IN VITRO INVESTIGATION OF BOVINE TUBERCULOSIS IN SULAYMANIYAH, IRAQ

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ABSTRACT

This study was conducted to detect bovine tuberculosis (TB) in Sulaymaniyah, Kurdistan Region of Iraq, using gamma interferon assay (GIA), and determine the prevalence in the governorate. The study was carried out among three groups of cattle: calves aged 6–12 months, heifers 13–24 months, and cows aged above two years. Bovine TB was detected by an indirect enzyme-linked immunosorbent assay (ELISA) using the GIA, carried out on 404 cattle within the target population (31173) from different districts around the governorate. A TB prevalence rate of 8.9% was detected. The highest prevalence rate (10.5%) was in the cattle above two years of age, and the lowest rate (2.7%) was recorded in 6–12 months old calves, while the rate was 9.5% in the 13–24 month-old cattle. The results revealed that the highest positive rate (15.2%) was in Bakrajo, and the lowest rate (3.2%) was in Chwarta. The blood samples of the infected and uninfected cattle were examined for some blood parameters such as hematocrit, total and differential leukocyte counts, and erythrocyte sedimentation rate (ESR). There was a decrease in the hematocrit and leukocyte counts and an increase in ESR in infected animals. We conclude that TB is still endemic in Sulaymaniyah, Iraq, and the GIA should be used instead of other standard tests for diagnosing bovine tuberculosis.

Keywords: Gamma interferon assay, infectious diseases, *Mycobacterium bovis*, TB

التحقيق المختبري في مرض السل البقري في السليمانية ، العراق

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المستخلص

أجريت هذه الدراسة للكشف عن مرض السل البقري في السليمانية ، إقليم كردستان العراق ، باستعمال مقايسة جاما إنترفيرون (GIA) ، وتحديد مدى انتشاره في المحافظة. أجريت الدراسة على ثلاث مجموعات من الأبقار: العجول التي تتراوح أعمارها بين 6–12 شهرًا ، والعجول من 13 إلى 24 شهرًا ، والأبقار التي يزيد عمرها عن عامين. تم الكشف عن السل البقري عن طريق مقايسة الممتز المناعي غير المباشرة المرتبطة بالإنزيم (ELISA) باستخدام GIA ، والتي أجريت على 404 من الماشية ضمن السكان المستهدفين (31173) من مناطق مختلفة حول محافظة المحافظة. تم الكشف عن معدل انتشار مرض السل بنسبة 8.9٪. أعلى معدل انتشار (10.5٪) كان في الأبقار فوق عمر السنتين، وأقل معدل (2.7٪) سُجل في العجول بعمر 6–12 شهرًا ، بينما كان المعدل 9.5٪ في عمر 13–24 شهرًا. ماشية. وأظهرت النتائج أن أعلى نسبة إيجابية (15.2٪) كانت في منطقة بكراجو ، وأقل نسبة (3.2٪) كانت في منطقة شوارتا. تم فحص عينات الدم من الأبقار المصابة وغير المصابة لبعض مقاييس الدم مثل الهيماتوكريت ، التعداد الكلي والتفاضلي للكريات البيض، ومعدل ترسيب كرات الدم الحمراء (ESR). كان هناك انخفاض في تعداد الهيماتوكريت والكريات البيض وزيادة في ESR في الحيوانات المصابة. نستنتج أن السل لا يزال مستوطنا في السليمانية ، العراق ، ويجب استعمال GIA بدلا من الاختبارات المعيارية الأخرى لتشخيص مرض السل البقري.

الكلمات المفتاحية: مقايسة جاما إنترفيرون، الأمراض المعدية، المتفطرة البقريّة

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INTRODUCTION

Bovine tuberculosis (TB), a chronic infectious disease caused by *Mycobacterium bovis*, affects all age groups of susceptible hosts and remains an economic and public health problem in several countries (17). Zoonotic tuberculosis caused by *M. bovis* has been shown to have a vast host range, as it was detected in 24 mammalian species, including impalas, buffalos, lions, leopards, cheetahs, and elephants (6). The most common route of infection is the inhalation of aerosols containing *Mycobacterium*, which infected animals may cough out. Other sources of infection are ingestion, through breaks in the skin, and infected semen. The disease can be transmitted from animals to humans and vice versa (22). The pathogenesis of bovine tuberculosis begins with bacterial entry into the host lungs by inhalation, and bacteria undergo phagocytosis by alveolar macrophages (20). Chronic infection is established due to mycobacterial virulence factors that allow it to enter and survive within the host phagocytic cells (24). Bovine tuberculosis is a chronic, generally respiratory disease that is clinically difficult to diagnose, although clinical signs of emaciation, loss of appetite, chronic cough, dyspnea, and roughened hair coat could be observed. It also has other signs of pneumonia which could be symptoms developing at relatively late stages of the infection in cattle (7). There are many methods for diagnosing bovine TB, including lymphocyte proliferation assay, GIA, ELISA, tuberculin skin test, and molecular methods like polymerase chain reaction (6). The control focus is on removing infected cattle at an earlier stage of infection (8). Slaughterhouse monitoring, movement control, and destruction of exposed animals have successfully eliminated the disease, except where a reservoir of infection exists outside the cattle population (21). Tuberculosis has been regularly found in Iraq and the Kurdistan Region for many years. The prevalence of bovine TB in cattle was about 5.1% in Sulaymaniyah, according to a study by Sulaiman R. R. *et al.* (23). Another study in Baghdad concluded that about 43.9% of cows on a farm were positive using the comparative tuberculin test, while the disease rate among the workers and veterinarians was 32% by ELISA (5). The present study aimed to detect bovine tuberculosis using GIA, determine the prevalence of bovine tuberculosis in some districts of Sulaymaniyah, and detect the changes in some blood parameters among study animals.

MATERIALS AND METHODS

Study area and sample collection: This study was carried out from the beginning of October 2020 to the end of March 2021. During that time, the researchers visited different villages and cattle farms. Visited locations were in five districts around Sulaymaniyah: Bakrajo, Chwarta, Piramagroon, Sharazoor, and Tanjaro. These districts comprise 256 villages (Figure 1), where cattle production by farmers continues on a small scale. There are also several privately owned dairy and beef cattle farms. The aim of the sample collection was explained to the cattle owners upon visiting, and verbal consent to participation was gained before each sample collection. Peripheral blood samples were collected from the jugular vein with a minimum volume of 8 mL. The blood was put into two collection tubes; one was heparinized (for measuring hematological parameters), and the other was a plain tube (for serum separation). The blood samples were transported as soon as possible to the laboratory in an icebox for indirect ELISA, GIA, and blood parameter analysis. The analyses were conducted at the College of Veterinary Medicine's Research Center at the University of Sulaimani. The duration of sample transports did not exceed four hours.

Release of gamma interferon and ELISA technique Gamma: interferon was released from the whole blood using a specialized kit manufactured by BioNote Company (South Korea), and the procedure was according to the manufacturer's instructions. The samples were then tested with an ELISA kit (BioNote Company, South Korea), following the kit manufacturer's recommendations. The IFN-γ ELISA test results were classified as negative or positive.

Analysis of blood parameters: The hematological parameters measured were the hematocrit, total and differential leukocyte counts, and erythrocyte sedimentation rate (ESR). The ESR was measured using the

Westergren method with special ESR equipment, which consists of an ESR rack and ESR tube calibrated from zero to 200 mm. The hematocrit was measured using a microcapillary tube. About two-thirds of the tube was filled with blood. A finger was placed over the "non-blood" end, and the opposite end was pushed into sealing wax. The microcapillary tube was placed in a hematocrit microcentrifuge

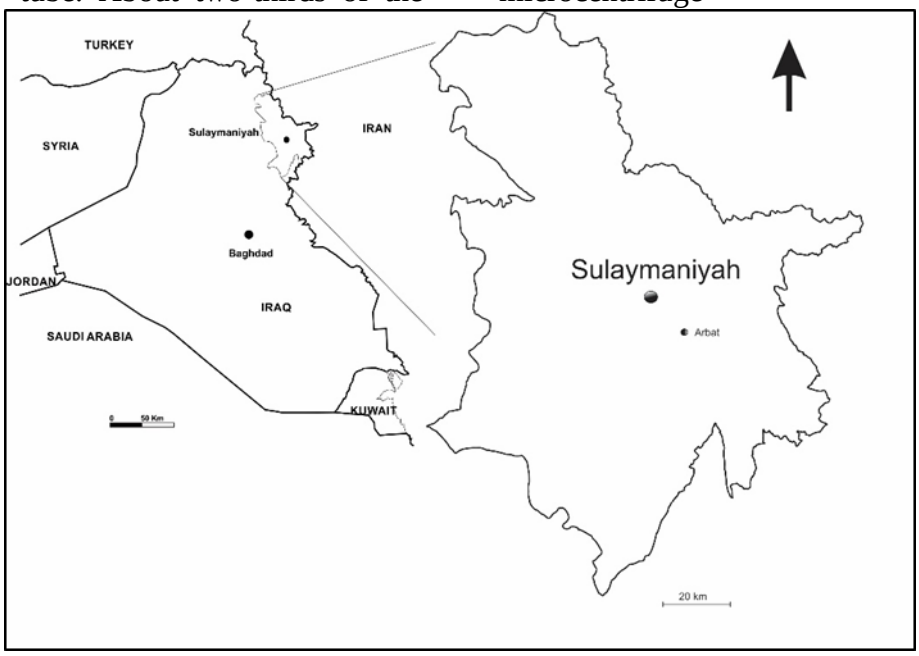

Figure 1. Map of Sulaymaniyah in Kurdistan Region, Iraq

(Hettich, Germany) with the clay end toward the periphery and centrifuged for five minutes at 10,000 rpm. The capillary tube was placed on a hematocrit reader to measure the percentage of packed cells in the tube.

Differential leukocyte count

The differential leukocyte count was done by a slide technique. The blood was collected in EDTA tubes. Blood smears were made for each of the samples and allowed to air-dry. The blood smears were fixed with absolute methanol for five minutes and later stained with Giemsa stain (Syrbio, Syria) for 20–30 minutes. The smears were washed with tap water and left to dry in the air. A drop of oil was placed over the slide, and the slide was examined under a light microscope using 1000× magnification. At least two hundred leukocytes were counted, and the number of each kind of cell was recorded manually.

Statistical analysis

All data were analyzed statistically using Statistical Package for Social Sciences (SPSS) version 24.0 (IBM, USA). Differences between the uninfected and infected groups were compared using an independent samples t-test. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The researchers visited 57 locations (villages and cattle farms) in five districts of Sulaymaniyah and collected blood samples from 404 apparently healthy cows (Table 1). The cows were of the local and crossbreed types. Sample collection lasted six months, starting in October 2020 and continuing until March 2021. The cows ncluded in the study represented about 1.3% of the target population. Infection was recorded in each of the five districts included in the study, ranging from 3.2% to 15.2%. As a whole, about 8.9% of the cattle were seropositive to bovine TB. About 15.2% of the cattle from the district of Bakrajo were seropositive for bovine TB, the highest infection rate in the study area. In contrast, the lowest infection rate was in cattle from Chwarta, as only 2/62 (2.3%) of the cattle were seropositive.According to their age, the animals were divided into three groups. About 18.1% of the cattle included in the sample collection were 6–12 months, and 20.8% were 13–24 months. The rest of the cattle were > 24 months. The highest infection rate was in cattle older than two years, about 3.9 times higher than in calves aged 6–12 months (Figure 2).

Table 1. Prevalence of bovine tuberculosis in the study area

District	No. of villages	Bovine population	No. of samples	No. infected*
Bakrajo	62	8,618	105	16 (15.2)
Chwarta	53	4,312	62	2 (3.2)
Piramagroon	42	5,842	76	4 (5.2)
Sharazoor	39	3,188	50	6 (12.0)
Tanjaro	60	9,213	111	8 (7.2)
Total	256	31,173	404	36 (8.9)

*** Numbers in the brackets represent the percentage of infected cattle according to the number of collected samples in each district**

Blood parameters

The hematocrit and ESR of the blood samples were done for all infected and uninfected cattle. The results showed a decrease in hematocrit and an increase in ESR in seropositive animals older than two years ($p \leq 0.05$), while they were within normal ranges in the uninfected animals (Table 2).

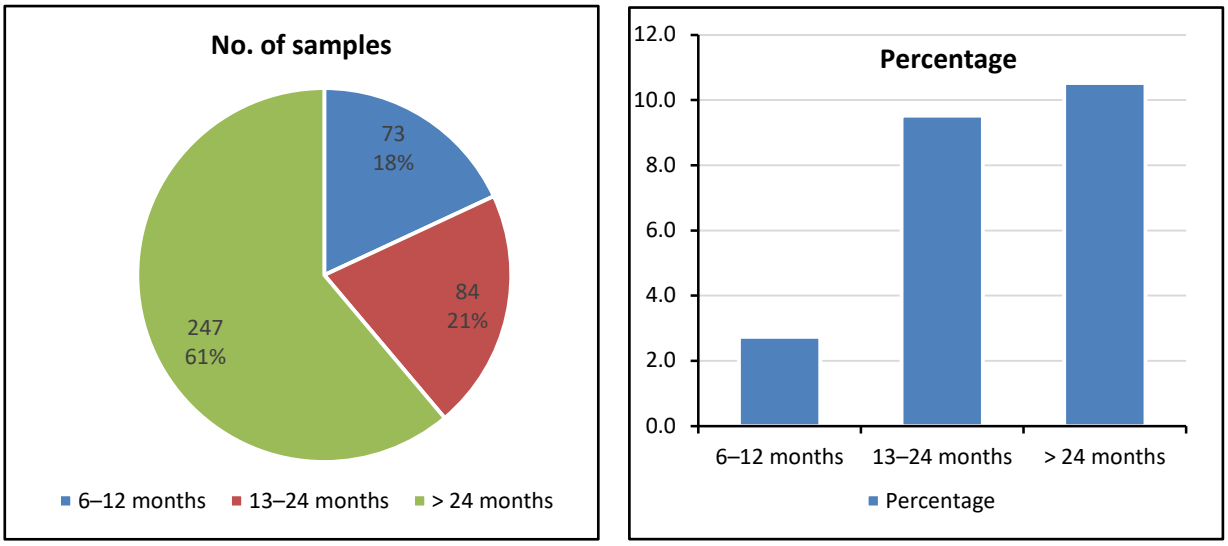

Figure 2. Number of cattle (left) and samples infected with bovine tuberculosis (right).

Table 2. Hematocrit and erythrocyte sedimentation rate (ESR) values in the infected and uninfected cattle

Age (months)	Infection status	ESR (mm)	Hematocrit (%)
6–12	infected	11.0	31.0
	uninfected	3.7 ± 0.7	44.0 ± 2.4
13–24	infected	9.0[a] ± 1.0	40.0 ± 2.1
	uninfected	4.2 ± 0.5	42.0 ± 1.3
> 24	infected	8.0[a] ± 1.1	35.0[a] ± 2.0
	uninfected	4.0 ± 0.8	46.0 ± 2.3

Values represent means ± standard errors. [a] means there is a significant difference ($p \leq 0.05$) within the same age group. Standard errors and statistical comparison were not recorded for calves aged 6–12 months since less than three animals were infected with bovine TB

Total and differential leukocyte counts

The total and differential leukocyte counts were measured in the blood samples of the infected and uninfected groups. The results revealed no differences in the leukocyte counts between the infected and uninfected cattle within the same age group (Table 3). The total leukocyte counts remained within the standard limits in the infected cattle. However, neutrophil concentrations were significantly higher in the older than one-year uninfected cattle. Also, the monocytes were significantly higher in the 13–24 month-old infected cattle, but the trend was not observed for the other age groups.

Table 3. Total and differential leukocyte counts in the infected and uninfected cattle

Age (months)	Infection status	Concentration (number × 10^3/µL) ± standard error					
		Leu.	Neu.	Eos.	Bas.	Lym.	Mon.
6–12	infected	8191.0	2418.5	234.5	0	4524.0	1014.0
	uninfected	8177.0 ± 57.0	3021.9 ± 112.0	88.9 ± 16.0	0 ± 0	4977.3 ± 21.5	88.9 ± 1.4
13–24	infected	8837.5 ± 354.0	1925.0[a] ± 41.3	175.0 ± 24.2	0 ± 0	5512.5 ± 132.6	1225.3[a] ± 31.0
	uninfected	9544.5 ± 778.0	2929.5 ± 78.0	378.0[a] ± 15.0	0 ± 0	5481.0 ± 278.0	756.0 ± 8.8
> 24	infected	6564.7 ± 481.0	2320.9[a] ± 347.0	132.6 ± 10.2	0 ± 0	3381.8 ± 188.0	729.4 ± 77.0
	uninfected	7247.3 ± 338.0	3206.2 ± 397.1	123.3 ± 529.4	0 ± 0	3260.1 ± 738.8	657.7 ± 27.5

Values represent means ± standard errors. [a] means there is a significant difference ($p \leq 0.05$) within the same age group. Standard errors were not calculated for the 6–12 month-old infected calves since only two calves were in the group

The bovine GIA is a diagnostic method that measures interferon-gamma (IFN-γ) released by lymphocytes in response to antigen stimulation. The test relies on measuring the differences in IFN-γ production *in vitro* in response to stimulation of blood with bovine purified protein derivatives (PPD-B) and avian purified protein derivatives PPD-A (16). In the current study, we used the IFN-γ assay to detect bovine TB for its high sensitivity (78.6%) and specificity (100%), according to the manufacturer. This assay provides many advantages for detecting bovine tuberculosis. First, it is highly sensitive and specific compared to other serological tests, like the tuberculin test (3). In a study, the sensitivity of the INF-γ assay ranged from 73.0% to 100.0%, and the specificity from 85.0% to 99.6%. In contrast, the sensitivity and specificity of a comparative tuberculin skin test were 69.0% and 97.0%, respectively (2). Second, the IFN-γ assay can be used for early identification of bovine tuberculosis, as early as 14 days post-infection (1), while the intradermal tuberculin test diagnoses the disease in 3–6 weeks post-infection (19). Finally, the IFN-γ assay provides the result 24 hours after blood collection and removes the operator's error (18). The rate of tuberculosis in the current study in Sulaymaniyah was 8.9%, which is higher than that previously reported by Sulaiman R. R. *et al.* (23) using a single intradermal comparative tuberculin test (5.1%). These outcomes indicate that bovine TB is endemic to the study area. The prevalence rate was lower than that obtained in Baghdad (43.9%) by Barak (5); however, the researcher selected animals with debilitating symptoms, while in the present study, animals were randomly selected. The prevalence rate was the highest in the Bakrajo district (15.2%), probably due to many factors. For example, the herd sizes are larger in Bakrajo, facilitating disease transmission. Continuous close contact between animals increases the probability of disease transmission due to increasing densities and limited pasture for grazing. Breeding and closely housing inversely affect the rate of disease transmission; the closer animals are packed together, the greater the chance that TB will be transmitted. Other factors that increase the risk of disease propagation include uncontrolled animal movement, unhygienic local habits which might facilitate transmission, and grazing sites and gathering of animals during drinking (13). The lowest tuberculosis prevalence rate was found in the Chwarta district (3.2%), possibly due to lower population densities, abundant natural pasture, and lower herd-herd or animal-animal contacts (4). Age has a significant effect on the rate of propagation of disease. The prevalence of the disease is elevated with the age of the cattle above two years (10.5%), while it was (2.7%) in 6–12 month-old calves. This result agrees with that found by other researchers (10, 15) Animal age is one of the primary individual risk factors for increasing the prevalence of TB, which may be related to increased duration of the exposure with age, with older cattle being more likely to have been exposed than the younger. Also, the low prevalence rate in young animals may be associated with the predominance of γδ T cells in calves that have been shown to play a relevant role in antimycobacterial immunity (14). *Mycobacterium bovis* produces tubercles and

adversely affects hemato-biochemical parameters as various diseases (12). The results of the hematological evaluation show that there was a significant increase in ESR in the infected cattle. This is possibly caused by anemia, in which ESR is increased due to fewer cells that settle more easily in large volumes of the fluid or alteration in plasma protein (9). Furthermore, there was also a decrease in hematocrit, which could be attributed to poor health status, anemia, chronicity of the disease, and bone marrow atrophy (11).

CONCLUSION

This study investigated the prevalence of bovine TB using the GIA instead of other common tests for diagnosing bovine tuberculosis in our region, and about 8.9% of the cattle in the study area were found infected. The highest incidence rate was in the Bakrajo district (15.2%), and the lowest was in the Chwarta district (3.2%), and the incidence was higher as the animal age increased. Compared with previous studies in Sulaymaniyah, the rate recorded in this study indicated that bovine TB is still endemic. More studies are required to determine the disease prevalence in other areas of Iraq. Also, based on testing and slaughtering, a national bovine TB control program by the Kurdistan Regional Government is necessary.

Competing interest

We have no competing interests.

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