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Molecular identification of *Crithidia* sp. from naturally infected dogs

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Abstract

The current study was performed to identify *Crithidia* sp., a blood microparasite, in canines in Al-Qadisiyah Province and its regional districts. The study included collecting blood samples from the cephalic vein from 50 street and domesticated dogs. The study included collecting data on age, sex, clinical signs, and sample collection regions. PCR and partial sequencing of the *18S rRNA* gene were performed. The findings revealed that the infection rates were 4/50 and 7/50 for age groups of >1 and ≤1 years old, respectively. Moreover, the rates were 10/50 and 1/50 for males and females. In addition, the rates were 4/50 and 7/50 for symptomatic and asymptomatic animals, respectively. Furthermore, the rates were 1(2%), 3(6%), 6(12%), 0, and 1(2%) for the city center of Al-Diwaniyah, Nuffar, Saniyah, Dagharah, and Sumar, respectively. The sequencing findings revealed that the isolates from the current study were similar to isolates from Czech and Russia. The findings suggest that *Crithidia* sp. exists in dogs, and these isolates might have a common ancestor of the parasite due to similarities between the isolates of the current study and Czech and Brazil isolates.

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Introduction

Crithidia is a protozoan with a heteroxenic life cycle, which means it needs more than one host. It has been found in reptiles, amphibians, and humans, and is a well-known parasite in honeybees. In dogs, *Crithidia* has already been described in sick animals or from both ill dogs and dogs without clinical signs (1-4). There is a little data on this potentially pathogenic trypanosomatid protozoa in dogs, which may infect the dog's retina, and why some animals become ill while healthy ones harbor the parasite in the retina and circulation. Therefore, our aim in this article was to conduct a comprehensive study on *Crithidia* infection in dogs, including molecular features like subspecies distribution (5-7). The global distribution of *C. canis* infection is still unknown in many countries, and enough evidence has yet to be accrued to confirm parasite distribution. In addition, the factors that determine disease distribution with latitude still need to be better understood. However, in recent studies, it has been noted that the distribution with latitude is increasing. In *Crithidia* spp. Infected dogs, altered C-reactive protein, and altered platelet profiles were observed depending on seasonal occurrence, amount of daily sunlight, and some climatic variables (5-9). These features determined the intensity and the courses of protozoal infectious diseases. The dynamics of euglenoid parasites could be influenced by the yearly rhythm and environmental temperature in the Mukogawa River basin, in Japan. Furthermore, other authors reported that in some cases, a proportion of infected dogs demonstrated increased clinical and laboratory abnormalities mainly due to colder temperatures, northerly latitudes, or both. Therefore, it is necessary to know the local prevalence, climatic conditions, and distribution of crucial blood and intracellular parasites in healthy agricultural/working dogs (10,11). The hemoflagellate protozoan *Crithidia canis* is thought to be particularly prevalent in cold northern and southern climates. Infection with this agent has been reported worldwide, primarily affecting working and hunting dogs. Although data concerning parasite distribution and prevalence are increasing, much remains unknown about the infection in many populations, particularly the risk factors associated

with *C. canis* infection (2,6). Some reviews have addressed the current state of knowledge of the parasite and some aspects of the problem, including its distribution, morphology, life cycles, infection, clinical and laboratory findings, diversity and polymorphism, diagnosis, and zoonotic significance. However, there is still a need for more comprehensive studies in canine populations, especially regarding the clinical and epidemiological status, pathology, immunophenotyping, and multilocus genotyping of different strains and in vitro cultivation techniques for vaccine production (12,13). When the mononuclear phagocyte system becomes overpopulated, the flagellates are found in the bloodstream, causing an overt infection. This fatal complication may cause the manifestation of chronically and acutely severe cases of the disease. When the host lice feed, they are frequently scratched by their hosts and die, releasing the amastigotes ingested by the next dog feeding the louse. When a canine host ingests infested lice, there might be a low probability of the transmission of amastigotes to host cells in the alimentary tract (14-16). These romanomermis nematodes infect the dog when ingesting the *C. canis* amastigote, likely reflecting or comprising normal parasitic life cycle progression. Feces containing yellowish to brown mucus are covered with an envelope filled with *C. canis* amastigotes. Infected cells, including those in the digestive tract, were observed to be easily lysed when fed to recipient naive blood-feeding lice, similar to but likely with a lower efficiency, resulting in flagellate transfer. However, the precise mechanism of horizontal transmission between dogs or environmental contamination and dog infection is uncertain and is currently under debate (17,18). *Crithidia canis* is likely transmitted from host to host via the intermediate host, the dog louse, *Heterodoxus spiniger*. Once the flea ingests the *C. canis* amastigote during feeding, the invasive metacyclic or extracellular form of the flagellate will develop and then be ingested during feeding of the uninfected dog, resulting in *C. canis* amastigote release (19-22). Thus, it is also possible, that the flagellate is transmitted through bite wounds caused by dogs in which the *H. spiniger* had lice that defecated containing infective metacyclic or extracellular forms of *C. canis*. The flagellate reproduces by binary fission in the canine host mononuclear phagocyte system, causing the recruitment of macrophages that can be found in the liver, leading to the occurrence of clinical cases associated with anemia (23).

The current study was performed to identify *Crithidia* sp., a blood microparasite, in canines in Al-Qadisiyah Province and its regional districts.

Materials and methods

Ethical approve

Write the name of the scientific or institutional board that gives the ethical approval to conduct this scientific work and provide the approval issue number and date. The study protocol was approved for animal care and use by the College of Veterinary Medicine, University of Al-Qadisiya, Iraq. The study was approved on November 24, 2022, under No. 1899.

Collection of samples

Blood samples of 3-5 ml per each from the cephalic vein were collected from 50 street and domesticated dogs; between February and October 2023. For the sample collection, the donors must hold still and either keep sitting or lie sideways if needed, a combination of Ketamine and xylazine is injected intramuscularly at a ratio of 1:1/2ml. EDTA-tubes were used to collect the blood samples.

Genomic material isolation

DNA from 50 samples was isolated from whole blood using a tissue DNA extraction kit (AddBio, Korea) and then quantified using a Quantus™ Fluorometer (Promega, US). After that, work was performed according to the protocol of the manufacturer. The extracted DNA samples were stored at -20 °C for later PCR and sequencing steps. The reaction kit for PCR technology, primers for the gene, and extracted DNA samples were used (24). Successful use of molecular DNA markers for the discriminating of genetic resources that are economically significant, such as poultry and other farm animals, requires an investigation of genetic variation and relatedness across or within species, populations, and individuals (25).

The primers used were designed using the NCBI service (accession number, MN757921) to the *18S rRNA* gene, F: GCCTCTAGGCTACCGTTTCG and GTAAGGTGGTAAAAGCGGGC, at 445 bp (26,27). Amplication reaction is a total of 20 µl, containing 10 µl AddBio master mix from Korea, 2 µl each forward and reverse primer, 4 µl PCR water, and 2 µl template DNA initial thermal conditions achieved on the thermal cycler system (BioRad, USA) and three minutes of initial denaturation at 95°C, then followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C, and extension and final extension at 72°C for 30 s and 60 s, respectively. The PCR products were examined using agarose gel electrophoresis. The amplicons from positive PCR samples were then selected for sequencing using the Sanger method in Macrogen, South Korea. After obtaining the sequences, they used to be trimmed to eliminate the noise signals and then deposited in the Gene Bank (NCBI). Once the accession numbers were deposited, the subject was phylogenetic analysis and comparison with other strains worldwide, which was done through Clusted W alignment, NCBI-Blast, and Maximum Likelihood method in MEGA 11 (28).

Statistical analysis

All statistical analyses were done using the software program SPSS (version-10). The results were shown within $P<0.05$ (29).

Results

Molecular results

All collected samples of 50 dogs were tested by PCR, and the results showed the total infection rate was 11(22%). The infection rate was correlated with the younger age of tested dogs. The rates were 4(8%) and 7(14%) for age groups of >1 and ≤ 1 year-old, respectively (Table 1). The rates were 10(20%) and 1(2%) for males and females, respectively. The collected samples belonged to 30 males and 20 females (Table 2). The rates were 4(8%) and 7(14%) for symptomatic and asymptomatic animals, respectively (Table 3). The rates were 1(2%), 3(6%), 6(12%), 0, and 1(2%) for the city center (Al-Diwaniyah), Nuffar, Saniyah, Dagharah, Summar, respectively (Table 4). The sequencing findings revealed that the isolates from the current study were similar to isolates from Czech and Brazil were explained in figures 1 and 2 (Table 5).

Table 1: Prevalence of *Crithidia* sp. on the age

Age	Infected	Healthy	Sum.
> 1 year	4(8%)	20(40%)	24(48%)
≤ 1 year	7(14%)	19(38%)	26(52%)
Total	11(22%)	39(78%)	50(100%)
x^2		0.765	
P value		0.382 (NS)	

NS: No significant difference at P<0.05.

Table 2: Prevalence of *Crithidia* sp. on the sex

Sex	Infected	Healthy	Sum.
Male	10(20%)	20(40%)	30(60%)
Female	1(2%)	19(38%)	20(40%)
Total	11(22%)	39(78%)	50(100%)
x^2		5.61	
P value		0018(NS)	

NS: No significant difference at P<0.05.

Table 3: Prevalence of *Crithidia* sp. on clinical signs

Clinical sign	Infected	Healthy	Sum.
Symptomatic	4(8%)	2(4%)	6(12%)
Asymptomatic	7(14%)	37(74%)	44(88%)
Total	11(22%)	39(78%)	50(100%)
x^2		7.92	
P value		0.005(HS)	

HS: Highly significant difference at P<0.01.

Table 4: Prevalence of *Crithidia* sp. on regions of sample collection

Clinical sign	Infected	Healthy	Sum.
City Center	1(2%)	9(18%)	10(20%)
Nuffar	3(6%)	6(12%)	9(18%)
Saniyah	6(12%)	13(26%)	19(38%)
Dagharah	0(0%)	6(12%)	6(12%)
Summar	1(2%)	5(10%)	6(12%)
Total	11(22%)	39(78%)	50(100%)
x^2		4.32	
P value		0.364(NS)	

NS: No significant difference at P<0.05.

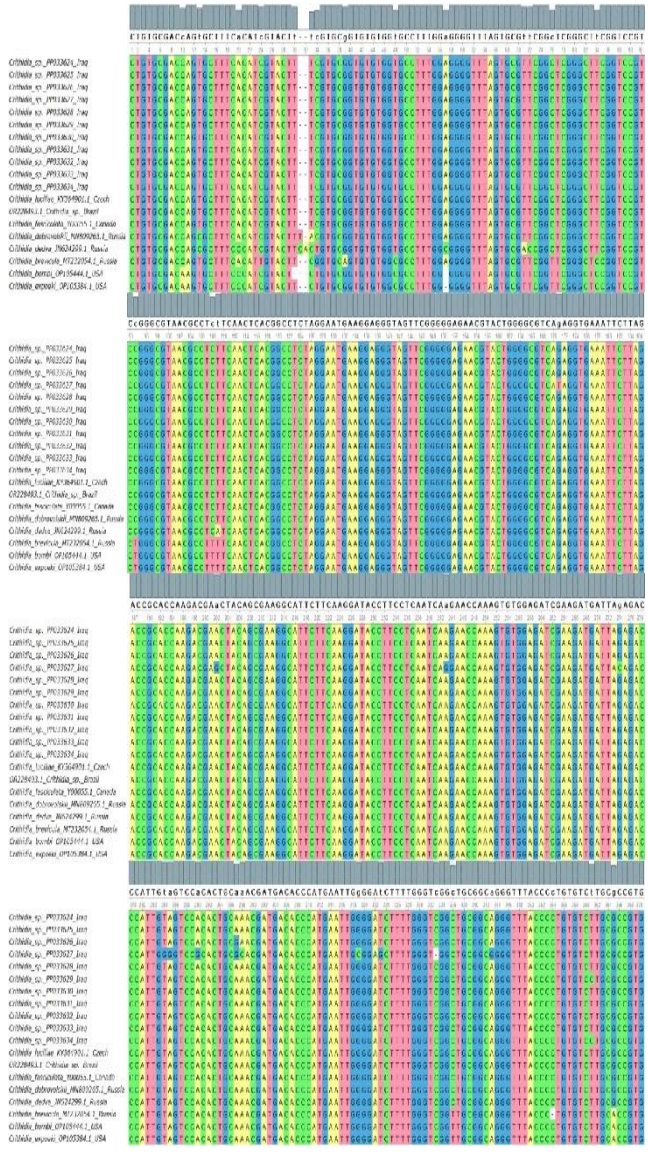

Figure 1: Multiple sequence alignment of the identified sequence based on the *18S rRNA* gene of *Crithidia* sp.

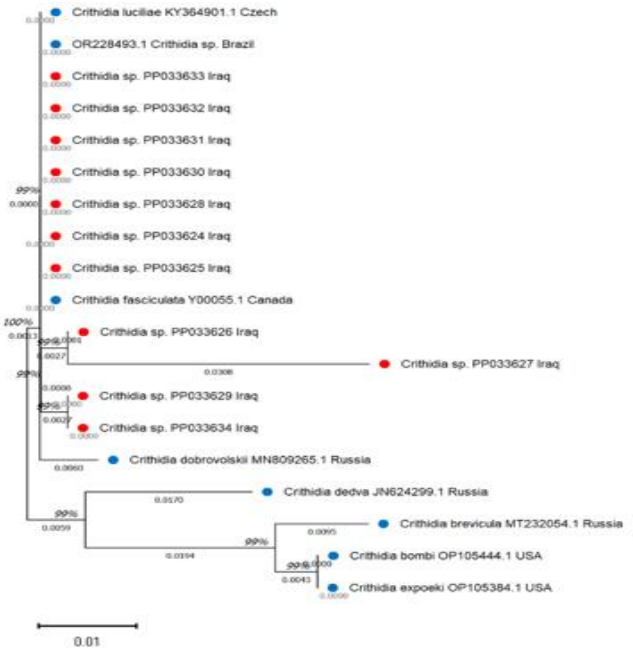

Figure 2: Evolutionary analysis of the identified sequences based on the *18S rRNA* gene of *Crithidia sp.* in dogs compared to other world sequences.

Discussion

It is known that the small flagellate of the genus *Crithidia* sp. is usually present in low numbers, in many mammals and is not accompanied by severe symptomatic disorder. This study, determined that infection rates of *Crithidia* sp. in dogs are much higher than reported up to the present date (30-36). The samples were collected in the field and there were difficulties in smearing and staining the slides. There was a long time between the collection and the samples' arrival at the lab, so the collected blood may be exposed to hemolysis. Low stage parasitemia during the clinical profile may result in a high percentage of false negatives associated with statistical difficulties in interpreting the results (32). The most sensitive and effective methods of detection consist of PCR; ELISA or immunobinding assay for the detection of specific antibodies; and culture of the flagellates, which can be identified subsequently by multiplex PCRs, PCR restriction fragment length polymorphism (RFLP) analysis and/or PCR DNA sequencing. Knowing the epidemiology of canine leishmaniasis for a given area is essential to interpreting the clinical significance of PCR results compared to microscopic observation and to the results of other methods for detecting *L. infantum* (37).

Table 5: The NCBI-BLAST homology sequence identity in local *Crithidia* sp. compared with other global sequences

Current study accession numbers	NCBI-BLAST Homology Sequence identity (%)			
	Identical to	GenBank accession numbers	Country	Identity (%)
PP033624	*Crithidia expoeki*	OP105384	USA	96.74
PP033625	*Crithidia bombi*	OP105444	USA	96.74
PP033626	*Crithidia brevicula*	MT232054	Russia	96.47
PP033627	*Crithidia dedva*	JN624299	Russia	94.05
PP033628	*Crithidia dobrovolskii*	MN809265	Russia	99.46
PP033629	*Crithidia fasciculata*	Y00055	Canada	99.73
PP033630	*Crithidia* sp.	OR228493	Brazil	100
PP033631	*Crithidia luciliae*	KY364901	Czech	100
PP033632	*Crithidia* sp.	OR228493	Brazil	100
PP033633	*Crithidia* sp.	OR228493	Brazil	100
PP033634	*Crithidia* sp.	OR228493	Brazil	99.73

The presence of sand flies is the most significant factor influencing the distribution of infection rates of *Crithidia* sp. in wild or kenneled dogs. Effective repellents against sand flies would be the most efficient way to block this effective transmission route of the parasites. Additionally, the number of sand flies found on the kenneled dogs was comparable to that on the other animals. Note that mosquitoes were found on almost all the kenneled domestic animals. Other reasons, such as variations in the physiological activities of different hosts for sand flies and host availability, may have contributed to the difference (38). The type of dog was another critical factor. Kenneled or hunting dogs are generally maintained at particular locations, and these could stimulate the presence of sand flies while caring for or feeding and the elimination of the animal's excrement. The increased amount of excrement deposited near air outlets may further attract sand flies to enter the kennel to carry away the excrement. Kinked hair in long-coated dogs may also play a role by providing a dense coat that could protect the sand flies from wind movement and reduce the awareness of the hosts (39).

The *Crithidia* sp. infection rate in domestic dogs depended on geographical area and population. In previous studies, *Crithidia* sp. infection transmission was associated with age and sex in domestic dogs; however, those findings varied. Focusing on other regions in Mexico, this study

found *C. lupi* more prevalent in male dogs. Another study revealed that dogs in rural areas more frequently harbored *C. canis* and *C. felis* infections than those in urban areas (40-42). Although the causes of these differences (e.g., sex and geographical area) are unclear, the high prevalence of infection can be attributed to poor management of dogs, especially in urban areas. Therefore, to reduce the actual *Crithidia* spp. regarding the infection rate in dogs, controlling the transmission and prevalence of *Crithidia*-infected sand flies in domestic dogs in each geographical area is essential. In addition, a multi-institutional collaborative project attempted to clarify the relationship between *Crithidia* infections and dogs, including their association and the influence of another protozoa (43,44).

Loss of cultivation land to desertification, recurrent droughts, and cultivation land to desertification, recurrent droughts and sandstorms, and declining agriculture are the of change in Iraq's fragile environment (45). The phylogenetic analyses revealed a close similarity of the current isolates with those from Czech, Russia, and Brazil. This could mean that all of these isolates may share the same ancestor, possibly due to the movement of humans and animals between countries (46-52). The result indicated that the parasite is active in transmission between countries and continents because higher genetic similarity between spatially distinct populations may indicate shared ancestry or recent genetic exchange between these strains (53). The molecular methods are potential tools for better understanding parasitic infections epidemiology and evolutionary dynamics (54). The difficulties in examining blood film due to misleading artifacts, nuclear debris, and/or cytoplasmic aggregation may require validation of the microscopic findings with more specific diagnostic tools such as serological tests and /or molecular methods such as PCR (55). The molecular methods are more robust regarding diagnostic sensitivity and specificity; the serological techniques may also result in false positive findings from cross-reaction with other species (56-60). The infection rate of dogs is different depending on climate conditions, sample size, vector control, contamination area, and model of living stray or pet animals (61).

Conclusion

The findings suggest that *Crithidia* sp. exists in dogs, and these isolates might have a common ancestor of the parasite due to similarities between the isolates of the current study and Czech and Brazil isolates.

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Conflict of interest

The authors declare that there is no conflict of interest in the manuscript.

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الكشف الجزيئي عن أصناف الكريثيديا من الكلاب المصابة طبيعيا

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الخلاصة

أجريت الدراسة الحالية للتعرف على أنواع الكريثيديا، وهو طفيلي دقيق في الدم، في الكلاب في محافظة القادسية ومناطقها المختلفة. شملت الدراسة جمع عينات دم من الوريد الرأسي من ٥٠ كلبا ضالا وكلبا مستأنسا. تضمنت الدراسة جمع بيانات العمر والجنس والعلامات السريرية ومناطق جمع العينات. تم إجراء تفاعل إنزيم البلمرة المتسلسل وتعاقب النيوكليوتيدات الجزئي لجين *18S rRNA*. كشفت النتائج أن معدلات الخمج كانت ٤ (٨%) و٧ (١٤%) لفئتي العمر <١ و≥١ سنة على التوالي. علاوة على ذلك، كانت المعدلات ١٠ (٢٠%) و١ (٢%) للذكور والإناث على التوالي. بالإضافة إلى ذلك، كانت المعدلات ٤ (٨%) و٧ (١٤%) للحيوانات التي تظهر أعراضا سريرية والتي لا تظهر أعراضا سريرية على التوالي. بالإضافة إلى ذلك، كانت المعدلات ١ (٢%)، ٣ (٦%)، ٦ (١٢%)، ٠، ١ (٢%) لمركز مدينة الديوانية، نفر، السنية، الدغارة، سومر على التوالي. كشفت نتائج التسلسل أن العزلات من الدراسة الحالية كانت قريبة في تشابهها مع العزلات من جمهورية التشيك والبرازيل. تقترح النتائج أن أنواع الكريثيديا موجودة في الكلاب وقد يكون لهذه العزلات سلف مشترك للطفيلي بسبب التشابه بين عزلات الدراسة الحالية وعزلات جمهورية التشيك والبرازيل.

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