

Detection of *Trypanosoma spp.* in Dogs Using Different Laboratory Techniques in Mosul City, Iraq

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Abstract

Canine trypanosomiasis is caused by a parasite of the genus trypanosome. It can be transmitted to different animals such as ruminants, horses, cats, in addition to human. Dogs play a crucial role in transmission of trypanosome, as they are the primary domestic animals that contribute to the parasite's transmission and maintenance cycles. Thus, the aim of this research was to estimate the prevalence of trypanosomiasis in dogs in Mosul city, Iraq using routine laboratory techniques and molecular approaches. The results of study showed that a total prevalence rates of *Trypanosome spp.* in 85 dogs were 18.8%, 24.7%, 38.8%, 7.05% using blood staining smear, microhaematocrit centrifugation technique (MHCT) and conventional PCR technique (ITS-1 gene and RoTat 1.2 VSG gene) respectively. A significant differences in the prevalence rate between males (18%) and females (20%) at $P \leq 0.05$, based on blood staining smear. While, no significant differences in the prevalence rate between males (24%, 36%, 16.6%) and females (25.7%, 42.8%, 20%) at ($P \leq 0.05$), based on MHCT, conventional PCR (ITS-1 gene and RoTat 1.2 VSG gene) respectively. The results finding indicated presence of significant differences among different ages of dogs using microscopical and conventional PCR ($P \leq 0.05$), A dogs from four to five years old have the highest prevalence rate (68.7%) when compared with others, and *Trypanosoma evansi* was determined in old dogs (3-5 years) only. results revealed that a perfect agreement between blood staining smear method and MHCT, and substantial agreement between blood staining smear method and conventional PCR and moderate agreement between MHCT and conventional PCR. In conclusion the prevalence of trypanosome infection in dogs (stray and housed) is very high in Mosul city, Iraq.

Key words: Prevalence, *Trypanosoma spp.*, Dogs, cPCR, Mosul, Iraq.

Introduction

Animal trypanosomiasis has both economic and biological importance for domestic animals worldwide. These haemoparasites can cause abortion in pregnant animals, and also have the potential to cause other reproductive disorders such as infertility in mature animals. In addition, these parasites can lead to delay of growth, loss of body weight and impaired physical conditions that may cause death of infected animals (1).

The disease caused by various species of genus *Trypanosoma*, but *Trypanosoma cruzi*, *T. caninum* and *T. evansi* (Nagana and Surra disease) are the most predominant species that are causing the disease in dogs (2), as well as *T. brucei brucei* and *T. congolense* (3).

In human, dogs act as a reservoir for American trypanosomiasis (Chagas disease), a disease caused by the species *T. cruzi*, and African trypanosomiasis (surra or sleeping sickness disease) a disease caused by *T. evansi* (4, 5).

Some species of trypanosome have a tendency to be host specific organisms such as *T. rangeli*, which is found to be a non-pathogenic organism in humans, but it has the ability to infect dogs (6).

These parasites are mechanically transmitted by various genera of hematophagous flies, including *Tabanus*, *Stomoxys*, and *Triatomids* bugs (7, 8). In fact, the source of infection in mammalian hosts is mainly originated from a bite of infected tsetse flies. However, dogs may acquire trypanosomiasis via ingestion of the infected carcasses, also, the parasitic infection can be established experimentally in dogs by oral route (9). The distribution of the disease worldwide is dependent upon the prevalence of vectors, which are involved in the transmission of trypanosomiasis in dogs and other hosts (10, 11).

Canine trypanosomiasis is clinically characterized by variable degrees of severity. The infection can range from acute, sub-acute and chronic disease. The disease is more common in stray dogs because they are more exposed to vectors “flies” compared to the housed animals (12).

Clinically, the infection is marked by increasing anemia, fever, inappetence, emaciation, weakness, head and throat edema, and lastly, stumbling gait and death (13, 14).

Identification of pathogenic trypanosome species in dogs is routinely done by the observation of the parasites in stained blood smears, wet blood film (WBF), or via the

micro-haematocrit centrifugation technique (MHCT). Nevertheless, the MHCT is considered simple, easy to use and inexpensive, but this technique has some drawbacks of low sensitivity, and it is unable to detect parasites in the late stages of infection especially at low parasitemia levels (15, 16). Other laboratory methods have also been implemented such as the serological techniques that are useful for detection the presence of *Trypanosoma* antibodies (for example, Enzyme-linked immune-sorbent assay). However, these serological tests are unable to detect antibodies in some cases due to serological latency (17). Recently the development of molecular tools in biology provides accurate and sensitive methods that enables researchers for specifically identify a variety of pathogens in general. One of these techniques is the polymerase chain reaction(PCR); it could assist in the confirmative diagnosis of the parasite presence in both acute and chronic infection, and also it can be useful for differentiating between species of this parasite (15, 1).

Thus, the purpose of this work was to determine the prevalence of trypanosomiasis in dogs in Mosul city by using different parasitological and molecular methods.

Materials and methods

Study area and sampling

This cross-sectional study was performed from December 2020 to June 2021 in deferent parts in Mosul city, Iraq. A total of 85 dogs from different species visiting veterinary teaching hospital and other private veterinary facilities were involved in this study (including 50 males and 35 females from one to five years old). All the dogs did not suffer from a clinical disease, but most dogs were emaciated and loss of appetite at the time of sampling. From each dog was blood sample (2–3 ml) drawn from the saphenous or cephalic vein, and then kept in tubes containing ethylene-diamine tetra-acetic acid (EDTA). After that, the blood tubes were transported to the laboratory of clinical pathology in department of internal and preventive medicine, university of Mosul for parasitological detections and molecular identification of the pathogen.

Parasitological Investigations

All blood samples were prepared for identification the presence of the protozoa in Giemsa-stained blood smear as described previously(18, 19), and in the micro haematocrit centrifugation technique

(MHCT); 70 µL of fresh blood was drawn by a capillary tube and then placed in a centrifuge for 5 min at 10,000 rpm. A light microscope at a magnification of 100× was used to check the capillary tubes for motile trypanosomes around the buffy coat (20).

Molecular detection of parasite using conventional PCR technique

1-Extraction of DNA from blood samples

DNA was extracted using the genomic DNA purification kit according to the manufacturer's procedure (Promega co. ®). To concentrate the parasites, 1 ml of whole blood was spun for 1 minute at 15,000 rpm with an anticoagulant and 300 µL of the leukocyte layer was separated for DNA extraction. The content of DNA (ng/µL) was quantified using spectrophotometry at 260 nm on 2 µL of sample via a Nano Drop.

2-Molecular Diagnosis

The first step of molecular analysis of PCR protocols, that applied to identify the presence of *Trypanosoma spp.* in the DNA samples, were used to amplify 480 base pair (bp) region of the ribosomal RNA internal transcribed spacer 1 (ITS-1). The primers that used for amplification of ITS gene including the forward and reverse primers, respectively ITS1 F(5'-

CCGGAAGTTCACCGATATTG-3') and ITS1 R(5'-TGCTGCGTTCTTCAACGAA-3'); these specific primers are crucial for detection the parasites of the genus *Trypanosoma spp.* (21), as described in table (1).

In the second step; all samples that tested positive with ITS-1 were proceeded to the second PCR test, which is a specific for *Trypanosoma evansi*. In this reaction, the RoTat 1.2 VSG gene fragment of *T. evansi* was targeted. Here, the sequences of the primers that have been used to amplify 151 bp fragment of the mentioned gene, were included the forward primer of RoTat 1.2 VSG (5'-CTGAAGAGGTTGGAAATGGAGAAG-3') and the reverse primer of RoTat 1.2 VSG (5'-GTTTCGGTGGTCTGTGTTG TTA-3') (22), as illustrated in table (1).

Statistical analysis

The software SPSS was used to conduct statistical analysis (V.17.0; IBM, USA). The chi-square test was applied to determine the association between the prevalence of infection and related risk variables such as gender and age. The significant difference was determined at P value ($p \leq 0.05$), the agreement between different tests used in this study was done according to kappa value (23).

Table (1): PCR steps for detection of *Trypanosoma spp.* and *Trypanosoma evansi* in the extracted DNA samples.

Target gene	Primer sequences 5'-3'	Product size\ Base Pair	Initial Denaturation C°/ min.	Amplification (40cycle)			Final Extension C°/ min.	Product Co.
				Secondary Denaturation C°/sec.	Annealing C°/ sec.	Extension C°/ sec.		
*ITS-1 gene	F(5'CCGGAAGTTCACCGATATTG-3') R(5'TGCTGCGTTC TTCAACGAA-3')	480	95/2	95/ 30	58/30	72/ 60	72/5	Bioingentech
*RoTat 1.2 VSG	F(5'CTGAAGAGGT TGGAAATGGAGA AG-3') R(5'GTTTCGGTGG TTCTGTTGTTGTT A-3')	151	95/2	95/ 30	58/30	72/ 60	72/5	Bioingentech

*ITS-1 gene: specific for genus trypanosome, Rotat 1.2 VSG gene: specific for *Trypanosoma evansi*

Results

The results of study showed that a total prevalence rates of *Trypanosome spp.* in 85 dogs were 18.8%, 24.7%, 38.8 %,7.05% using blood staining smear, microhaematocrit centrifugation technique and conventional PCR technique (ITS-1 gene and RoTat 1.2 VSG gene) respectively. A significant differences in the prevalence rate between males (18%) and females (20%) at $P \leq 0.05$, based on blood staining smear. While, no significant differences in the prevalence rate between males (24%,

36%,16.6%) and females (25.7%, 42.8%, 20%) at $P \leq 0.05$, based on MHCT, conventional PCR technique (ITS-1) and conventional PCR test (RoTat 1.2 VSG gene) respectively (Table 2).

Our findings indicated presence of significant differences among different ages of dogs using microscopical and conventional PCR technique ($P \leq 0.05$), A dogs from four to five years old have the highest prevalence rate (68.7%) when compared with others, and *Trypanosoma evansi* was determined in old dogs (3-5 years) only (Table 2).

Table (2): Prevalence rate of trypanosome infection in dogs according to sex and age by microscopical and conventional PCR technique.

Categories	Microscopical Examination					Conventional PCR Technique			
	No.	Smear	%	MHCT	%	ITS-1	%	RoTat 1.2 VSG	%
Sexes									
Male	50	^a 9	18	^a 12	24	^a 18	36	^a 3	16.6
Female	35	^b 7	20	^a 9	25.7	^a 15	42.8	^a 3	20
Ages/Years									
1-2	22	^a 1	4.54	^a 2	9.09	^a 5	22.7	^a 0	0
2-3	18	^a 3	16.6	^a 5	27.7	^a 8	44.4	^a 0	0
3-4	29	^b 8	27.5	^a 6	20.6	^a 9	31.03	^a 2	22.2
4-5	16	^a 4	25	^b 8	50	^b 11	68.7	^a 4	36.3
Total	85	16	18.8	21	24.7	33	38.8	6	7.05

Letters different vertically indicate a statistically significant difference between categories at value of $P \leq 0.05$.

The results of study revealed a perfect agreement between blood staining smear method and MHCT base on kappa value(0.828) for diagnosis of trypanosome infection (Table 3), and substantial agreement between blood staining smear

method and conventional PCR technique base on kappa value(0.535) (Table 4), and moderate agreement between MHCT and conventional PCR technique base on kappa value(0.682) (Table 5).

Table(3): Agreement between blood staining smear method and microhaematocrit centrifugation technique based on kappa value for diagnosis of Trypanosome infection.

		Blood staining smear method		
		Infected	Uninfected	Total No.
Microhaematocrit centrifugation technique	Infected	5	*16	21
	Uninfected	**64	0	64
		69	16	85

*Means false positive. **Means false negative. Kappa value was (0.828) perfect agreement.

Table(4): Agreement between blood staining smear method and conventional PCR technique based on kappa value for diagnosis of trypanosome infection.

		Blood staining smear method		
		Infected	Uninfected	Total No.
Conventional PCR technique	Infected	16	*17	33
	Uninfected	**0	52	52
		16	69	85

*Means false positive. **Means false negative. Kappa value was (0.535) substantial agreement.

Table(5): Agreement between microhaematocrit centrifugation technique and conventional PCR technique based on kappa value for diagnosis of trypanosome infection.

		microhaematocrit centrifugation technique		
		Infected	Uninfected	Total No.
Conventional PCR technique	Infected	21	*12	33
	Uninfected	**0	52	52
		21	64	85

*Means false positive. **Means false negative. Kappa value was (0.682) Moderate agreement.

The polymerase chain reaction approach with the ITS-1 primer set revealed that 33 of 85 dogs (38.8%) tested positive for the trypanosome gene (Fig. 1). To narrow our diagnosis to the species level, the RoTat 1.2

VSG gene was detected by the PCR technique on (33 positive samples with ITS-1.) Our results showed that six samples were positive for *T. evansi* (Fig. 1).

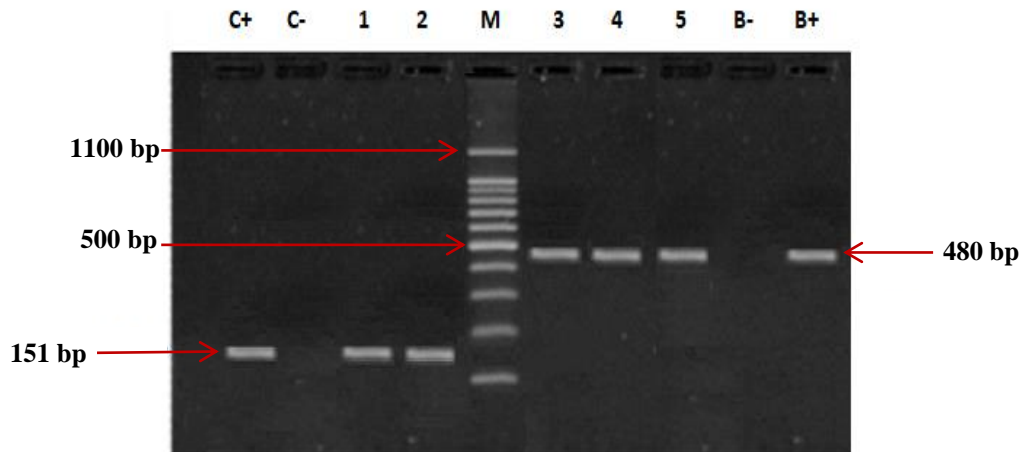


Figure 1/ The amplified DNA bands revealed by the agarose gel electrophoresis (2%) by using internal transcribed spacer (ITS-1) from right side and RoTat 1.2 VSG gene from left . M: molecular size marker 100-1100 base pair. Lane B+: the positive control. Lane B-: the negative control. Lanes 3,4,5: DNA amplified bands of *Trypanosoma sp.* isolated from dogs at 480bp. Lane C+: the positive control. Lane C-: the negative control. Lanes 1,2: DNA amplified bands of *Trypanosoma evansi* isolated from dogs at 151bp.

Discussion

The results of our study suggested that while microscopical examination was inadequate for confirming trypanosome infection and the molecular diagnostic methods (ITS1 PCR test) were more accurate in confirming *Trypanosoma* spp. infection in dogs, which is consistent with previous studies (24, 25, 26). The results revealed a perfect agreement between blood staining smear method and microhaematocrit centrifugation technique base on kappa value(0.828) for diagnosis of trypanosome infection, and substantial agreement between blood staining smear method and conventional PCR technique base on kappa value(0.535) and moderate agreement between microhaematocrit centrifugation technique and conventional PCR technique base on kappa value(0.682), this results were agree with (9,27). The study's findings indicated that the sex of dogs have no significant effect on the trypanosome infection ($P \leq 0.05$); both male and female dogs were similarly susceptible to the parasitic infection. Truly, these findings corroborate those of prior research conducted in the different regions around the globe (28, 16). The low number of positive samples for presence of *T. evansi* reported in the results of our study using the RoTat 1.2

VSG gene in comparison to the outcomes of amplification of ITS1 gene are consistent with the prior scientific reports (22, 29, 30, 31).

Additionally, the findings of our study revealed that ages of animals yield a significant differences to trypanosome infection rate($P \leq 0.05$) and the dogs 4-5 years recorded a highest prevalence rate. This results disagree with the findings of (32, 33, 16, 25). The fact of these finding may be due to the differences of immune status and previous exposure between ages, as well as the stray dogs roaming on the animal carcasses that dead because trypanosome infection, in compared with housed dogs. specially the disease become endemic in domestic animals in Mosul city in the last years ago, this facts may be lead to increase the incidence of trypanosoma infections in adult dogs.

Conclusion: The prevalence of trypanosome infection in dogs (stray and housed) is very high in Mosul city, Iraq. The microscopical examination is inadequate for confirming trypanosome infection. The molecular diagnostic methods (ITS1 PCR test) are more accurate in confirming *Trypanosoma* spp. infection in dogs. Further studies are necessary to determine other

epidemiological factors that can impact trypanosome infection in different farm animals, particularly in dogs, notably the zoonotic nature of the disease.

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Conflict of interest

The author declare no conflicts of interest.

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الكشف عن انواع المثقبيات في الكلاب باستخدام تقنيات
مختبرية مختلفة في مدينة الموصل- العراق

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الموصل، الموصل، العراق

داء المثقبيات في الكلاب يسببه طفيلي من جنس الخلاصة:
المثقبيات. يمكن ان ينتقل الى مختلف انواع الحيوانات
كالمجترات، الخيول، والقطط، فضلا عن الانسان. تلعب
الكلاب دورا مهما في نقل الطفيلي، كما ان الحيوانات
الاليفة تساهم في نقل الطفيلي والحفاظ على دورة حياته.
وبالتالي كان الهدف من الدراسة هو تقدير انتشار داء
المثقبيات في الكلاب في مدينة الموصل- العراق باستخدام
التقنيات المخبرية الروتينية والتقنيات الجزيئية. اظهرت
نتائج الدراسة ان نسبة الانتشار الكلية للمثقبيات في 85
كلبا كانت 18.8%، 24.7%، 38.8%، 7.05%
باستخدام تقنية المسحة الدموية، تقنية الطرد المركزي
الدقيقة، وتقنية تفاعل البلمرة المتسلسل التقليدي للجين
(على التوالي. ITS-1 وRoTat 1.2VSG والجين ITS-1)
لوحظت فروقات معنوية في معدل انتشار الطفيلي بين
الذكور(18%) والاناث (20%) عند مستوى معنوية
بالاعتماد على تقنية المسحة الدموية بينما لم $p \leq 0.05$
توجد فروق معنوية بين الذكور(24%)، 36%، 16.6%)
والاناث (25.7%)، 42.8%، 20%) عند مستوى معنوية
بالاعتماد على تقنية الطرد المركزي الدقيقة $p \leq 0.05$
ITS-1 وتقنية تفاعل البلمرة المتسلسل التقليدي للجين ()
على التوالي. وأشارت النتائج ITS-1 وRoTat 1.2VSG والجين
الى وجود فروق معنوية بين الاعمار المختلفة للكلاب
باستخدام التقنية المجهرية وتقنية تفاعل البلمرة المتسلسل
، وكان اعلى معدل $p \leq 0.05$ التقليدي عند مستوى معنوية
انتشار للطفيلي في عمر 4-5 سنوات(68.7%) مقارنة
بالاعمار الاخرى التي شملتها الدراسة، وكان النوع
هو الاكثر انتشارا وشخص في *Trypanosoma evansi*
الاعمار 3-5 سنوات فقط. اظهرت النتائج وجود اتفاق تام
بين تقنية المسحة الدموية وتقنية الطرد المركزي الدقيق،
واتفاق حقيقي بين تقنية المسحة الدموية وتقنية تفاعل
البلمرة المتسلسل التقليدي، واتفاق معتدل بين تقنية الطرد
المركزي الدقيق وتقنية تفاعل البلمرة المتسلسل التقليدي.
استنتج من هذه الدراسة ان انتشار عدوى المثقبيات في
الكلاب السائبة مرتفع في مدينة الموصل-العراق.

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