Research Article

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Detection of Non-tuberculous mycobacteria *hsp65* in some ruminant's subclinical mastitis milk and rheumatoid arthritis patient's blood

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Abstract

The main aim of this study was to detect non-tuberculous mycobacteria hsp*65* in animal's subclinical mastitis milk and Rheumatoid arthritis patients blood. Modified White Side Test (MWT) was used as a preliminary screening test for subclinical mastitis in 70 cows ,50 ewes and 30 goats milk samples. The results of this test revealed that 58(82.9%), 38(76%) and (46.7%) of cows, ewes and goats milk samples respectively were positive with significant difference($P<0.05$). Statistically significant ($P<0.05$) varying score of positivity was observed by this test, as a follow; score (+++) in cows (37.93%) and ewes (28.9%) milk samples only **,** score (++) in all cows, ewes and goats milk samples at different percentage(18.96,21.1 and14.3%, respectively) and score (+) in cows **(**43.11%,), ewes (50%) and goats(85.7%) milk samples. Molecular detection of non-tuberculous mycobacteria (NTM)16SrRNA gene in Milk samples revealed that 3**(**6%**)** and 11 **(**22%**)** cows and ewes milk samples respectively showed positive results with significant difference($P<0.05$). The NTM hsp65 gene (441 bp) was detected in 50**(**71.4%**)** and10 **(**20%**)** milk samples of cows and ewes respectively with significant difference($P<0.05$).This gene also detected in 3(33.3%) rheumatoid arthritis patients blood samples with an overall percentage of 7.5% and negative results for this gene was observed in non rheumatoid arthritis patients blood samples with significant difference($P<0.05$). The hsp65 gen sequencing results confirmed high sequence similarity (99%) of the current *hsp65* (441 bp) sequences with *Mycobacterium avium* retrieved DNA sequences (GenBank acc. NZ_CP009360.4).

Keywords:NTM, subclinical mastitis, Rheumatoid arthritis

Introduction

Mastitis in cows causes greatest losses in dairy production, and bacteria is the most common agents (1; 2; 3; 4; 5). Some udder pathogens causes of human's infections or food-borne infections and intoxications (6; 7; 8; 9). The atypical or non-tuberculous mycobacteria (NTM) also referred to as mycobacteria other than tuberculosis complex are mostly ubiquitous free-living saprophytes (10). They have been isolated from water, soil, livestock, wild animals, milk, meat products and humans. The species of NTM are geographically diverse, and *M. avium* complex is the most frequently isolated followed by rapidly growing mycobacteria such as *M. fortuitum* and *M. abscessus*; (11). Information about NTM from Iraq is limited. However, Winthrop *et al.,* (12) reported that NTM are not considered notifiable by most countries' public health authorities reporting and global statistics of NTM are lacking. NTM are opportunistic pathogens to animals and humans, including fish and poultry (13).

Coinfection with different members of *M. tuberculosis* complex and *M. avium* subspecies or with other mycobacterial species combinations is not rare in animal and human hosts (14; 15). This adds more difficulties to the process of diagnosis, and thus, the study of the ecology of such widespread bacteria becomes even more complicated. In addition, the epidemiologic significance of wildlife-livestock-human interface is expected to increase together with human population growth (16).Rapid, specific, and sensitive methods for the detection and identification of mycobacteriosis are needed to avoid unnecessary delay in making appropriate decisions. Molecular methods can advantageously complement and accelerate this process by minimizing the need to wait for a culture report (17).

Unlike the most frequent agents of bovine mastitis, represented by staphylococci, streptococci and enterobacteria, which commonly cause an ascending infection of the teat canal, mycobacteria reach the mammary gland by systemic dissemination, probably after the spread of the primary infection by respiratory or digestive route (18.). Occasionally, infection can come from the environment of farms or by contamination of cannulas used in intramammary treatments. The occurrence of clinical or subclinical mastitis and the frequency of mycobacteria shedding into milk is controversial (19). In raw milk, the risk of mycobacteria elimination in lactating cows was investigated in Tanzania using phenotypic and molecular methods. Among 805 milk samples, there was

a predominance of atypical mycobacteria such as *M. flavescens* (n=13), *M. terrae* (n=7), *M. smegmatis* (n=4), *M. fortuitum* (n=2), *M. gordonae* (n=1), whereas only two isolates were identified as *M. bovis.* Despite the low prevalence of *M. bovis*, the presence of atypical mycobacteria in the milk of animals is a public health concern, because of the habit of consuming raw milk by these people. (20).

There is sufficient evidence showing a link between different microbial pathogens and RA development and progression (21). Rheumatoid arthritis (RA) is a chronic autoimmune disease, with a reported prevalence ranging between 0.5–1% worldwide (22; 23;24). RA clinical manifestations are usually not confined to musculoskeletal system, but also involve cardiovascular system, kidneys, lungs, liver and skin (25).

Mycobacterium is a potent immunogen, often causing uncontrolled immune responses that are likely to play a role in RA pathogenesis (26). Mycobacterium antigens, such as MTBC, are associated with autoimmune diseases, such as autoimmune arthritis, sarcoidosis, systemic lupus erythematosus (27). where the most prevalent mycobacterial antigen detected was the heat shock protein 65 (HSP65) (28). HSP65is an immunodominant protein similar to several human proteins, such as lactoferrin, transferrin, alphaB-crystallin in terms of sequence and conformation (29). Bo *et al*., (30) confirmed the presence of antibodies (Abs) directed against two proteins of Mycobacterium avium subsp. paratuberculosis (MAP) in sera of RA subjects, which are crucial for the survival of the pathogen within macrophages. Thus, the main aim of this study was to detect NTM *hsh65* gene in animals mastitis milk and Rheumatoid arthritis patients blood .

Materials and method

Selection of patients and animals

This study was conducted through period extended from October 2019 to December 2020 in different areas of Basrah and Thi-Qar provinces. south of Iraq.Cow,sheep and goat's milk samples were collected randomly from cows(70),sheep(50) and goats (30) with different age and breed. Forty blood samples of arthritis patients selected from the Private laboratories. They were considered as two groups1st group consisted of 9 patients with rheumatoid arthritis, 31 non rheumatoid arthritis patients compromised the 2nd group. After cleaning the udder, 10 ml of milk was collected from the 4 quarters into sterile plane

tube by hand milking, the first 3 jets were discarded... Blood was collected from each individual (human) by venipuncture into tubes with anticoagulant (K2-EDTA). All studied samples aseptically placed into sterilized test tubes and transported with ice in cooler box to the laboratory for subsequent analysis.

Screening for mastitis

Modified white side test was performed according to method of Kahir *et al.,* (31). Briefly, 100 µL of sodium hydroxide solution 4% was added to 250 µL of cold milk on slide on black background and the mixture was stirred vigorously for 20 seconds. No reaction was observed in normal milk after addition of sodium hydroxide solution and remains uniformly opaque. While the mastitis milk showed reaction, this reaction was scored as follow:

Negative(N): opaque, milky mixture, no precipitant

(+): Clumping of slight degree is present.

(++): Mixture thickness, coagulated materials are present

(+++): Large mass of precipitants

Molecular detection of NTM in milk and blood samples

This study used the sequence analysis of the hsp65 and 16S rRNA genes to identify the NTM. A fragment of *hsp65* and *16S rRNA* genes were amplified with primer sets according to Telenti et al. (32) ; Wilton and Cousins et al.., (33), respectively. The extraction of genomic DNA from animals milk and human blood samples was done according to manufacturer's instructions (gSYNC™ DNA Extraction Kit / Genaid / Korea). The extracted DNA was verified on a 1% agarose gel stained with GreenStar™ Nucleic Acid Staining BioNeer (Korea). Subsequently the concentration and DNA purity was estimated by Nanodrop spectrophotometer (Quawell, USA) at 260/280 nm.

AccuPower PCR premix (Cat # K-2012, Bioneer, Korea) was used as PCR reaction premix which contained 1 U of Top DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$. The reaction mixture (20µl) was composed of 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in PCR thermocycler (, Bioneer, Korea). PCR was carried using the Mycobacterium species 16S rDNA bacterial primers F (5'- AGA GTT TGA TCC TGG CTC AG 3') and R (5'- TGC ACA CAG GCC ACA AGG GA -3') targeting a region of 1030bp in the NTM 16S rDNA (Wilton and Cousins *et al*.., 1992). To confirm the presence of NTM DNA, the primers Tb11 (5′-

ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5′ CTTGTCGAACCGCATACCCT-3′). targeting a region of 441 bp in the *hsp65* Gene of Mycobacterium species was used in the PCR amplification .

PCR cycle conditions for amplification of the two genes (hsp65 and 16S rRNA) were as follow: 94 ^{o}C for 5 min followed by 30 cycles of 94 ^{o}C for 30s, 61 ^{o}C for 30 s, and 72 ^{o}C for 50 s, and then final extension at 72 ^{o}C for 10 min. Analysis of 3 µl of the PCR products (amplicon) was done by electrophoresis on 1.5% gel agarose. After electrophoresis and gel staining with ethidium bromide (0.5 mg/ml) DNA stains, the fragments were visualized under UV light in the gel documentation system (Gel Doc, ATP Co).The resolved hsp65 PCR amplicons were commercially sequenced from both forward and reverse termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). The sequencing results of the PCR products were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed nucleic acids were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. Each detected variant within the *Mycobacterium* genes was annotated by SnapGene Viewer ver. 4.0.4 (https://www.snapg ene.com).

Statistical analysis

Descriptive tests like frequency and Chi-square as atest of significance were applied to analyze the data using SPSS v.16 software. Statistical significance limit being set at 5%.

Results

Modified white side test Screening results

Modified White Side Test (MWT) was used as a screening test for subclinical mastitis in 70 cows ,50 sheep and 30 goats milk samples. The results of this test revealed that 58(82.9%), 38(76%) and (46.7%) of cows, sheep and goats milk samples respectively showed positive results in the preliminary screening for subclinical mastitis (Table 1). Chi-square test was used to estimate the statistical significance. Accordingly, the difference among the current results MWT positive results was considered to be statistically significant (γ^2 : 14.338 df:2 p-value:0.0007)

Varying degree of the positive milk samples MWT reactions presented in table (4-2), the score of positivity (+++) was observed in cows (37.93%) and sheep (28.9%) milk samples only **.**while all cows, sheep and goats

milk samples showed the positivity score (++) at different percentage(18.96,21.1 and14.3%, respectively).The positivity score (+) was detected in cows **(**43.11%,), sheep(50%) and goats(85.7%) milk samples . The difference among the current results of positivity score was statistically significant (γ^2 : 35.541 df:8 p-value:0.00002). According to *hsp65* based PCR results NTM preset in 3 rheumatoid arthritis patients blood samples with a percentage of 33.3% within rheumatoid arthritis (1st group) and **7.5**% as total percentage within arthritis patients blood. Negative *hsp65* based PCR results for NTM presence in nonspecific arthritis patients (2nd group) blood (Table 5).

Table (1): Modified White Side Test results of milk samples

Milk sources	Examined n(%)	MST +ve n(%)	MST -ve n(%)	p-value
Cows	70	58(82.9)	12(17.1)	
Sheep	50	38(76)	12(24)	
Goats	30	14(46.7)	16(53.3)	<0.05

Table (2): Varying scores of White Side Test positive reaction

Milk sources	Total positive milk samples	Positive results n.(%)			p-value
		+	++	+++	
Cows	58	25(43.11)	11(18.96)	22(37.93)	<0.05
Sheep	38	19(50)	8(21.1)	11(28.9)	
Goats	14	12(85.7)	2(14.3)	0	

Molecular detection of NTM spp in milk and blood samples

The amplified product of 16SrRNA gene sequence (1030 bp) was observed in 3**(**6%**)** milk samples of cows and 11 **(**22%**)** samples of sheep's milk, while goat's milk samples showed negative results (Figure1; Table 3). According to Chi-square test there was statistical significance difference among cows, sheep and goates milk positive *16SrRNA* based PCR results ($\gamma^{2:}$8 .772: df:2 : p-value: 0.01).

The results of *hsp65* based PCR confirmed the presence of NTM in tested milk samples of cows, sheep and goats by using Tb11: Tb12 primers. The amplified product of hsp65 gene sequence (441 bp) was detected in 50**(**71.4%**)** milk samples of cows. The hsp65 gene detected in 10 (20%) samples of ewe's milk. While this gene was not detected in goat's milk samples (Figure 2; Table 4). The difference among these results of positive hsp65 based PCR was statistically significant (γ^2 : 57.143 : df:8 : p-value:0).

According to *hsp65* based PCR results NTM spp preset in 3 rheumatoid arthritis patient's blood samples with a percentage of 33.3% within rheumatoid arthritis (1st group) and 7.5% as total percentage within arthritis patient's blood. Negative *hsp65* based PCR results for NTM presence in nonspecific arthritis patients (2nd group)) blood (Table 5). The difference between the results of two groups was considered to be statistically significant (γ^2:8 . 6.883: df:1: p-value:0.009).

The hsp65 gen sequencing results confirmed high sequence similarity (99%) of the current *hsp65* sequences (441 bp) with *Mycobacterium avium* retrieved DNA sequences (GenBank acc. NZ_CP009360.4), the exact positions and other details of the retrieved PCR fragments were displayed in figure (3).

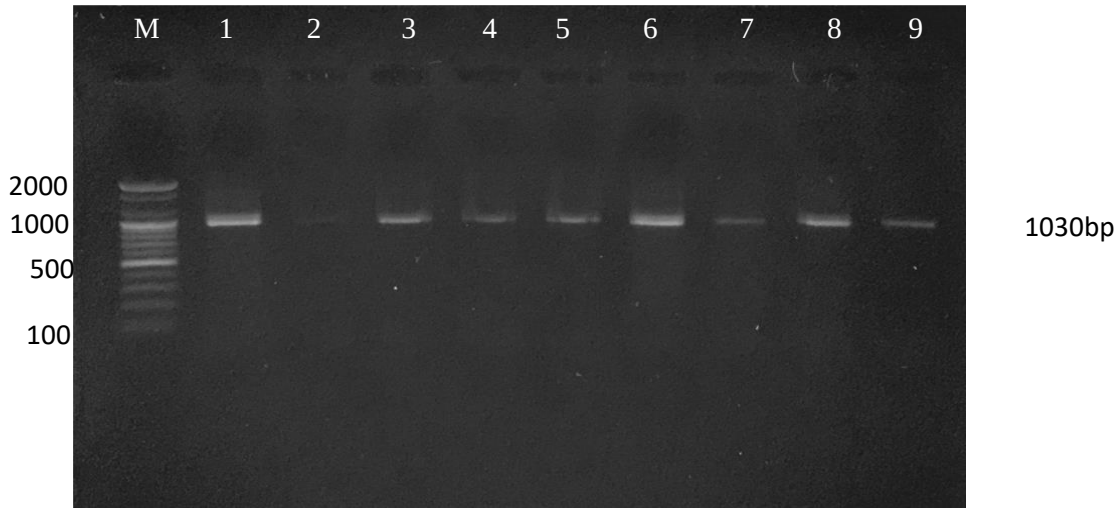

Figure (1) *16SrRNA* PCR amplification results of milk and blood samples. Lane 1-9: 16SrRNA gen (976bp.M: DNA Ladder. The16SrRNA amplicons was electrophoresis on 1.5% agarose

Table (3): The results of 16SrRNA PCR in in three milk sources

Milk sources	Examined milk Samples n.	16SrRNA based PCR +ve n.(%)	16SrRNA based PCR -ve n.(%)	P-value
Cows	50	3(6)	47(96)	<0.05
Sheep	50	11(22)	39(78)	
Goats	30	0	30(100)	

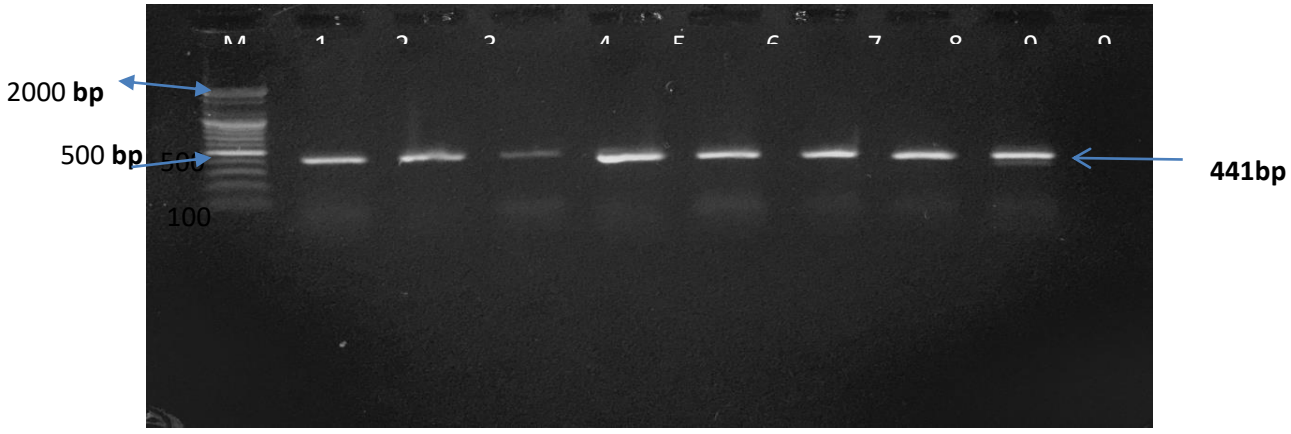

Figure (2) PCR amplification of hsp65 gen in milk and blood samples. The product was electrophoresis on 1.5% agarose. M: DNA Ladder; Lane (1-9) hsp65 gen(441bp).

Table (4): The results of hsp65 based PCR 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)	<0.05
Ewes	50	10(20)	40(80)	
Goats	30	0	30(100)	

Table (5): The results of hsp65 based PCR in arthritis patients

Patients	Examined n(%)	PCR+ve n(%)	PCR -ve n(%)	p-value
Rheumatoid arthritis	9	3(33.3)	6(66.7)	
Nonspecific arthritis	31	0(0)	31(100)	<0.05
Total	40	3(7.5)	37(92.5)	

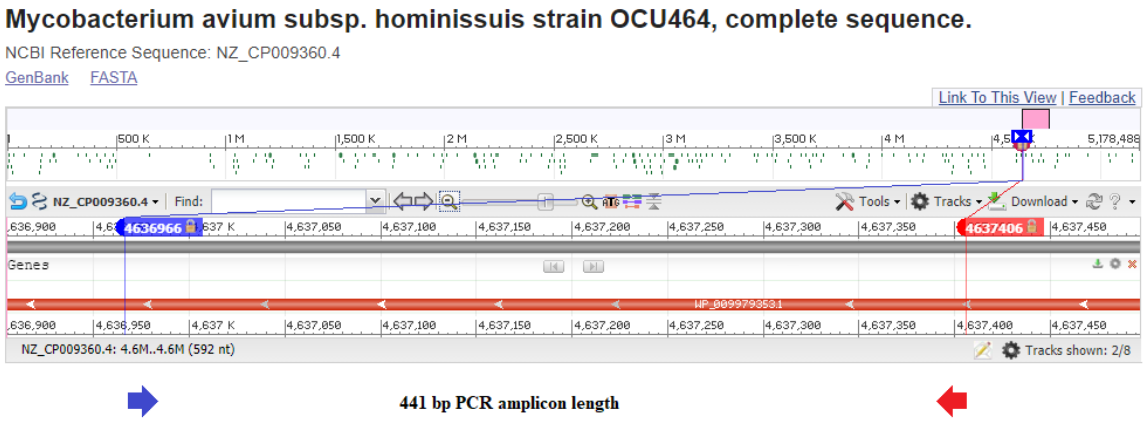

Fig. (3). The exact position of the retrieved 441 bp fragments that partially covered the *Hsp65* genetic sequences within *Mycobacterium avium* genomic sequences (GenBank acc. no. NZ_CP009360.4). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

Discussion

The isolation of NTM in body secretions was previously considered to be caused by contamination; however, recently they have been linked to disease (34). Despite the increasing international interest in NTM infections, information on the occurrence and the diversity of NTM in human, cows,sheep and goats, is ill restricted in Basra- Iraq. The available literature is mainly focused on the *Mycobacterium avium* complex (MAC) and its subspecies paratuberculosus, (35; 36).While other studies also previously conducted in the same province involve NTM derived from clinical samples suspicious for MTBC (37 ;38) On other hand Alsaimary (39)studied MTBC in Tuberculosis Suspected Patients .The current study was the first report of the NTM spp presence in cows, ewes and goats milk and arthritis patients blood samples by using conventional PCR and gene sequencing .The present results revealed an overall percentage of NTM spp in cows and ewes to be 71.4 and 20%, respectively which is far from the primary screening results (82.9 and76%) estimated based on MWT. In contrast, goats showed negative results to *hsp65* and 16S rRNA based PCR but 14(46.7%) of these goats were positive in MWT. Also this study has shown that, 3(33.3%) of Rheumatoid Arthritis Patients blood samples were hsp65 based PCR positive. Comparison of the NTM PCR detection results in this study indicate significant difference have been found among the studied milk and blood samples. The distribution of NTM analyzed by previous studies has been shown to be variable, Alzaidi, (40) confirmed the presence of NTM

spp in tested human blood samples of 31 (20.7%) and 3(12%) cows fecal samples in Basrah and Thi-Qar provinces south of Iraq .Katale et al (41) supported the present results by reporting very wide range of NTM from humans and animals and understanding their transmission dynamics.

NTM are uncommon causes of bovine mastitis. Some old reports have described these bacteria as etiological agent of mastitis, but without definitive diagnostic . However Franco et al., (42); Machado *et al.,(*43**)** had been reported that the *M.smegmatis* group species may cause clinical mastitis in ewes and dairy cows . In Turkey, 35 samples of raw milk were evaluated for the presence of mycobacteria using phenotypic methods and confirmed by Polymerase Chain Reaction - Restriction Pattern Analysis (PCR-PRA), resulting in the detection of *M. terrae, M. kansassii, M. agri*, and *M. haemophilum (44*). While Aydin et al., *(*45) in the same country, identified. *M. genavense, M. simiae, M. szulgai, M. bovis* and *M. fortuitum*were in bovine raw milk Cvetnić *et al*., (46) concluded that all cases of mastitis in cows with unsuccessful antimicrobial treatment or negative bacteriological tests for the usual bacterial causative agents, *Mycobacteriae* should be suspected as the possible causative agent.

NTM described in this study originated from cows and ewes milk containing an increased number of somatic cells and displaying an altered consistency of milk. Beside that, the present study depended PCR detection of 16SrRNA and hsp65 genes which were demonstrated the accurate identification of the mastitis milk NTM spp. Some previous studies supported the current results, Siqueira *et al.,* (47) performed phenotypic and molecular characterization which were demonstrated the accurate identification of the mastitis milk isolates as NTM in one adult Holstein cow with rare bovine mastitis. Franco et al., (42) in Brazil described NTM spp in milk. Similarly, Jayasumana *et al.* (48) confirmed the presence of NTM spp in two milk samples (0.8%) according to PCR results. Alzaidi, (40) concluded that the hsp65 gene detection is a better identification tool for differentiation of Mycobacterium species and is useful to complement diagnosis of NTM spp. Cvetnić *et al*., (46) confirmed the presence of NTM spp in One mastitis milk sample by Sequenceing *hsp65*, *rpo*B, 16S rRNA genes.

Although *M. tuberculosis* and *M. bovis* act as the major pathogenic species, NTM have become increasingly important in causing human infections (49). HSP65 region between aa180–188 can stimulate auto-reactive T

lymphocytes that react with cartilage-resident self-proteins (50). HSP65 increases the responses of mononuclear cells (51). There are reports of some species of Mycobacterium associated with RA disease that make it difficult to treat the disease. There have been case reports of Mycobacterium species in infections and arthritis and other parts of the body in RA patients around the world (52; 53; 54; 55; 56). Furthermore, the risk of death in RA patients with mycobacterial infection was higher than that in patients without infection. (57).

Conclusion

PCR-based identification assays may be used to investigate the presence of NTM spp. in milk samples. According to current findings, Iraqi RA patients may have been exposed to NTM spp. through the intake of raw bovine milk and related dairy products.

Conflict of Interest: The authors state that there is no conflict of interest.

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الكشف عن بروتينات الصدمه الحراريه (hsp65) للمتفطرات غير السلية في حليب التهاب الضرع تحت السيري لبعض المجترات و دم مرضى التهاب المفاصل الرثوي

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الخلاصه

كان الهدف الرئيسي من هذه الدراسة هو الكشف عن hsp*65* للمتفطرات غير السلية في حليب التهاب الضرع تحت السريري الحيوانات ودم مرضى التهاب المفاصل الرثوي. استخدم اختبار الجانب الأبيض المعدل(MWT) كغربله اوليه لالتهاب الضرع تحت السريري في عينات حليب 70 بقرة و 50 نعجة و 30معزه. اظهرت نتائج هذا الاختبار أن 58 (82.9٪) و 38 (76٪) و (46.7٪) من عينات حليب الأبقار والنعاج والماعز على التوالي كانت موجبه مع وجود فرق معنوي (P<0.05). لوحظت درجات إيجابية متفاوتة ذات دلالة إحصائية (P<0.05) من خلال هذا الاختبار ، على النحو التالي : درجة (+++) في عينات حليب الأبقار (37.93٪) والنعاج (28.9٪) فقط ، درجة الإيجابية (++) بنسب 18.96 و 21.1و,14.3% في جميع عينات حليب الأبقار والنعاج والماعز على التوالي ودرجة (+) في عينات حليب الأبقار (43.11٪) و النعاج (50٪) والماعز (85.7٪) .

الكشف الجزيئي عن المورثه16SrRNA للمتفطرات غير السلية (NTM) في عينات الحليب اظهر أن 3 (6٪) و 11 (22٪) عينة حليب ابقار ونعاج على التوالي كانت موجبه مع اختلاف معنوي (P<0.05). تم الكشف عن المورثه(hsp65(441 bp للمتفطرات غير السليه في 50 (71.4٪) و 10 (20٪) عينة حليب ابقار ونعاج على التوالي مع اختلاف معنوي (P<0.05) . كما تم اكتشاف هذه المورثه في 3 عينات من دم مرضى التهاب المفاصل الرثوي وبنسبة 33.3٪ مع نسبه كليه(7.5٪) و لوحظت نتائج سلبية لـهذه المورثه في مرضى التهاب المفاصل غير الرثوي مع فرق معنوي (P<0.05). اكدت نتائج التسلسل الجيني للمورثه hsp65 نسبة تشابهًه عاليًه (99 ٪) مع تسلسل الحمض النووي المسترجع من GenBank acc. NZ_CP 009360.4 لجرثومة *Mycobacterium avium*

الكلماث المفتاحيه : NTMو التهاب الضرع تحت السريري والتهاب المفاصل الرثوي