsp65 gene (17). The *hsp65* gene was amplified using the following PCR conditions: 94ºC for 5 min, followed by 30 cycles of 94ºC for 30s, 61ºC for 30s, and 72ºC for 50 s, with a final extension at 72ºC for 10 min. Electrophoresis on 1.5% gel agarose was used to analyze 3 µl of PCR products. After electrophoresis and gel staining with GreenStar^{TM} Nucleic Acid Staining/ BioNeer (Korea), fragments in the gel documentation system were observed under UV light (Gel Doc, ATP Co).

The resolved hsp65 PCR amplicons were commercially sequenced from both the forward and reverse termini according to the sequencing company's instructions (Macrogen Inc. Geumchen, Seoul, South Korea). Using BioEdit Sequence Alignment Editor Software (version 7.1), the sequencing results of the PCR products were edited, aligned, and assessed as long as they matched the appropriate sequences in the reference database (DNASTAR, Madison, WI, USA). The nucleic acids found in the PCR amplicons and their respective locations in the reference genome were numbered. SnapGene Viewer (version 4.0.4) (https://www.snapg ene.com) was used to annotate each discovered mutation within the Mycobacterium genes.

The discovered variants were matched with their nearby homology reference sequences using the NCBI-BLASTn service, and the results were analyzed (18). Thereafter, using the neighbor-joining strategy, an inclusive tree spanning the observed variance was built and exhibited using the iTOL suit, resulting in a traditional clade-building tool. Sequences from each phylogenetic species group were tagged appropriately in the complete tree.

2.4. Enzyme-Linked Immunosorbent Assays

The manufacturer's instructions were followed in using the Bovine Interleukin 6 ELISA Kit (USA). In brief, 100 µl milk samples and diluted standards were pipetted into an enzyme-linked immunosorbent assay (ELISA) plate pre-coated with antibody, mixed gently on the sides, and incubated at room temperature for 45 min. The plates were hand-washed three times using wash buffer. In each well, 100 µl of detection antibody was placed and then incubated at room temperature for 30 min. Thereafter, the wells were washed and blotted as aforesaid. Each well received a pipette of diluted HRP-streptavidin (100 µL). The plate was covered and incubated at room temperature for 30 min. The wells were cleansed and blotted as before. Each well was filled with 100 µL of TMB substrate solution and incubated at room temperature for 10 min in the dark. The chromogenic process was stopped with 100 µL of stop solution, and the optical density values were read with a plate reader at 450 nm (Micro ELISA auto reader, Biotek, USA). The average of duplicate readings was calculated for each standard to interpret the ELISA results, and the data were utilized to create a Standard Curve. The values for the test samples were extrapolated from the standard curve.

2.5. Statistical Analysis

The chi-square test and the t-test were performed to assess the correlation between the data, with the level of significance set at 5%. The data were analyzed in SPSS software (version 11).

3. Results

3.1. Modified Whiteside Test Screening Results

Varying degrees of the positive milk samples Modified Whiteside Test (MWT) reactions are presented in table 1, the score of positivity (+++ve) was only observed in the milk samples from 22 (37.93%) cows and 11(28.9%) sheep, while the milk samples of all cows, sheep, and goats demonstrated the positive score (++ve) at different percentages (18.96,21.1 and 14.3%, respectively). The positivity score (+ve) was detected in the milk samples of cows **(**43.11%,), sheep (50%), and goats (85.7%). The difference in the current results of the positivity score was statistically significant (γ^2: 35.541; df:8; *P=0.00002*).

3.2. Polymerase Chain Reaction Detection of NTM *hsp65* in Milk Samples

The results of *hsp65*-based PCR confirmed the presence of *hsp65* NTM in tested milk samples from cows, sheep, and goats by using Tb11: Tb12 primers. The amplified product of the NTM hsp65 gene (441 bp) was detected in 50 (71.4%) and 10 (20%) milk samples of cows and goats, respectively. Nonetheless, this gene was not detected in the milk samples from goats (Figure 1; Table 2). The difference among the positive NTM *hsp65*-based PCR was statistically significant (γ^2 : 57.143; df:8; *P=0*).

3.3. Polymerase Chain Reaction Detection of NTM *hsp65* Gene in Fecal Samples from Cows, Sheep, and Goats

The amplified product of the NTM *hsp65* gene sequence (441 bp) was detected in the fecal samples from 48 cows, 11 sheep, and 21 goats (70). A higher percentage of NTM *hsp65* gene amplification was observed in cows (80%), followed by goats (70%), while the lower percentage (22%) was observed in sheep milk samples (Figure 1; Table 3). The difference among the positive NTM *hsp65*-based PCR was statistically significant (γ^2: 40.04; df: 2; *P=0*).

Table 1. Varying scores of modified Whiteside test positive reaction

Milk sources	Tested number	Total positive n. (%)	Positive results n. (%)			*P*-value
			+ ve	++ ve	+++ ve	
Cows	70	58 (82.9)	25 (43.11)	11 (18.96)	22 (37.93)	
Sheep	50	38 (76)	19 (50)	8 (21.1)	11 (28.9)	<0.05
Goats	30	14 (46.7)	12 (85.7)	2 (14.3)	0	

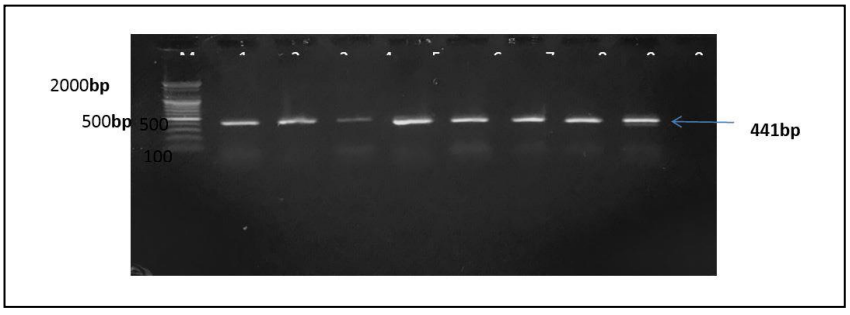

Figure 1. Polymerase chain reaction amplification of NTM *hsp65* gene in cow milk and fecal samples the product was electrophoresis on 1.5% agarose. M: DNA Ladder; Lane (1-9) NTM hsp65positive (441bp)

Table 2. Results of NTM *hsp65*-based polymerase chain reaction in three milk sources

Milk sources	Examined milk Samples n.	*hsp65* based PCR+ve n. (%)	*hsp65* based PCR-ve n. (%)	*P*-value
Cows	70	50 (71.4)	20 (28.6)	
Ewes	50	10 (20)	40 (80)	<0.05
Goats	30	0	30 (100)	

Table 3. Results of *hsp65* PCR in fecal samples from three sources

Animals	Tested number	*hsp65* PCR positive n (%)	*hsp65* PCR negative n (%)	*P*-value
Cows	60	48 (80)	12 (20)	
Sheep	50	11 (22)	39 (78)	<0.05
Goats	30	21 (70)	9 (30)	

3.4. Sequence Analysis and Phylogenetic Tree of *hsp65*

The present study reported two new *Mycobacterium avium hsp65* in Gene Bank according to the sequence comparison, types of nucleotide substitution, and amino acid changes among the current NTM (*hsp65*) gene and other Gene Bank reported NTM (*hsp65*) genes. The currently identified NTM (*hsp65*) was deposited in Gene Bank as *Mycobacterium avium* NTM (*hsp65*). (Accession number: LC636294 and LC636295). The current NTM (*hsp65*) had 99% similarity with Mycobacterium avium *hsp65* sequences (GenBank acc. NZ CP009360.4). The phylogenetic analysis of the NTM (*hsp65*) sequences demonstrated that the new IraqiNTM (*hsp65*) was placed in a group with the previously reported *Mycobacterium avium* clade (Figure 2).

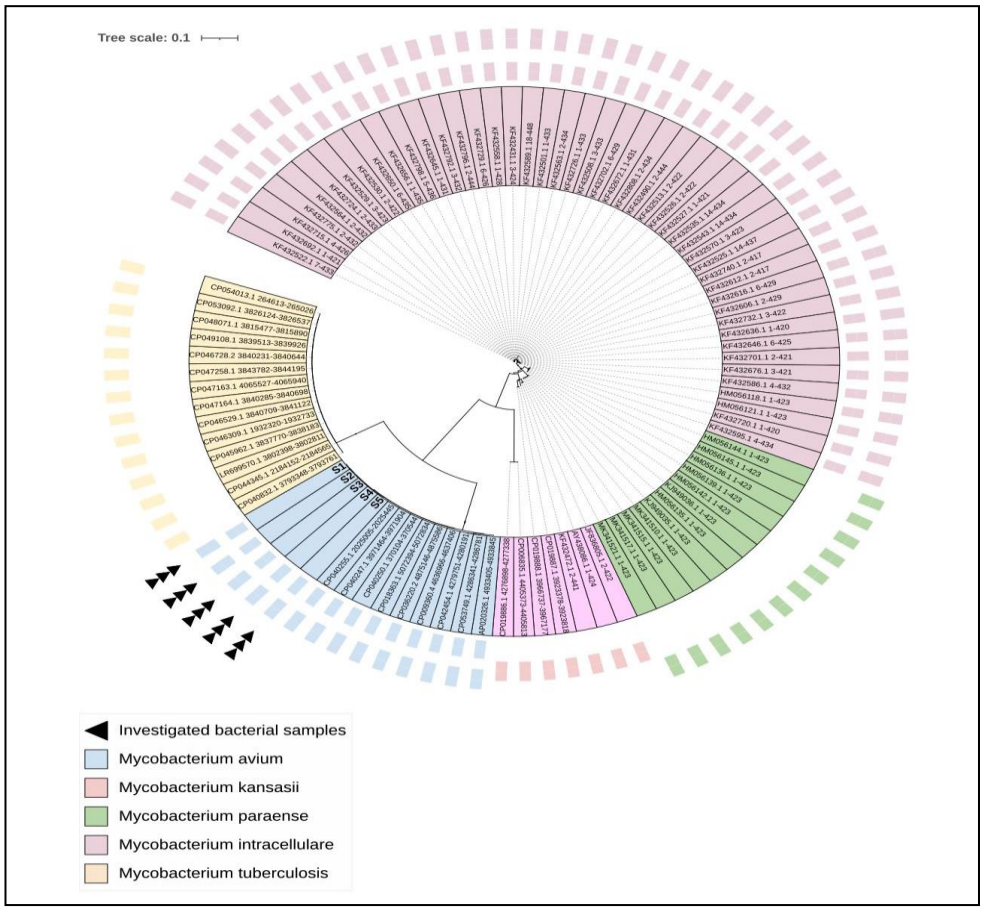

Figure 2. Amplicon (441 bp) phylogenetic tree partially covers *Hsp65* sequences inside the M.avium genomic sequences. The varying colors represent the different arrangements of the examined variations within their Genbank-deposited sequences. The number "0.1" at the upper left of the tree describes the degree of scale range among the creatures classified by the thorough tree. The numbers in the tree represent the degree of phylogenetic distances between the bacterial species studied. The symbol "S" stands for the code of the specimens examined in this study

3.5. ELISA Detection of IL6 Concentration in Cow's Milk

Table 4 demonstrates variable IL-6 concentration values (pg/mL) in cow's mastitis milk which appeared with different modified MWT scores. The IL-6 concentration was statistically calculated as a median, mean, and standard deviation. A statistical t-test was also used to determine the significant difference among IL-6 concentration means. A highly significant difference was observed between the mean of IL-6 concentration in cow's mastitis milk with MWT scores (+++ve) and the mean of IL6 concentration in each MWT scores (++ve), MWT scores (+ve), and -ve MWT cow's milk (*P<0.001*).

Table 4. Concentration of IL-6 in milk from cows with mastitis caused by nontuberculous mycobacteria

Modified Whiteside test Scores	No.	Median	Mean	Standard Deviation
+++	22	9.948217	10.01397[abc]	6.955264
++	11	2.455613	4.634477[b]	3.618207
+	25	2.268625	2.751798[a]	1.816182
-ve	11	2.455613	3.917968[c]	2.707616

a, A Referred to significant differences between IL-6 concentration means of MWT scorer (+++ve) and MWT scores (+ve) (*P<0.0001*).
b, B Referred to significant differences between IL-6 concentration means of MWT scorer (+++ve) and MWT scores (++ve) (*P*<0.001).
c, C Referred to significant differences between IL-6 concentration means of MWT scorer (+++ve) and MWT scores (-ve) (*P<0.0001*).

4. Discussion

The isolation of NTM in body fluids was previously assumed to be due to pollution; nonetheless, they have been recently linked to the presence of a disease (19). Despite worldwide interest in NTM infections, Basra-Iraq still lacks data on the prevalence and diversity of NTM infections in livestock. The current study was the first to use standard PCR and gene sequencing to detect NTM in milk and fecal samples from cows, sheep, and goats. The findings of this study demonstrated that the overall percentage rates of NTM in cows and sheep were 71.4% and 20%, respectively, which is significantly lower than the primary screening results (82.9% and 76%) calculated using MWT. Goats, on the other hand, tested negative for *hsp65*, although 46.7% of them tested positive using the MWT.

The NTM are infrequent causes of mastitis in cows, sheep, and goats. These bacteria have been described as the etiological cause of mastitis in several older papers. Nevertheless, the NTM described in this study was from milk of cows, ewes, and goats, and had a higher amount of somatic cells and an abnormal consistency. Since the affected animals' medical histories did not reveal any previous occurrences of mastitis, it was unable to verify whether NTM was the major cause of the mastitis. However, the current study relied on PCR detection of *hsp65* genes, signifying that the mastitis milk NTM could be accurately identified.

Previous studies validated the findings of the current research. Siqueira, Lopes (20) and Escobar-Escamilla, Ramirez-Gonzalez (21) used phenotypic and molecular characterization to demonstrate the correct identification of mastitis milk isolates as NTM in one adult Holstein cow. Furthermore, in the studies by Franco, Paes (22) and Machado, Gressler (23), the M. smegmatis group species have been found to produce clinical mastitis in sheep and dairy cows. In addition, Jayasumana, Galappaththi (24) verified the presence of NTM in two milk samples (0.8%). According to PCR data. The detection of the *hsp65* gene provides a better identification tool for differentiating Mycobacterium species and could help with diagnosis.

Clinical microbiology aims to offer timely and precise information on the presence or absence of microorganisms that could be implicated in the progression of an infectious disease. As a result, slow-growing infections, such as mycobacteria, require several weeks to isolate and detect. Therefore, instead of using traditional microbiological approaches, *in vitro* amplification of mycobacterial nucleic acids can be used. In this study, NTM *hsp65* PCR and Sequencing analyses, as well as a phylogenetic tree, were used. The amplified product of the NTM *hsp65* gene sequence

(441 bp) was found in the feces of 48 (80%) cows, 11 (22%) sheep, and 21 (70%) goats for a total proportion of 57.14% (80/140).

In contrast to the current findings, K.K.J. Alzaidi (25) in Basrah, Iraq, reported that 6% of 50 cow dung samples which were evaluated by MAP-*hsp65* based PCR, gene sequencing, and phylogenetic tree analysis were positive. Sumiyah, Deepti (13) found that 5.5% of 200 fecal samples tested positive for non-tuberculous mycobacteria using a PCR assay targeting the *hsp65* gene. Leão (26) found Map IS900 nested real-time PCR positive in 83% of 155 fecal samples from goats, cattle, and sheep, and 26% of 98 milk samples from cattle. According to NTM *hsp65* genes previously reported in Gene Bank, sequencing and phylogenetic tree analysis revealed the presence of two novel NTM *Hsp65* genes (GenBank acc. LC636294 and LC636295).

By comparing its nucleotide sequence to reference sequences, several prior investigations validated the importance of mycobacteria's 65-kDa heat shock proteins in the identification of species (25, 27). The present sequenced NTM *hsp65* and Mycobacterium avium *hsp65* sequences (GenBank acc. NZ CP009360.4) have a marked sequence similarity (99%). The current findings were consistent with those reported by K.K.J. Alzaidi (25) who discovered 98%-99% sequence homology between *hsp65* and MAP *Hsp65* nucleotide sequences. In contrast to the results of the present study, in the research by Joao, Cristovao (28), *hsp65* nucleotide sequences from seven isolates (E4, E17–E19, and E28–E30) were determined to share 96% homology with Mgordonae ATCC 14470.

Concerning the proinflammatory cytokine IL-6 concentrations, in milk, intramammary infections cause various changes in milk composition. Inflammatory proteins, such as interleukins, are significantly increased rather than chemical composition (29). Inflammatory cytokines, such as IL-6, are linked to inflamed mammary glands at the beginning of mastitis, according to Sakemi, Tamura (30). Clinical samples, on the other hand, had higher IL-1 and IL-6 levels than sub-clinical samples. According to Sakemi, Tamura (30), inflammatory cytokines, such as IL-6, are linked to inflamed mammary glands at the beginning of mastitis. Clinical samples, on the other hand, had higher IL-1 and IL-6 levels, as compared to sub-clinical samples.

The results of the current study revealed that there was a significant difference between the mean of IL-6 concentration in cow's mastitis milk with MWT scores (+++ve) and the mean of IL6 concentration in each MWT scores (++ve), MWT scores (+ve), and negative MWT cow's milk. These findings pointed out that the amount of IL-6 is related to the severity of inflammation, pointing to a relationship between IL-6 levels and inflammation severity. In line with the current findings, Nayan (31) found that IL-1 and IL-6 expression was higher in subclinical mastitis, as compared to that in control samples.

Furthermore, the presence of IL-6 in milk indicated the presence of subclinical mastitis before the presence of increased SCC. As a result, IL-6 detection in milk could be used as a future predictor of subclinical mastitis. In comparison with somatic cell count, Bayoumi, Amer (32) discovered that IL-6 determination had superiority and sensitivity for the detection of subclinical mastitis. In conclusion, IL-6 concentrations in milk are linked to inflammation and the number of somatic cells. The NTM was identified as a rare ruminant mastitis causative agent in ewes, dairy cows, and goats in this investigation.

Authors' Contribution

Study concept and design: N. R. A.
Acquisition of data: F. A. A.
Analysis and interpretation of data: N. R. A.
Drafting of the manuscript: N. R. A.
Critical revision of the manuscript for important intellectual content: F. A. A.

Statistical analysis: F. A. A.
Administrative, technical, and material support: F. A. A.

Ethics

The protocol was approved by the University of Basrah, Basrah, Iraq review board.

Conflict of Interest

The authors declare that they have no conflict of interest.

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