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Prevalence of ovine theileriosis in Mosul city, Iraq

M.M. Hamid and Q.T. Al-Obaidi

Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

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Correspondence:
Q.T. Al-Obaidi
qaestalb1976@uomosul.edu.iq

Abstract

The present study aimed to determine the prevalence of ovine theileriosis (OT) in sheep in Mosul city, Iraq using microscopic examination (ME) of the blood smears stained with MGG- Quick stain and conventional polymerase chain reaction technique (c-PCR) to compare between c-PCR technique and ME as techniques for the diagnosis of disease, and to investigate the pattern and type of infections based on multiplex polymerase chain reaction technique (m-PCR). From October 2021 to May 2022, one-handed eighty-five Blood samples were drawn randomly from sheep in various regions of Mosul city. The overall prevalence of OT was 42% (22.7 out of 185) and 52.4% (97 out of 185) using microscopic examination and c-PCR technique, respectively. A slight agreement was observed between ME of blood smears and c-PCR technique according to Kappa value 0.190, with low sensitivity, specificity, and accuracy of ME method was 30%, 88.6%, 58.4%, respectively, compared with c-PCR technique. The prevalence of mixed infection 22.7% and single infection with *T. lestoquardi* 20% were significantly higher (P<0.05) than single infection with *T. ovis* 9.7%. This study concludes that OT is widespread in Mosul city, Iraq, and the c-PCR technique is more reliable and suitable for detecting Theileria infection in sheep than the ME method.

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Introduction

Ovine theileriosis (OT) is a serious protozoal disease that affects sheep and goats, transmitted by ticks (1,2). It causes significant economic losses due to the disease's high mortality rate, decreases in production and the cost of treatment, and controls the disease's vectors (3). The morbidity rate of the disease is 100%, while the mortality rate is more than 40% was shown in naturally infected sheep with *T. hirci* in Iraq (4). At least six species of Theileria caused the disease in sheep and goats, such as *Theileria separate*, *T. ovis*, *and T. recondite*. Low or nonpathogenic protozoan caused a benign form of the disease called benign ovine theileriosis (5). Moreover, *Theileria hirci* (*T. lestoquardi*) and *Theileria spp. chaina1 and china* 2, which are highly pathogenic hemoprotozoan, cause a malignant form of the disease called malignant ovine theileriosis (6,7). These parasites can be biologically transmission via Ixodidae ticks from the genera: Hyalomma, Haemaphysalis, and Rhipicephalus, Amblyomma (8-10), also can be mechanically transmission by stinging flies or blood-sucking insects and blood-contaminated syringes and needles (11,12), Moreover, through vertical transmission (Transplacental transmission) from the ewe to the fetus (9). Ovine theileriosis has been reported in various geographic regions, including Central Asia, North and East Africa, and Southern and Eastern Europe (13). Sheep infected with the disease are suffering from weakness, loss of appetite, fever more than 40ºC, pale mucous membrane, enlargement of superficial lymph nodes, exophthalmos, conjunctivitis, dyspnea, diarrhea mixed with blood, dehydration, and presence of ticks on different body parts of the animal (4,6). Ovine theileriosis can be diagnosed based on clinical signs, laboratory tests, microscopic examination of stained blood and lymph smears, and direct or indirect enzyme-linked

immunosorbent assays (14,15) and different polymerase chain reaction techniques (16,17).

Ovine theileriosis Studies concerning ovine Theileriosis in Mosul need more knowledge and epidemiological investigations. Hence, the objectives of the present study were to determine the prevalence of OT in sheep in Mosul city, Iraq, with a comparison between the c-PCR technique and ME as techniques for disease diagnosis and to investigate the pattern and type of parasites.

Materials and methods

Ethical approval

This work was ethically permitted by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, University of Mosul, (UM.VET.2021.18) on the 6th of September 2021.

Animal and sample size

This work was conducted on 185 sheep of both sexes, of different ages, breeds, and origins, and from various areas of Mosul city-Iraq, representing 22 fields (1859 sheep), at a rate of 10% of animals from each field. The number of animals were calculated based on an earlier study of the seroprevalence of OT in Kurdistan, Iraq, was 13.43% (18). Using the following formula, the expected prevalence of the disease was 13% with a confidence level of 95% and an absolute error of 5% (19).

Samples collection

From October 2021 to May 2022, one-hundred eighty-five blood samples 2.5 ml were withdrawn via the jugular vein using a 3ml sterile syringe. Then, they were dispended into tubes with anticoagulant ethylene diamine tetraacetic acid (EDTA), which is used for preparing thin and thick blood smears (2 smears from each animal) if it is not possible to do it via the ear vein of the sheep, for the initial microscopically investigated in the sheep infected with Theileria parasite. The rest of the blood was kept in the freezer at -20ºC until the molecular examination was performed using c-PCR and m-PCR techniques to distinguish species of Theileria parasite. Moreover, lymph smears were prepared from lymph biopsy of enlarged prescapular lymph nodes (1 smear from animals suffering from enlargement lymph nodes) (20).

Microscopic examination of blood and lymph smears

A total of 442 examined smears comprising (185 thick blood smears and 185 thin blood smears) and (72 lymph smears) were prepared, air dried, then stained with MGG-Quick stain (Bio-Optic, Italy) and examined under a light microscope at (X1000) with immersion oil (Leitz, Germany) (21). For the initial investigation of *Theileria spp*. in erythrocytes and lymphocytes. Moreover, the calculation of the percentage of the Theileria parasite (Parasitemia) in the blood and lymph follows the equations of Al-Obaidi and Alsaad (20) and Altay *et al.* (22), respectively.

DNA extraction

A ready kit was used to extract genomic DNA from 185 sheep whole blood samples: FavorPrep™ Blood/ Cultured cells Genomic DNA Extraction Mini Kit (FAVORGEN Biotech Corporation, Taiwan). Using Nano-drop (BioDrop, England), the concentration of extracted DNA regarded at wavelength 260nm ranged between 37.6 - 322.7 ng/ µl. While, by calculating the ratio of (A260 nm to A280 nm), the purity was found to be between 1.5 and 1.9.

DNA amplification

Two reactions were used to amplify the 18S rRNA gene of *Theileria spp.*: The first reaction by c-PCR technique, to identify the positive sheep for all *Theileria spp*. in approximately band size 1098bp, using universal primers (989-F and 990-R). While the second reaction by m-PCR technique was done to differentiate between *T. lestoquardi* and *T. ovis* in all positive samples in the first reaction, using specific primers (T170-F and T670-R) for *T. ovis* in approximately band size 520bp and (TF2 and TR2) for *T. lestoquardi* in approximately band size 230bp, all primers were provided by (Macrogen Inc. South Korea), (Table 1). Conventional PCR technique was done with a total volume of 25 µl composing 12.5 µl of master mix (2X), 1µl (10 pmol) of each primer (989-F and 990-R), 3 µl of template DNA, and 7.5µl of PCR-Grade water. At the same time, the m-PCR technique was done with a total volume of 25 µl consisting of 12.5 µl of master mix(2X), 1µl (10 pmol) of each primer (T170-F and T670-R) for *T. ovis*, and (TF2 and TR2) for *T. lestoquardi*, 3µl of template DNA and 5.5µl of PCR -Grade water. In the m-PCR technique, positive control was consisting the same components above. Instead of the extracted DNA sample, the DNA sample of the known parasite type was placed, obtained from a clinically infected laboratory sheep. Moreover, a negative control consistend of the same components without template DNA.

The thermocycler (BIO-RAD/ USA) was set as follows: 5min at 95°C for the predenaturation step (1 cycle), 1 min at 95°C for the denaturation step, 45s at 55°C for the annealing step, and 1 min at 72°C for extension step (35 cycles), with a 5 min at 72°C for final extension step (1cycle), according to Radwan and El Kelesh, (23) with some modification in annealing step. PCR yields were electrophoresed in a 1.5% agarose gel stained with Midori green. UV transillumination (BIO-RAD/USA) was used to visualize the resulting bands.

Comparison between techniques used in this work

Kappa value was used to determine the agreement between ME of blood smears, and the c-PCR technique was determined. If the Kappa value < 0, this indicates no agreement between the two tests, If the Kappa value is 0.0 - 0.20, the agreement is slight; if the Kappa value is 0.21 -

0.40, the agreement is fair; if the Kappa value is 0.41 - 0.60 the agreement is moderate; if the Kappa value is 0.61 - 0.80 the agreement is substantial; and if the Kappa value is 0.81 - 1 the agreement is almost perfect (27). Moreover, sensitivity, specificity, and accuracy of ME were calculated in comparison with the c-PCR technique (28).

Table 1: Oligonucleotide primers of *Theileria spp*., *T. lestoquardi*, and *T. ovis* used in this work

Type of Parasite	Primers	Sequences 5'-3'	Size (bp)	References
Theileria spp.	989-F 990-R	AGTTTCTGACCTATCAG TTGCCTTAAACTTCCTTG	1098	(24)
*T. lestoqua*rdi	TF2 TR2	GACACAGGGAGGTAGTGACAAG CTAAAGAATTTCACCTTTCTGACA	230	(25)
T. ovis	T170-F T670-R	TCGAGACCTTCGGGT TCCGGACATTGTAAAACAAA	520	(26)

Statistical analysis

This study's data were analyzed using IBM-SPSS Version 19 (Inc., Chicago, USA), which included the Chi-square 2x2 table and the Kappa value. The data was deemed statistically significant when the P value was 0.05.

Results

In the present work, the overall prevalence of OT in sheep in Mosul city using microscopic examination of blood smears and conventional PCR technique was 22.7% (42 out of 185) and 52.4% (97 out of 185), respectively (Table 2). For primary identifications of Theileria parasite, microscopic examination of 360 thin and thick blood smears staining with MGG quick stain demonstrated that *Theileria spp*. is seen singly with different shapes such as coma, round and anaplasma-like shapes within erythrocytes of infected animals with parasitemia ranged between 2%-18% with the mean 9.4% (Figure 1A). In contrast, microscopic examination of 72 lymph smears observed the parasite in the form of macroschizontes and macroschizontes (Koch's blue bodies) within lymphocytes, with the percentage of intra-lymphatic parasites (Parasitemia) ranging between 1% - 15%, with the mean of 7.3% (Figure 1B). Moreover, the results were based on conventional PCR for amplified DNA fragments of the 18S rRNA gene of *Theileria spp*. using universal "catch-all" primers (first time in Mosul city) in 185 blood samples from sheep demonstrated a positive band approximately at 1098 base pair (bp) (Figure 2).

Table 2: Ovine theileriosis prevalence in sheep using microscopic examination and conventional PCR technique

Used test	Number of exanimated samples	Number of positive (%)
Microscopic examination	185	42 (22.7)
Conventional PCR technique		97 (52.4)

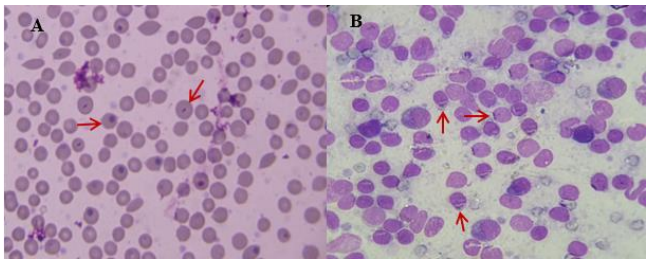

Figure 1: A- MGG-Quick stained blood smear showed Theileria parasite in the sheep erythrocytes. B- Lymph smear stained with MGG-Quick stain showed microschizontes and macroschizontes (Koch's Blue bodies) inside sheep lymphocytes under oil immersion at (1000X).

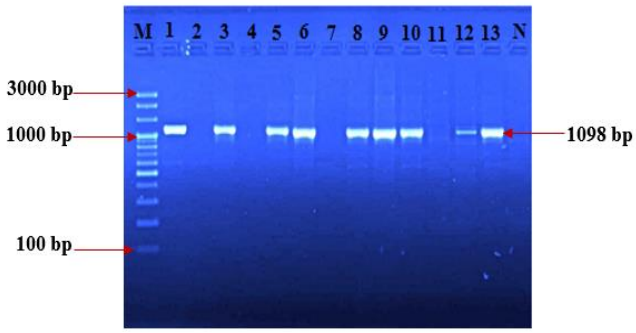

Figure 2: Image representing: lanes M: DNA ladder, Lane 1.3.5,6,8-10,12,13: c-PCR assay detected *Theileria* spp. using universal primers in a band size 1098 bp, Lane N: DNA extracted from Theileria-free sheep as a negative control.

Based on the Kappa value of 0.191, the current study also found a slight agreement between ME of staining blood smears and the c-PCR technique in diagnosing *Theileria spp*. in sheep, and the sensitivity, specificity, and accuracy of the microscopic examination of blood smears were 30.9, 88.6, and 58.4%, respectively, when compared to the c-PCR technique (Table 3).

Table 3: Based on kappa value comparison between microscopic examination (ME) and conventional PCR technique (c-PCR), with the calculation of sensitivity, specificity, and accuracy of ME for diagnosing OT

		Conventional PCR technique		
		Infected	Uninfected	Total No.
Microscopic examination	Infected	30 a	10 b	40
	Uninfected	67c	78 d	145
Total		97	88	185

(a) True positive samples, (b) False positive samples, (c) False negative samples, (d) True negative samples. Kappa value was (0.413). Sensitivity = a/(a+c) x 100 = 30.9%. Specificity = d/(b+d) x 100 = 88.6%. Accuracy= (a+c)/ (a+c+ b+d) x 100 = 58.4%.

Results based on the multiplex PCR technique observed that the prevalence of single infection *T. ovis* was 9.7% (18 out of 185), with the positive bands at approximately 520 bp (Figure 3), and the prevalence of single infection *T. lestoquardi* was 20% (37 out of 185), with the positive bands at approximately 230 bp (Figure 4), while the prevalence of both parasites (Mixed infection) was 22.7% (42 out of 185), with the positive bands at approximately 520 bp. and 230 bp (Figure 4). Prevalence of mixed infection of both parasites and *T. lestoquardi* single infection was significantly (P<0.05) higher compared to *T. ovis* single infection (Table 4).

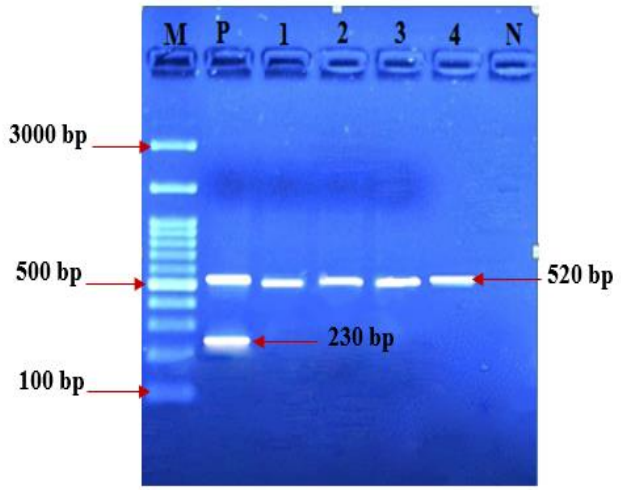

Figure 3: Image representing: lane M: DNA ladder, Lane P: Positive control DNA for *T. ovis* and *T. lestoquardi* was extracted from a clinically infected animal, Lane 1-5: m-PCR assay detected only *T. ovis* in a band size 520 bp, Lane N: Negative control DNA was extracted from Theileria-free sheep.

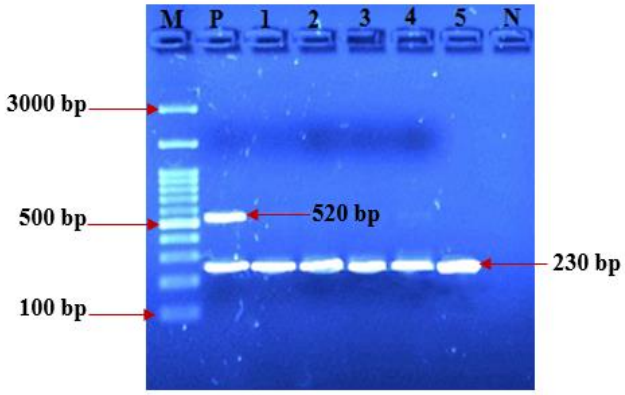

Figure 4: Image representing: lane M; DNA ladder, Lane P: Positive control DNA for *T. ovis* and *T. lestoquardi* was extracted from a clinically infected animal, Lane 1-4: Multiplex PCR technique detected only *T. lestoquardi* in a band size 320 bp; Lane N: Negative control DNA was extracted from Theileria-free sheep.

Table 4: Prevalence of the pattern and type of *Theileria spp.* in sheep in Mosul city using multiplex PCR technique (n*=185)

Pattern of infection		Multiplex PCR technique	
		No. of positive	Percentage (%)
Single	*T. ovis*	18	[a] 9.7
	T. lestoquardi	37	[b]20
Mixed	*T. ovis* with *T. lestoquardi*	42	[b] 22.7
Total		97	52.4

Different superscript letters (a,b) were assigned to values that were significantly different (P <0.05). n= Total number of animals used for calculating the prevalence of *Theileria spp.*

Discussion

In the present study, the overall prevalence of OT in sheep in Mosul city was 21.7% by ME of blood smears and 52.4% by c-PCR technique. These results are lower than the prevalence reported in other studies of OT in Iraq. A Jalil *et al.* (29) reported that the prevalence of *Theileria spp*. in sheep at Al-Diwaniyah city was 90.1% using ME of stained blood smears. Based on the c-PCR technique, the prevalence of OT was 75.8% in Al-Kut South Iraq (30). While the prevalence of OT in the current study was higher than those reported in Mosul city, which was 0.03% using the microscopic examination method (1), in the middle region of Iraq was 4.3% using the c-PCR technique (31), and in Kurdistan regions of Iraq was 13.43% and 17.91% using microscopy test and polymerase chain reaction technique respectively (18). The variance in the prevalence of OT in sheep among

regions in the same country may be related to field management practices, type of diagnostic test, presence of tick vectors in the field and/or on the animals, sampling size and variant climatic factors, which effect on the tick's population (1).

Other studies conducted around the world revealed varying prevalence rates of *Theileria spp*. in sheep using different laboratory techniques, such as: in Iran, was 47.27% (32), in Turkey, it was 7% (33), in Saudi Arabia was 57.8% (34), in Egypt was 21.7% (35), in Sudan was 12.9% (36), in Pakistan was 24.6% (13), and in China was 57.53% (37). The reasons for the difference in the prevalence of *Theileria spp*. among countries could be the following: different management practices, efficient diagnostics techniques used, the inefficiency of ticks control programs, presence of competent tick vectors, and climatic variations (2,38,39).

In this study, ME of staining blood smears showed *Theileria spp*. singly with different shapes inside the erythrocytes, the mean of parasitemia was 9.4%, which is consistent with that demonstrated by Tylor *et al.* (40) and Rahmani *et al.* (41). Furthermore, ME of lymph smears seen in the Theileria in the form of macroschizontes and macroschizontes (Koch's blue bodies) inside the lymphocytes, with the mean of parasitemia was 7.3%, which agrees with El Imam and Taha, (4).

Results of the current study indicate a slight agreement between ME of blood smears and c-PCR technique according to Kappa value 0.190, with low sensitivity, specificity, and accuracy of ME method compared with c-PCR technique. This finding agrees with the results of Sharifi *et al.* (42). An optical detection of piroplasms in the erythrocytes by microscopic examination of stained blood smears is probable during an acute form of OT. In contrast, through subclinical, persistent, and /or chronic infection, piroplasms are rarely detected because of very low parasitemia in the infected animals (13,43). Despite the low sensitivity, easiness, quickness, and cheapness of microscopic examination, it should be confirmed by other more sensitive and accurate techniques such as serological and molecular techniques (42,44). Today, the use of PCR assay to detect the DNA from infected animal piroplasms is due to compassion, specificity, and ability to investigate the DNA from 2.5µl of blood with an estimated parasitemia of 0.000001% (45,46).

In the present work, the prevalence of single infection with *T. ovis* and *T. lestoquardi* and mixed infection of both parasites in sheep was 9.7, 20, and 22.7%, respectively. This result was lower than A'aiz and Dhaim's (31) result, which found that the prevalence was 63.2, 48.2 and 45.9% in sheep in Al-Kut South Iraq. Results also showed that the prevalence of mixed infection and single infection with *T. lestoquardi* was significantly higher and more risk than a single infection with *T. ovis*. This finding corresponds with Zhao *et al.* (2) and Zarei *et al.* (47), who mentioned that *T. lestoquardi* caused malignant ovine theileriosis, while *T. ovis* caused benign Ovine Theileriosis and it is a nonpathogenic type of parasite.

Conclusions

It has been concluded that OT is familiar in Mosul city, Iraq, and the c-PCR technique is more reliable and suitable for detecting both *T. ovis* and *T. lestoquardi* infection in sheep than the microscopic examination method. Furthermore, a mixed infection of both parasites and a single infection with *T. lestoquardi* in sheep is more risk than a single infection with *T. ovis.*

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Conflict of interest

The authors declare that there are no conflicting interests in the article.

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انتشار مرض الثايليريوسز في ضأن مدينة الموصل، العراق

محمد محمود حامد و قيس طالب العبيدي

فرع الطب الباطني والوقائي، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

الخلاصة

هدفت الدراسة الحالية تحديد مدى انتشار مرض ثايليريوسز الأغنام في ضأن مدينة الموصل- العراق باستخدام الفحص المجهري للمسحات الدموية المصبوغة بالصبغة السريعة تفاعل البلمرة المتسلسل التقليدي، وكما تم المقارنة بين تقنية تفاعل البلمرة المتسلسل التقليدي والفحص المجهري كتقنيات لتشخيص المرض، وأيضا تم الكشف عن نمط ونوع الإصابة اعتمادا على تفاعل البلمرة المتسلسل المتعدد. استغرقت الدراسة الفترة من تشرين الأول ٢٠٢١ إلى أيار ٢٠٢٢، تم سحب ١٨٥ عينة دم بشكل عشوائي من الأغنام في مناطق مختلفة من مدينة الموصل. وبلغت نسبة الانتشار الكلي للمرض في الضأن ٢٢,٧٪ (٤٢ من أصل ١٨٥) و ٥٢,٤٪ (٩٧ من أصل ١٨٥) باستخدام الفحص المجهري وتقنية تقنية تفاعل البلمرة المتسلسل التقليدي على التوالي. لوحظ توافق طفيف بين الفحص المجهري للمسحات الدم وتقنية تفاعل البلمرة المتسلسل التقليدي اعتمادا على قيمة كابا التي بلغت ٠,١٩٠، مع انخفاض في حساسية ونوعية ودقة طريقة الفحص المجهري والتي كانت ٣٠، ٨٨,٦ و ٥٨,٤% على التوالي مقارنة بتقنية تفاعل البلمرة المتسلسل. وكان مدى انتشار الإصابة المختلطة ٢٢,٧٪ والإصابة المفردة بطفيل الثايليريا ليستوكواردي ٢٠%، أعلى معنويا من الإصابة المنفردة بطفيلي الثايليريا البقرية ٩,٧%. استنتج من هذه الدراسة إلى أن مرض ثايليريوسز الأغنام منتشر في الضأن في مدينة الموصل-العراق، وأن تقنية تفاعل البلمرة المتسلسل التقليدي هي تقنية أكثر موثوقية ومناسبة للكشف عن طفيلي الثايليريا في الضأن من الفحص المجهري.