Research Article

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PCR detection of Non-Tuberculous Mycobacteria 16s rDNA and its Relation to Bovine IL6 Concentration in Subclinical Mastitis Cow's Milk

Rawaa Bannay Zubairi[1], Moaed H. Sayhood[2].

1-Sothern Technical University/Basrah Technical Institute, Basrah, Iraq.

2-Department of Public Health, College of Veterinary Medicine, University of Basrah, Basrah, Iraq.

Corresponding Author Email Address: rawa@stu.edu.iq

ORCID ID: https://orcid.org/0000-0002-2007-5961

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Abstract

The mammary tissue gets inflamed when it is affected by mastitis. Inflammation is indicated by changes in the appearance and color of clots and an increase in milk cell counts. The current study tried to find out how leukocytes change the levels of interleukin (IL) 6 in milk and what role nontuberculous mycobacteria (NTM) plays as an accusatory agent in subclinical mastitis (SCM). The major screening method for subclinical mastitis in samples of cow's milk was the Modified Whiteside Test (MWT). Prevalence of SCM according to different MWST reaction scores was estimated in 70 cow' milk samples. The SCM was observed in 58 cows (82.9%). The highest prevalence (35.7%) was observed in the cow's milk sample with MWST reaction scorer 1+, followed by a prevalence (31.5 %) of cow's milk sample with MWST reaction scorer 3+. The 16s rRNA-based polymerase chain reaction (PCR) has been used to confirm the existence of NTM spp. in the examined cow's milk samples. Out of 70 milk samples, 8 (11.4%) had positive PCR results. An ELISA test was used to evaluate how the somatic cell counts affected IL-6 levels in cow's milk. The test's findings showed that there had been a significant variation ($P<0.05$) in the average concentration of IL-6 in SCM milk. Cow's milk samples with MWST reaction scorer 2+ had a higher mean IL-6 concentration (474.5670), while milk samples with MWST reaction scorer 1+ had a lower mean IL-6 concentration (243.2944).

Keywords: Cow's Milk, Polymerase chain reaction, non-tuberculous mycobacteria

Introduction

The acid-fast, rod-shaped, non-motile NTM does not produce spores. Because of the high lipid content of their cell walls, members of such genera resist to host defenses, which can lead to chronic disease. Those micro-organisms are distinguished by their capacity to generate mycolic acid that provides pathogenicity, virulence, acid-fast cell wall properties, and resistance to host cell defenses (1). Both animals and humans, including poultry and fish, could be harmed by the opportunistic NTM infections (2).

Dairy products and milk are the primary protein sources for most individuals in countries with low to middle incomes. Buffalo and cattle farming account for much of the world's milk production (3). However, despite the modern animal-raising methods and genetic upgrades, production per head has remained low (4). The mammary tissue gets inflamed when it is affected by mastitis. Increases in the number of cells in the milk, together with changes in the appearance and color of clots, are all signs of inflammation (5). Mastitis can be identified by searching for high somatic cell counts (SCCs) in milk, typically done in conjunction with the bacteriological testing with the use of either phenotypic (milk cultures) or genotypic PCR techniques (6). Numerous indirect tests, such as the MDST (Mastrip), California Mastitis Test (CMT), Modified White Side Test (MWST), and Bromothymol Blue Card Test (BTB), are utilized to identify subclinical mastitis (SCM). The CMT and the Whiteside test share the same foundation, which uses the extra leukocytes in milk as a marker of inflammation. Compared to CMT, Whiteside test is easy, quick, and affordable (7). Immune mediators involved in the inflammatory response to intramammary infections were evaluated in several earlier studies. Milk from mastitic udders included cytokines as well as acute phase proteins (8).

Geographically diversified NTM species include the most commonly isolated *M. avium* complex and fast-growing mycobacteria like *M. abscessus* and *M. fortuitum* (9). There are few trustworthy NTM statistics globally, and reporting requirements are inadequate since public health agencies in most nations do not view NTM as a communicable disease (10). Molecular methods, which are sensitive, rapid, and specific, were created to identify Mycobacteria because of the shortcomings of older methods. Molecular methods for mycobacteria identification have significantly improved over the past 20 years (11). 16S rRNA replication is the most important and precise technique for identifying NTM in clinical specimens. Through sequencing the 16S rRNA gene, *Mycobacterium* species could occasionally be identified down to the level of the species (12,13). Except for a few studies that have been carried out on *Mycobacterium avium* sub sp. paratuberculosis, the causative Johne's disease agent, carried out in Basrah, Iraq, reports of NTM-caused animal infections are rather uncommon in Iraq (14). The NTM has been found in the clinical samples that have been obtained from patients who are suspected of having tuberculosis and were thought to contain Mycobacterium tuberculosis complex (MTC) in another Iraqi study (15). Because of these issues, the current study tried to find out how somatic cell counts affect the amount of IL-6 in cow's milk and whether 16S rDNA could be used for quick PCR detection of NTM in milk samples.

Methods and Material

Milk sampling: The current study was conducted between October 2023 and December 2023 in several different Basra province regions. Visibly abnormal milk, like clots, flakes, or serous milk—signatures of inflammation in the mammary gland—

was present in the milk sample. Milk samples from 70 crossbreed cows of various ages have been collected through cooperation with livestock owners. The samples were taken with the use of 70% alcohol-moistened cotton, following the udder being cleaned with a piece of cloth. The first milk flow was stopped, and 10ml was collected into a sterile tube before being taken to the lab with an ice box (16).

Screening for SCM through Modified Whiteside Test: Mastitis screening using (MWST) was performed as per the modified test described by (7). The reagent used for the modified Whiteside test is a 4% sodium hydroxide solution. Well-mixed milk (250 µl) was placed on a glass plate with a dark background, and 100 µl of 4 % sodium hydroxide was added to the milk. The mixture of milk and reagent should be stirred rapidly with an applicator stick for 20 to 25 seconds; milk from normal animals will have no change after adding sodium hydroxide. Milk from a cow suffering from acute or subacute mastitis will become thick and viscid. However, an animal with a chronic case may have only a few white flakes. The basis of MWST and CMT is the same, reflecting the presence of excess leukocytes in milk as an indicator of inflammation. The scores for the MWST reactions were as follows:

Negative (N): No precipitant is present, and the combination is opaque and milky. 1+: The background is less opaque but still slightly milky, with larger particles of coagulated materials present and densely dispersed across the area. A minor amount of clumping is seen. 2+: The background appears waterier, with numerous clumps of coagulated material present. If the stirring was rapid, fine threads or strings could be present. 3+: The background is extremely watery and whey-like, with enormous amounts of coagulated material forming into strings and shreds.

Detection of Non-Tuberculous Mycobacteria in Milk with the Use of Molecular Approaches: PCR as well as sequence analysis were used to identify 16S rRNA gene in both milk and fecal samples. Primer sets for amplifying the 16S rRNA gene have been used, as advised by (17). The gSYNCTM DNA Extraction Kit (Genaid, Korea) has been utilized for extracting samples of the genomic DNA from milk in accordance with ’manufacturer's recommendations. GreenStarTM Nucleic Acid Staining BioNeer (Korea) was used for validating extracted DNA on a 1% agarose gel. Nano-drop spectrophotometer (Quawell, US) has been used in order to measure ’DNA's concentration and purity with the use of Telenti. This work used the AccuPower PCR reaction premix (Cat # K2012, Bioneer, Korea) that included 1U of Top DNA polymerase, 250µM of dNTPs, 30mM of KCl, 10mM of Tris-HCl (pH 9.0), and 1.5mM of MgCl2. Taq PCR Premix (5µl), Tb12 (1µl), Tb11 (1µl), nuclease-free water (10 µl), and DNA (3 µl) made up the reaction mixture (20 µl). Targeting a 441bp fragment in 16SrRNA gene of Mycobacterium species, the primers Tb-11 (5-ACCAACGATGGTGTGTCCAT-3) and Tb12 (5 CTTGTCGAACCGCATACCCT-3) have been utilized for the amplification the

16SrRNA gene using a PCR thermocycler (Bioneer, Korea) (18). Initial denaturation at a temperature of 94 ºC for 5min., 30 cycles of 94ºC for 30 sec., 61 ºC for 30sec., and 72 ºC for 50sec., and a final extension at 72ºC for 10 min. were the conditions for the PCR. A total of µl of PCR products have been subjected to electrophoresis on a 1.50% agarose gel. Following electrophoresis and gel staining using GreenStarTM Nucleic Acid Staining/BioNeer (Korea), observations of gel documentation system have been carried out under ultraviolet light (Gel Doc, ATP Co).

Enzyme-Linked Immunosorbent Assays (ELISA): As directed by the manufacturer, the Bovine Interleukin 6 ELISA Kit (USA) was used. To summarize, an ELISA plate that had already been coated with antibodies was filled with 100 µl of milk samples and diluted standards. After a 45-minute incubation period at room temperature, the contents were gently stirred on the sides. The plates were then manually cleaned three times with a washing buffer. After adding 100µl of detection antibody to every one of the wells, the wells were incubated for 30 mins at room temperature. After an additional round of blotting and washing, 100µl of diluted HRP-streptavidin was added to every well. After covering the plate, it was left to incubate for another half hour at room temperature. Following a second round of cleaning and blotting, 100µl of TMB substrate solution was added to every well, and the wells were then left to sit at room temperature for ten minutes in the dark. A 450 nm ELISA reader (Micro ELISA auto reader, Biotek, USA) was used to assess the optical density values after stopping the chromogenic process with 100µl of stop solution. For interpreting ELISA results, the average of the duplicate readings for each one of the standards was computed, and a Standard Curve was created by using the data. The standard curve was used to extrapolate the values for test samples.

Statistical Analyses: A one-way ANOVA and the least significant difference test (LSD) were used to evaluate the differences between groups of means, and the level of significance was chosen at less than 0.05 to show any association between the results. The statistical software SPSS V. 22 (SPSS Inc.) was used to perform all statistical computations.

Results

Modified Whiteside Test Screening Results: The prevalence of SCM according to different **MWST** reaction scorers was estimated in 70 cow's milk samples. The SCM was observed in 58 cow's (82.9%). The highest prevalence (35.7%) was observed in cow''s milk sample with **MWST** reaction scorer 1+, followed by a prevalence (31.5 %) of cow''s milk sample with **MWST** reaction scorer 3+ .(Table 1)

PCR Detection of *16SrRNA* in Milk Samples: Tb11: Tb12 primers, 16SrRNA-based PCR results verified the existence of NTM 16SrRNA in the examined Tb11: Tb12 primers, 16SrRNA-based PCR results verified the existence of NTM 16SrRNA in the examined cow's milk samples. In 8/70 (11.4%) milk samples, the 16SrRNA gene's amplified product (441 bp) was found (Table 1, Figure 1)

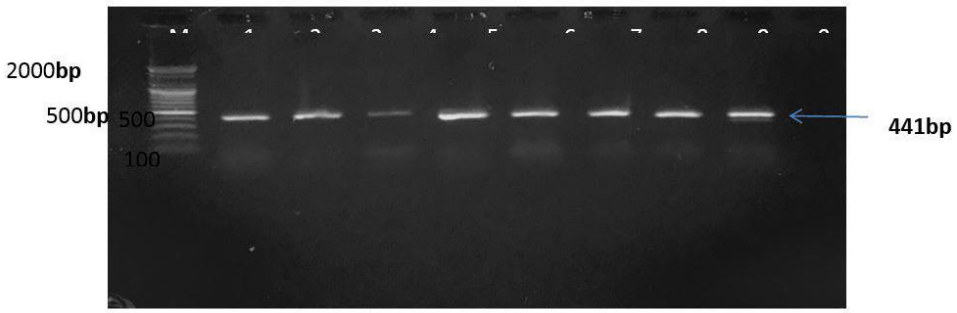

Figure 1. PCR reaction amplification of *16SrDNA* gene in samples of 'cow's milk.

Table (1): Modified White Side Test (MWST and *16S* rRNA PCR results of cow' 'milk samples

Test	Examined N.	MWST Reaction scorer			
		Positive n. (%)			Negative n. (%)
		1+	2+	3+	N
MWST	70	25(35.7)	11(15.7)	22(31.5)	12(17.1)
***16S*rDNA PCR**	70	0	0	8(11.4)	62(88.6)

ELISA Detection of IL-6 Concentration in Cow Milk: ELISA test was used to evaluate how the somatic cell counts affected IL6 levels in cow's milk. The test's findings showed that there had been a significant variation ($P<0.05$) in the average concentration of IL-6 in SCM milk. Cow's milk samples with MWST reaction scorer 2+ had a higher mean IL-6 concentration (474.5670), while milk samples with MWST reaction scorer 1+ had a lower mean IL-6 concentration (243.2944) (Table2).

Discussion

Other than *Mycobacterium avium* subsp. paratuberculosis, the incidence of NTM is rarely investigated in veterinary medicine (19). Thus, NTM isolation is often a follow-up outcome of TB testing. As a result, in the context of TB monitoring programs, most studies describe the isolation of numerous NTM species. Although commercially available kits are good at identifying Mycobacteria's most common clinical species, they are costly and poor at detecting rare or novel species (20). Other options should be created in this situation to give a precise diagnosis that most labs can provide. To correctly identify NTM, this research employed the highly conserved gene 16S rRNA for identifying mycobacteria. 16S rRNA was utilized in numerous investigations to identify NTM.

Table2. The effects of the somatic cell count on IL6 mean Concentration in SCM **'cow's milk**

MWT reaction scores	IL-6 -ELISA results				95% Confidence Interval for Mean	
	Positive n.	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound
1+	4	243.2944*	124.32505	41.44168	147.7297	338.8591
2+	10	474.5670*	218.13715	68.98102	318.5211	630.6129
3+	10	449.0173*	177.81169	45.91078	350.5485	547.4862
N	34	317.3236*	190.01505	32.58731	-383.6230	-251.0242

***: The mean difference is significant at 0.05 level**

16S rRNA is the gold standard and has proven to be more effective at detecting the most prevalent NTM, so it must be investigated first for accurate NTM identification (18, 19). In addition, the 16S rRNA was used in several earlier studies to identify this bacterium (21- 23). The PCR examination of milk samples revealed that 11.4% of cows had subclinical mastitis. Compared to (19) and (23), the current research's reported percentage of cows with subclinical mastitis was lower at 71.4%. In contrast, the current result was higher than the percentage (6%) recently reported by (19). Other studies, like (24) and (25), backed up this study's results by showing that isolates of mastitis milk have been correctly identified as NTM in a single adult Holstein cow. These isolates cause clinical mastitis in dairy cows and ewes, as reported by (26) and (27). Furthermore, (28) acquired 26% positive NTM out of 98 samples of cattle milk, and (29) demonstrated NTM presence in 2 milk samples (0.80%) depending on the results of the PCR. Numerous factors, such as lactation stage, animal age, number of lactations, sample time, milk production, and frequency of daily milking, can be responsible for the disparity between these results (30). In this investigation, MWST was utilized for primary screening of subclinical mastitis in addition to PCR testing of milk samples. The test's findings showed that the percentage of cows (82.9%) was significantly lower than the PCR's projected results. Another researcher (7) confirmed the frequency of SCM in cows, supporting the existing MWST's overall proportion of 58%, which was 54% among all cattle breeds. However, some prior reports indicated a higher frequency of SCM. According to other authors (31), the prevalence was lower than what was reported (32). The limited size, sample mix (cross breeds only), and SCM detection techniques may be the causes of these variances (17). WST's sensitivity and specificity were

comparatively lower than those of other tests, according to most authors (33). Some writers say WST is a better test than CMT (34).

In terms of the levels of proinflammatory cytokine IL-6 in milk, intra-mammary infections alter the composition of milk in several ways. Instead of chemical composition, there is a marked rise in inflammatory proteins, including interleukins (35). When mastitis first appears, inflammatory cytokines like IL-6 are associated with the inflamed mammary glands. Compared with sub-clinical samples, IL-6 and IL-1 levels have been higher in clinical samples (36). The current research's findings showed that there has been a significant variation ($P<0.05$) in the mean content of IL6 in SCM milk. Cow's milk samples with moderate SCM reaction (++ve) had a higher mean IL-6 concentration (474.5670), followed by milk samples with high SCM reaction (+++ve) with an IL-6 concentration (449.0173), and milk samples with slight SCM reaction (+ve) with a lower mean IL-6 concentration (243.2944). These results demonstrated a correlation between levels of IL-6 and the degree of inflammation, indicating that IL-6 levels are correlated with inflammation severity. (37) discovered that subclinical mastitis had higher levels of IL-1 and IL-6 expression than control samples, consistent with the present results. Additionally, the presence of IL-6 in milk suggested that subclinical mastitis existed before elevated SCC. Therefore, the identification of IL-6 in milk may be one of the potential indicators of subclinical mastitis. (38) found that the determination of IL-6 has sensitivity and superiority for identifying subclinical mastitis compared to somatic cell count. This study concluded that IL-6 levels in milk are associated with inflammations and somatic 'cells' amounts and that NTM is an uncommon ruminant subclinical mastitis causative agent in dairy cows.

Conclusion

The 16S rRNA was utilized in numerous investigations to identify NTM. The PCR examination of milk samples revealed that 11.4% of cows had subclinical mastitis. Numerous factors, such as lactation stage, animal age, number of lactations, sample time, milk production, and frequency of daily milking, can be responsible for the disparity between these results. When mastitis first appears, inflammatory cytokines like IL6 are associated with the inflamed mammary glands. IL6 levels in milk are associated with inflammations and somatic cells' amount, and NTM is an uncommon ruminant subclinical mastitis causative agent in dairy cows.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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الكشف عن 16s rDNA للبكتريا غير السلية بأستخدام تفاعل البلمرة المتسلسل وعلاقته بتركيز (IL-6)البقري في عينات حليب الابقار المصابة بألتهاب الضرع تحت السريري

رواء بناي زبيري[1] , مؤيد حنون صيهود [2].

1-الجامعة التقنية الجنوبية، المعهد التقني، البصرة' العراق.

2-فرع الصحة العامة, كلية الطب البيطري ،جامعة البصرة, البصرة, العراق.

الخلاصة

تلتهب أنسجة الضرع المصابة بألتهاب الضرع. يُشار إلى الالتهاب من خلال التغيرات في مظهر ولون الجلطات، وكذلك من خلال زيادة عدد خلايا الحليب.يسعى البحث الحالي الى تحديد كيف تغير كريات الدم البيضاء تركيز (IL-6)ودور البكتريا غير السلية كعامل مسبب لالتهاب الضرع تحت السريري. كانت طريقة الفحص الرئيسية لالتهاب الضرع تحت السريري(MWT) المعدلة. تم تقدير البكتريا غير السلية في الاختبار المعدل كعامل رئيسي في التهاب الضرع تحت السريري ل 70 عينة من الحليب البقري.لوحظ وجود البكتريا غير السلية في 58 عينه (28.9%).اعلى معدل انتشار كان (35%)لعينات +1 تليها نسبة (31.5%)لعينات +3. تم استخدام تفاعل البلمرة المتسلسل القائم على 16s rRNA لتأكيد وجود انواع البكتريا غير السلية في عينات حليب البقر المفحوصة. اظهرت 8(11.4%) نتيجة موجبة لاختبار البلمرة المتسلسل .تم استخدام الاختبار المناعيا(ELISA) لتقييم كيفية تأثير الخلايا الجسدية على مستويات (IL-6) في عينات الحليب. اظهرت نتائج الاختبار وجود تباين كبير ($P<0.05$) في معدل تركيز (IL6).كان لعينات +2 اعلى معدل تركيز بينما اظهرت عينات +1 اقل معدل تركيز.

الكلمات المفتاحية: حليب البقر، تفاعل البلمرة المتسلسل، البكتريا غير السلية.