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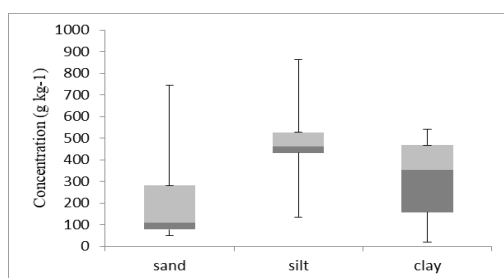
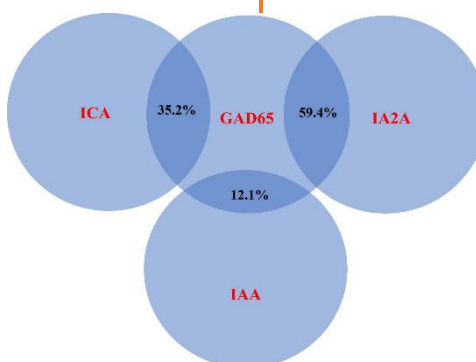
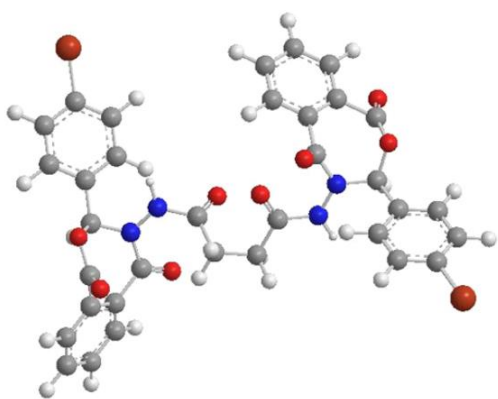
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Serological, Morphological and Molecular Diagnosis of *Mycobacterium bovis* in Cattle at Sulaimani Province of Iraq

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Abstract

Background: *Mycobacterium bovis* (*M. bovis*) causes bovine tuberculosis (BTB), is an endemic disease in cattle and poses a high risk of spreading to humans. **Objective:** This study aimed to determine *M. bovis* in cattle and assess the similarities between cattle and humans through molecular methods and histopathological examinations. **Methodology:** Randomly, blood samples from 411 healthy appearance cows (1% of the target population) in five districts in Sulaimani province, Iraq, were collected from January to March 2022. Sera were obtained immediately and used for ELISA test to determine *M. bovis*. Additionally, the disease prevalence was confirmed by gross lesions at the slaughterhouse and histopathological examination of collected lymph nodes. Moreover, a PCR assay was used to detect *M. bovis* in suspected cow samples and previously diagnosed human samples. Gene sequencing and phylogenetic tree analysis were also done to determine the molecular differences between animal and human *M. bovis*. **Results:** Using an ELISA test, 46 (11.11%) of 414 samples were positive, while 368 (88.89%) were negative without significant differences between the districts ($p > 0.05$). According to postmortem lesions at the slaughterhouse, only three cows were infected with TB, and typical gross lesions were calcified necrotic and multiple well-demarcated granulomas. The molecular test using two primers (CSB2 and oxyR gene) revealed that *M. bovis* was found in animal and human extra-pulmonary lymph nodes with no molecular change. **Conclusion:** Healthy cows harbored *M. bovis*, the causative agent of a contagious disease that spreads and causes a persistent health problem in humans.

Introduction

Mycobacterium bovis (*M. bovis*) is a rod-shaped Gram-positive bacterium that causes bovine tuberculosis (BTB), which is an infectious disease of progressive character and chronic evolution (Paes & Franco, 2016; Pérez-Lago et al., 2014), In addition, it belongs to the *M. tuberculosis* complex (MTBC), which contains several closely related species responsible for human tuberculosis and zoonotic tuberculosis (Shah et al., 2007).

In several countries, zoonotic tuberculosis still severely threatens public health and the economy, with new cases and deaths increasing every year. Bovine tuberculosis has been classified as a notifiable animal disease by the World Organization for Animal Health (Loiseau et al., 2020; WHO, 2013). Despite long-standing

international efforts to eradicate tuberculosis, 1/86 billion people are infected worldwide (Chakravorty et al., 2005).

Humans are infected with *M. bovis* by directly handling infected animals or their products. The most common route is aerosol inhalation, followed by ingestion (Ibrahim et al., 2010). At the same time, breaks in the skin and congenital routes through infected semen are also considered. Transmission could be from animal to human and vice versa (Barak, 2012; Dürr et al., 2013).

Tuberculosis as a chronic disease has been detected in Iraq, including the Kurdistan region, for many years. For example, the prevalence of BTB in cattle was approximately 5.1% in Sulaimani province (Sulaiman, 2005), while in Baghdad, it was found that 43.88% of cows were positive using the comparative tuberculin test (Barak, 2012). Generally, the disease rate among workers and veterinarians was 32% using the ELISA test (Herrera-Rodríguez et al., 2013).

A presumptive diagnosis of BTB in cattle is generally made based on the history, clinical results, tuberculin skin examinations, isolating and immediate slaughtering, and necropsy to look at tuberculosis-associated lesions (Tessema et al., 2011; Wedlock et al., 2002). In addition, *in vitro* lymphocyte assays, including an enzyme-linked interferon-gamma assay, have been designed to detect disease in cattle and other animals exposed to *M. bovis* (Špičić et al., 2012). Recently, *M. bovis* immune complex proteins have been used to diagnose BTB (Hadi et al., 2018).

Histopathologically, the development of white granulomatous lesions, often described as tubercles, in tissues results from this cell-mediated immunity response (Domingo et al., 2014). These lesions develop with early domination $\gamma\delta$ T cells with cytokine responses recruiting more macrophages for phagocytosis and other antigen presentation of *M. bovis* (McGill et al., 2014). Usually, small lesions can develop seven days after *M. bovis* infection, but gross lesions are not detected until the bacilli multiply rapidly 14 days after infection (Domingo et al., 2014).

Using polymerase chain reaction (PCR) assay to differentiate between groups of mycobacteria and the use of DNA amplification for the detection of Mycobacterium in formalin and fixed paraffin-embedded tissue samples would be helpful in cases in which diagnosis depends on tissue examination (Jafarian et al., 2008; Lorente-Leal et al., 2019). Thus, this study was designed to detect *M. bovis* in cattle by assessing the similarities between cattle and humans through molecular methods in combination with histopathological examinations in Sulaimani province.

Materials and Methods

Area of the study

This study was conducted from January 1st to March 31st 2022, confined to five different districts in Sulaimani province, including Tanjaro, Bakrajo, Peramagrun, Chwarta, and Sharazor. These areas include nearly 273 villages and contain 38661 cattle. Around 414 healthy cows were randomly tested, representing about 1% of the target population, according to the Veterinary Directory of Sulaimani (Table 1).

Sample collection for serology and histopathology

About 10 mL of blood samples were collected from each cow for quantitative interferon-gamma (IF- γ) detection using ELISA test special kit (TB-Feron ELISA Plus, BioNote company, South Korea). Then, sera were obtained and frozen at -20° C until use. Conversely, the lymph node (LN) tissues were collected from the cow at the Karagol slaughterhouse in Sulaimani province. LN was removed aseptically and examined for tuberculosis lesions (enlargement and necrosis). Histopathological examination was performed after the tissue fixation in a 10% neutral buffer formalin. Then, LN were embedded in paraffin wax and stained with hematoxylin and eosin.

Table 1: The study area, the target population, and the number of samples.

District	Number of villages	Bovine population	Number of samples
Tanjaro	60	5883	68
Bakrajo	65	12670	130
Peramagrun	58	13391	134
Chwarta	48	2732	32
Sharazwr	42	3985	50
Total	273	38661	414

Human samples

For human tissues, 12 paraffin-embedded LN tissues that were previously diagnosed as positive for mycobacterium species were obtained from Shorsh General Hospital and Smart Health Towers, Sulaimani province. First, the tissue samples from each paraffin block were obtained by sectioning (10 mm thickness) using a microtome (R. Jung/Heidelberg, Germany), then paraffin was removed (Olert et al., 2001; Sen Gupta et al., 2003) and dewaxed with xylene and absolute ethanol and finally preserved at -20° C for extracting genomic DNA.

PCR assay

DNA samples were extracted from 12 obtained human deparaffinized LN tissues (25 mg) and three cattle LN using a DNA extraction kit (AddPrep Genomic, Korea). Then two sets of specific primers were selected, encoding CSB2 (168 bp) (F5'-TTCCGAATCCCTTGTGA-3'); (R-5'-GGAGAGCGCCGTTGTA-3') (Bakshi et al., 2005), and oxyR (548-bp) specific primers to distinguishing *M. bovis* isolates from other members of Mycobacterium (F-5'-GGTGATATATCACACCATA-3'); (R-5'-CTATGCGATCAGGCGTACTTG-3') (Sreevatsan et al., 1996). The PCR master mix was prepared by adding 1.0 µL of each forward and reverse primer (CSB2 and oxyR separately), 8.0 µL of the DNA sample, and the volume was completed to 20 µL using distilled water. The PCR cycles included an initial denaturation for 5 min at 94° C, followed by 35 cycles of denaturation (30 sec at 94° C), annealing (1 min at 53.3° C for CSB2 and one min at 54° C for oxyR), pre-extension (1 min at 72° C), and final extension for 5 min at 72° C. Later on, the PCR product (10 µL) from each gene was stained with 7.0 µL Gel stain dye (Safe Gel Stain Dye, Taiwan) and run on a 1.8% agarose gel using a Gel electrophoresis system (Cleaver, UK) at an electrical potential of 90 volts for 60 min. DNA bands were visualized under ultraviolet light using a molecular imager (UV Transilluminator, Ingenious, USA). The size of the amplified DNA strand was estimated by comparing it with a 100 bp DNA ladder (GenDirex, USA).

Sequencing of PCR products and phylogenetic construction

The CSB2 genes, including sh1, sh2, sh3, and sh4 and oxyR genes, including Sul-3, Sul-2, and Sul-1, were selected for sequencing at the Macrogen genome centre in South Korea. Then, sequence alignment was performed using the Applied Biosystems 3500 Genetic Analyzer program. The result was published in GenBank under accession numbers of OP716210, OP716211, OP716212, OP716213 for sh1, sh2, and sh3 and OP503571, OP503572, OP503570 for Sul-3, Sul-2, and Sul-1. The sequence of the CSB2 and oxyR genes of the samples was comparatively analyzed, and a phylogenetic tree was constructed using the Neighbor-Joining method with MEGA 10 software (version 10.0.5) (Kumar et al., 2016). In the bootstrap test (1000 replicates), the percentage of duplicate trees in which the related taxa clustered together was

determined (Felsenstein, 1985). The Kimura 2-parameter method was used to calculate the evolutionary distances (Kimura, 1980).

Ethics approval and consent to participate

This study was approved by the Ethical Committee of the College of Veterinary Medicine, University of Sulaimani, Iraq (no. 7/29/1623 UoS).

Statistical analysis

All analyses were done using SPSS, version 25. The histograms were used to determine the normal distribution status of the data. The chi-square test was used to compare variables, while frequency and percentage were calculated for categorical data. Mean±SD estimated for numerical variables. P<0.05 was considered statistically significant.

Results

Serology

In this study, and based on the ELISA assay, a total of 46 (11.11%) cow blood samples were positive for *M. bovis*, while 368 (88.89%) were negative (Figure 1). Bakrajo district was the most affected area, while Chwarta district was the least affected area, but without significant difference (p>0.05) (Table 2 and Figure 2).

Table 2: The number and rate of infection in the five examined district at Sulaimani province.

District		ELISA Results		Total	P- value
		Negative	Positive		
Bakrajo	Count	111	19	130	0.541
	%	85.4	14.6	100.0	
Chwarta	Count	30	2	32	
	%	93.8	6.3	100.0	
Peramagrwn	Count	122	12	134	
	%	91.0	9.0	100.0	
Sharazwr	Count	44	6	50	
	%	88.0	12.0	100.0	
Tanjaro	Count	61	7	68	
	%	89.7	10.3	100.0	
Total	Count	368	46	414	
	%	88.9	11.1	100.0	

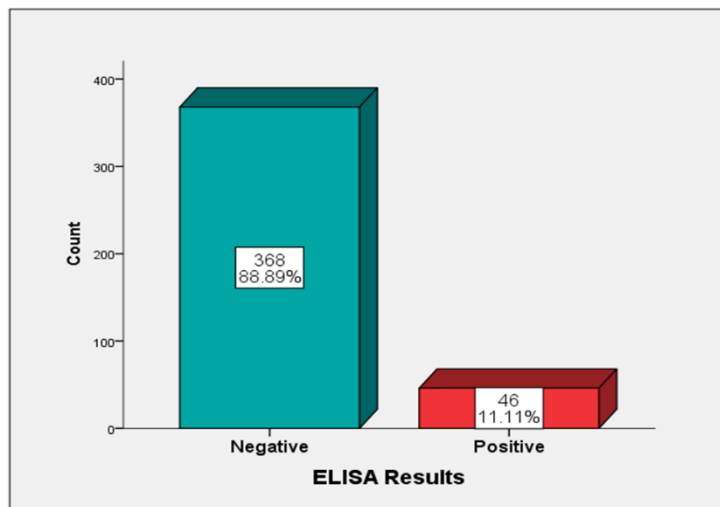


Figure 1: The number of infected and uninfected cows with *M. bovis* using ELISA assay.

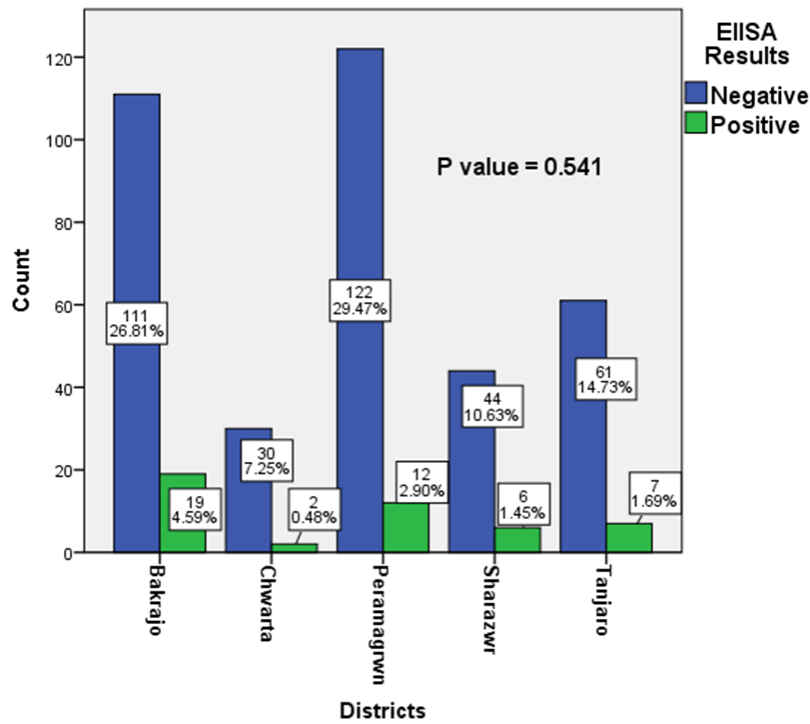


Figure 2: The number and rate of *M. bovis* infection in five studied districts in Sulaimani province using ELISA assay.

Gross examination

According to postmortem lesions at the slaughterhouse, only three cows were infected with tuberculosis, and typical gross lesions were various nodular granulomas/tubercles in different organs, more specifically, a typical caseous granuloma in the retropharyngeal and mediastinal LNs (Figure 3).

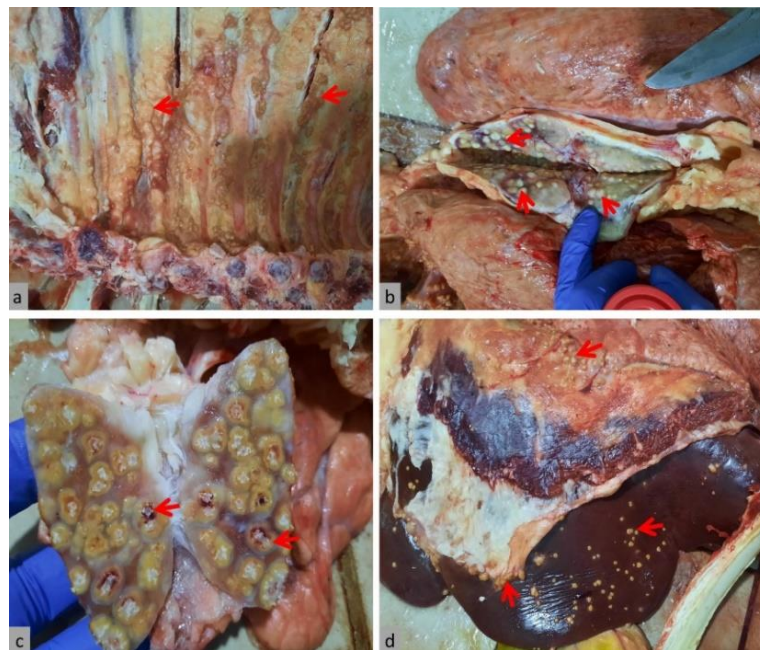


Figure 3: Gross lesions of bovine tuberculosis in different organs. **A:** Multiple-variable sized pyogranuloma (red arrows) diffusely distributed throughout the intercostal muscle, **B:** Variable-sized calcified necrotic granuloma in the mediastinal lymph node (red arrows), **C:** Retropharyngeal lymph node edematous and contained multiple well-demarcated granulomas bordered by a red rim (red arrows), and **D:** Multiple small-sized necrotic foci (granuloma nodule) on the dorsal surface of the liver and peritoneum (red arrows).

Histopathological examination

The sections of the cow's LN revealed typical TB granulomas that were composed of homogeneous oval irregular eosinophilic caseous necrosis in the centre area with calcification surrounded by a layer of epithelioid cells (macrophages similar to the epithelium arrangement), then infiltrated by a large number of lymphocytes, macrophages, and fibroblasts with Langhans-type multinucleated giant cells. The entire lesions were partially or entirely encapsulated in collagenous connective tissue, with localized penetration of the granulomatous process of the capsule extending the lesion into the surrounding parenchyma (Figure 4).

Molecular diagnosis

M. bovis species were specifically detected using two primers, one for the CSB2 gene (168 bp), specific for rapid *M. bovis* detection. All three cow samples were positive for *M. bovis*. In contrast, only 4 out of 12 human samples were positive for *M. bovis* (Figure 5). The second primer set was for detecting the oxyR gene (548 bp), which was specific for the *M. bovis* diagnosis distinguishing from other MTBC, non-tuberculosis bacteria (NTB). Again, it was found that all three cow samples were *M. bovis* positive, and only four human samples were positive (Figure 5).

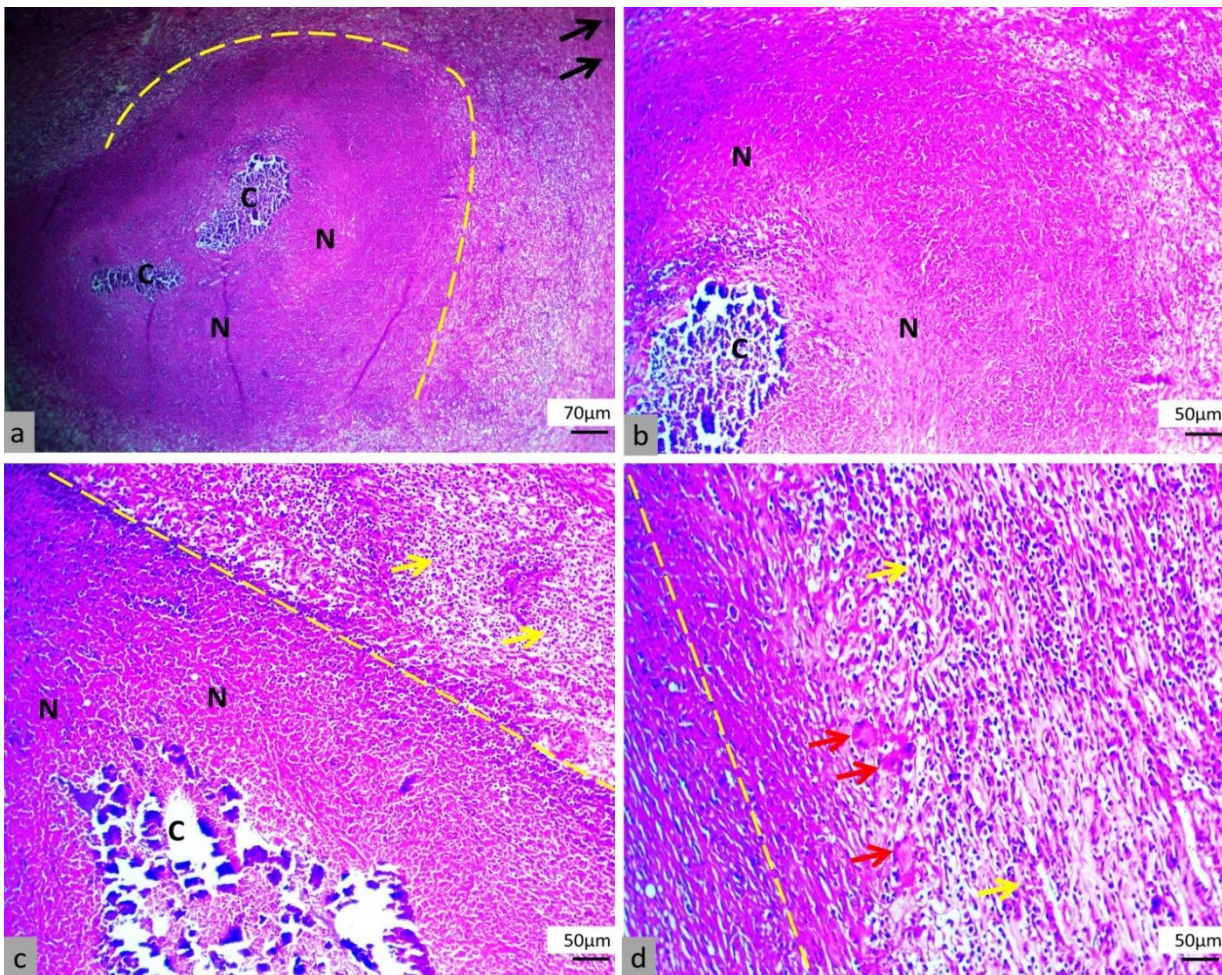


Figure 4. Microscopic sections of the cattle lymph node showed typical stage IV TB granulomas. **A and B:** Coalescence of a large number of nodular granuloma with completely mineralized centers with caseous necrosis, surrounded by a thick fibrous capsule (black arrows). **C and D:** Granulomas contain epithelioid macrophages infiltrates (yellow dash lines), mixed with lymphocytes, macrophages, and fibroblast (yellow arrows) with multinucleated langhan's giant cells (red arrows), C: Caseation, N: Necrosis, (H & E stain).

DNA sequencing and phylogenetic analysis

According to the oxyR gene analysis, three *M. bovis* isolates had an adenine at position 285. On the contrary, other strains of *M. tuberculosis* had guanine in their sequences at position 285. In addition, additional single nucleotide substitution in the field isolate at position 125 (thymine instead of cytosine) was also found, a rare and sporadic variation of that condition (Figures 6 and 7).

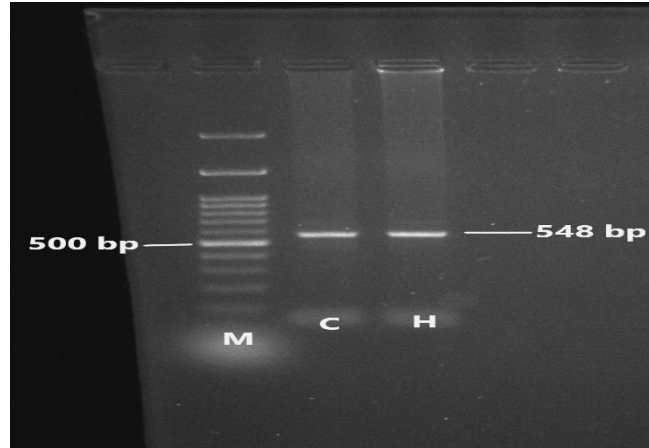


Figure 5: Detection of *M. bovis* (oxyR gene 548-bp) by PCR. Line M: Marker of 100-bp ladder, C: Positives for *M. bovis* in cattle, and H: Positive for *M. bovis* in human.

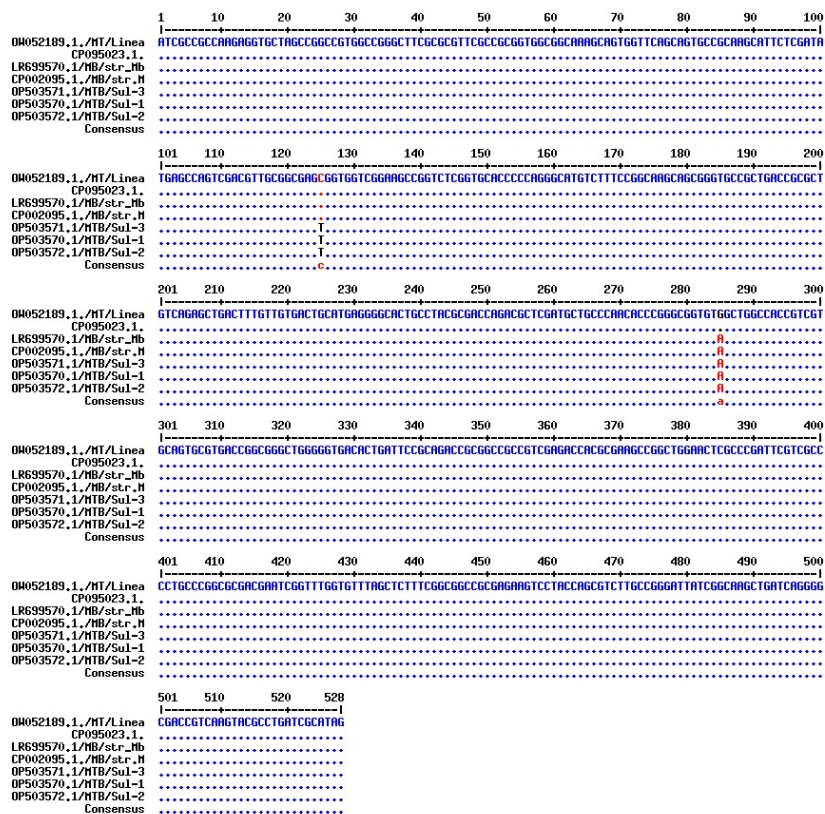


Figure 6: Multiple sequence alignment of the partial DNA sequence of the methyl transferase domain region of four field isolate (*M. bovis*), MT/ bovis reference strains. All field isolate 100% matched with *M. bovis*.

The three mycobacterium species nucleotide sequences found were identical and did not show diversification. The highest identities, 99.62% and 99.81% were shown by field sequences isolated from *M. tuberculosis* and *M. bovis*, respectively. In order to evaluate and confirm the evolutionary ancestry of the field samples, phylogenetic analysis was used to compare partial sequences of the oxyR gene from field strains and representative strains from GenBank. It was revealed that the three field isolates were associated with other *M. bovis* in cluster 1 (*M. bovis*). However, these field isolate sequences are distinct from cluster 2 (*M. tuberculosis*) (Figure 8).

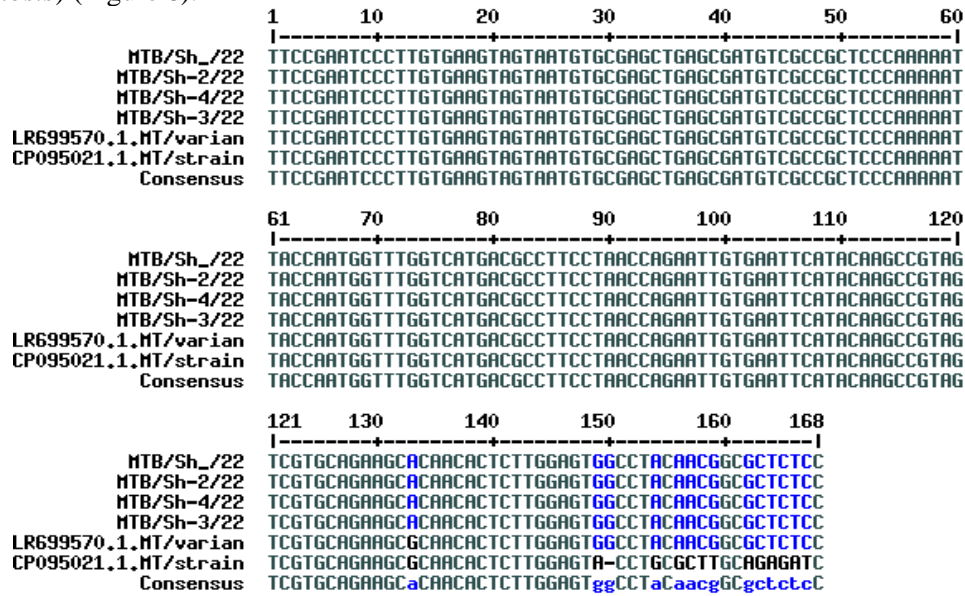


Figure 7: Multiple sequences alignment of field isolate (OP503570-MTB/sul-1/2022-OP503572.MTB/sul-2/2022) from bovine source, and from human source (OP503571.MTB/sul-3/2022) with other reference strains [(CP095023 & OW052189-MT/human source) and (LR699570 & CP002095-MB/Bovine source)].

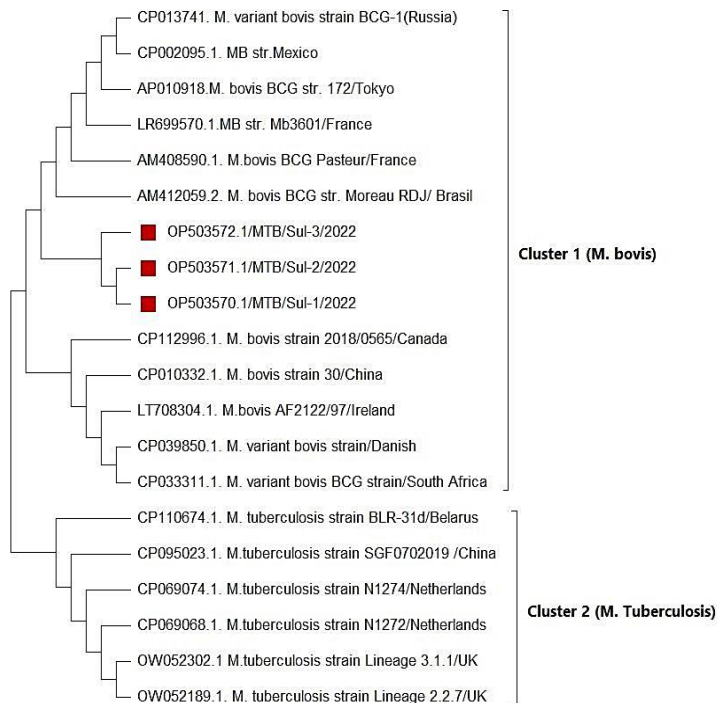


Figure 8: Neighbor-Joining method. Phylogenetic tree of mycobacterium spp. Based on oxyR gene sequences (*M. tuberculosis* and *M. bovis*). The Iraqi sequences identified in this study are represented by a red square.

Discussion

Tuberculosis is a disease of animals and humans caused by pathogenic members of *Mycobacterium* and characterized by granulomatous lesions or tubercles (Thoen et al., 2009). Bovine TB is among the most severe zoonosis worldwide, causing enormous economic losses in the livestock sector and posing serious health risks to humans, particularly in developing countries where bovine TB management programs are lacking (WHO, 2009).

The current study used a serological test (ELISA assay) to detect *M. bovis* to screen bovine tuberculosis in mid to late-state cattle. Only 11.11 % of cattle blood was found samples were infected. These outcomes were higher than the previous report (5.1%) using a single intradermal tuberculin test to determine *M. bovis* prevalence in Sulaimani province (Sulaiman, 2005). There have never been any control measures for BTB in the Kurdistan region. In a farm with no prevention program and a large herd size, even if one animal is infected, the disease is highly likely to spread (Ameni et al., 2003). However, the results of the present study are lower than those obtained (Barak, 2012) in Baghdad (43.88%) because the present study chose animals randomly instead of those with debilitating symptoms.

According to statistical data from Sulaimani province, the prevalence rate in the Bakrajo district (19%) was the highest. However, the different number of samples collected between districts leads to different ratios from each district, which is insignificant. In addition, some factors, including herd size, continuous close contact between animals (as the animal population has increased in small grazing areas), breeding and close housing (the closer animals are packed together), uncontrollable animal movement, unhygienic local habits that could facilitate transmission, as well as grazing sites and animal gatherings during drinking, all play a role in disease prevalence (Mwakapuja et al., 2013; Santos et al., 2012).

For gross tissue inspection, tuberculous granuloma in the tissues and LN of the upper respiratory tract were found, similar to that found in another study (Cassidy et al., 1998). In histopathological examination, four broad developmental stages in the granuloma and haemorrhage were often observed, with infiltration of lymphocytes, neutrophils, and often Langan's multinucleated giant cells. Sometimes there were minimal necrotic areas, generally composed of necrotic inflammatory cells; the granulomas were fully encapsulated, with central necrotic areas, which were caseous and mineralized. These results agreed with another study (Wangoo et al., 2005).

A good choice enabling direct detection and concurrent strain-typing for diagnosing *M. bovis* in veterinary specimens is PCR which could be used routinely. *M. bovis* was detected in extrapulmonary lesions of cattle and paraffin-embedded tissues of humans using the CSB2 and oxyR genes. These findings agreed with another study (Heinmöller et al., 2001; Roring et al., 2000). Moreover, PCR results obtained in this study indicated that the primer sequences designed to differentiate closely related *M. bovis* and *M. tuberculosis* are 100% specific. These findings are similar to another study in India (Bakshi et al., 2005).

According to the analysis, the oxyR gene of *M. bovis* had an AGCT sequence beginning at the adenine residue located at nucleotide 285. In contrast, *M. tuberculosis* strains had guanine in their sequences at position 285 with no significant changes. With additional single nucleotide substitution in the field isolate at position 125 (thymine instead of cytosine), this is a sporadic, uncommon variation of that condition.

The highest identities (99.62% and 99.81%) were shown by field sequences isolated from *M. tuberculosis* and *M. bovis*, respectively. In order to evaluate and confirm the evolutionary ancestry of the field samples, phylogenetic analysis was used to compare partial sequences of the oxyR gene from field strains and representative strains from GenBank. A phylogenetic tree also revealed that the three field isolates were associated with other *M. bovis* in cluster 1 (*M. bovis*). However, this field isolate sequences are distinct from cluster 2 (*M. tuberculosis*).

Conclusions

We concluded that tuberculosis is an endemic disease in the Sulaimani region and could be transmitted from animals to animals and from animals to humans due to poor control programs among farmers and poses a

high risk of spreading to people. This study demonstrated that *M. bovis* was found in different districts in Sulaimani province according to the ELISA interferon assay. Furthermore, the prevalence of BTB varied between these districts according to the size of the herd. According to the PCR test, humans and animals were infected with *M. bovis* using two sets of primers specific for identifying *M. bovis* in the LN tissues. Therefore, we strongly recommend conducting more studies on the source of bovine tuberculosis due to the high rate in cattle and humans.

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Conflict of interest

The authors confirm that they are not affiliated with or involved in any organization or entity with financial interests.

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