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Study on *Leishmania* infection in cats from Ahar, East Azerbaijan Province and North West Iran by parasitological, serological and molecular methods

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PEER REVIEW

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Comments

This is a good and interesting report on tropical veterinarian science. The work reports the data from the area without previous report. The report can be linked to the human problem in the area and the epidemiological data can fulfill the presently absent knowledge of leishmaniasis in the Middle East. The data can be useful in epidemiological study for future followers.
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ABSTRACT

Objective: To study *Leishmania* infection in cats and its potential role in transmission of the disease to human by parasitological, serological and molecular methods in Ahar District, East Azerbaijan Province.

Methods: In this study, 65 cats from different parts of Ahar Province were trapped. The cats were anesthetized with chloroform and blood samples were taken from jugular vein and tested by direct agglutination test. Spleen and liver smear samples were prepared in order to microscopically examine these organs, and also cultured in Novy-MacNeal-Nicolle and Roswell Park Memorial Institute 1640 media. Finally, spleen tissue DNA was extracted to perform polymerase chain reaction analysis.

Results: In direct agglutination test, 4 (6%) cats had a positive titer, while 14 (22%) cats had a titer of 1:80 which was suspected for an infection and 47 (72%) cats were negative. Culture results were negative and in polymerase chain reaction no amplification was observed.

Conclusions: We found no case of feline visceral leishmaniasis. It needs more extensive studies by using a larger number of cats to firmly establish leishmaniasis in this area.

KEYWORDS
Cat, Ahar, *Leishmania*

1. Introduction

Leishmaniasis is a vector-born disease triggered by obligate intracellular protozoa and as a parasitic disease manifests itself mostly in three clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis and mucocutaneous leishmaniasis[1]. VL is caused by various species of the *Leishmania donovani* complex [*Leishmania donovani*, *Leishmania infantum* (*L. infantum*), *Leishmania chagasi* (*L. chagasi*)], and is a deteriorating disease in dogs and humans which is frequently fatal if left untreated. The genus *Phlebotomus* and *Lutzomyia* play an important role in disease transmission[1]. About 200 000 to 400 000 new

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cases of VL happen worldwide each year, and most of the them occur in countries such as Bangladesh, Brazil, Ethiopia, India, and South Sudan[2]. VL has been reported sporadically from different parts in Iran, but it is endemic in northwestern and southern areas of the country with about 100–300 new cases stated annually[3,4]. Clinical symptoms in human include fever, continuous and prolonged enlarged liver and spleen, weight loss, anemia and gradually death. *L. infantum* is the agent of VL in children in Mediterranean countries such as Iran[5]. Dogs are the main reservoir of zoonotic *Leishmania* caused by *L. infantum* with the 63%-80% prevalence in endemic areas[6]. In addition to carnivores, VL has been recognized in numerous mammalian species, including primates, bats, rodents and horses[7]. Domestic dogs and some rodents are the main sources of human infection in America and in the Mideast, respectively. The ratio of infected dogs live in an area where canine VL is endemic and has main public health significance. It is confirmed that asymptomatic dogs are the source of parasite for phlebotomine sand fly vector and therefore play a principal role in the transmission of *L. infantum*[8]. In Iran, several epidemiological studies have been performed in recent years in the different cities on the disease agent, animal reservoirs, human infection and vectors, and it was demonstrated VL in human and some animal reservoirs[9]. Also several other studies demonstrated infection of rodents with *Leishmania* parasites[10,11]. Seroprevalence of the disease in different parts of Iran has been reported to be from 10% to 37%[12]. Currently, sporadic clinical cases of *Leishmania* infection with *L. infantum* have been described in cats from some countries where canine leishmaniasis is endemic. The first cat infection was reported in 1912 from a domestic cat where the pet dog and the children were suffering from leishmaniasis. Studies on DNA isolated from various tissues of cats in endemic areas indicate that *L. infantum* is the causative agent[13,14]. So cats have been proposed as a secondary reservoirs host in areas where *L. infantum* is endemic and it is required to evaluate their infection status and play a role in the epidemiology of zoonotic leishmaniasis. The diagnosis of leishmaniasis has been made by classic microbiological methods. However, there are some limitations such as low number of parasite and low sensitivity. Direct agglutination test (DAT) has been established as a simple and inexpensive technique in serodiagnosis and seroepidemiological studies of VL. The diagnostic performance of DAT for VL is considerable[15]. Currently, new methods for the detection of parasites, such as polymerase chain reaction (PCR) have been tried and provided an influential attitude to the application of molecular techniques for the diagnosis of leishmaniasis[16]. In previous studies, Ahar Province in Iran has been reported to be endemic for VL. Although dogs and wild carnivores such as jackals, foxes and cats have been supposed to be reservoirs for parasites particularly in regions where sporadic cases of disease have been reported, there is no study conducted on feline leishmaniasis in this area[17,18]. This is the first study conducted on cats in this area and main objective of this investigation was to study *Leishmania* infection in cats by serological, parasitological and molecular examination in Ahar Province, an endemic part of East Azerbaijan.

2. Materials and methods

2.1. Ethics statement

The study was conducted in agreement with Research Ethics Committee of Tabriz University of Medical Sciences (tbzmed).

2.2. Study area

The study was conducted throughout 2012-2013 in suspicious districts of East Azerbaijan Province including Ahar District which is 103 km far from Tabriz City and the capital of East Azerbaijan.

2.3. Sampling and testing

The study was conducted in coordination with Office of Environmental and Health Center of Ahar. A total of 65 cats were hunted by using live traps. The hunted cats were transferred to medical entomology laboratory and anesthetized with chloroform. Trapped live cats were immediately necropsied and approximately 5 mL of blood were taken by syringe into tubes by jugular venipuncture. After 6-10 h, the samples were centrifuged at 2 500 r/min and serum was separated. Finally, for DAT test, sera were kept in -20 ℃. To perform DAT, *L. infantum* antigen was used, which was developed in center of infectious disease prevention laboratory based on the method of Harith *et al*[19]. For DAT test in reservoirs, *Leishmania* antibody titers of 1:320 or greater was positive and below 1:80 was suspected. Titers lower than 1:80 were considered negative. To perform autopsy from the spleen or liver, firstly, two contact slides were obtained and fixed immediately with 95% methanol and then they were stained with 10% Giemsa and samples examined microscopically for the presence of *Leishmania* amastigote. A small portion of the spleen and liver of animals were cultured in *L. infantum* special culture medium: Novy-MacNeal-Nicolle (NNN) and Roswell Park Memorial Institute (RPMI) 1 640 with 20% fetal calf serum. For molecular testing DNA was extracted by means of a commercial kit (PGDNEX CO.). PCR (ASTEC kit) reaction was performed in a final volume of 25 μL by using the following reagents: 12.5 μL of the master mix, 1 μL of each forward and reverse primers solution, 3 μL DNA and nuclease free water to complete the final volume. The forward (5'-GGG GTT GTT GTA AAA TAG GG-3') and reverse primers LINR17 (5'–TTT GAA CGG GAT TTC TG-3') were designed in Tabriz Infectious and Tropical Disease Research Center for both *L. infantum* and *Leishmania tropica* (*L. tropica*) and *Leishmania major* with OligoV7.56 software (primers can recognize *L. tropica*, too). The amplification mixture was incubated in a master cycler gradient (Eppendorf) by using the following cycling profile with little modification: initial denaturation at 94 ℃ for 4 min, followed by 35 cycles of denaturation at 94 ℃ for 1 min, annealing 65 ℃ for 5 min, extension at 72 ℃ for 1 min and final extension at 72 ℃ for 4 min[20]. The amplified products were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide. The amplified DNA fragments were visualized in an image analyzer.

3. Results

A total of 65 cats from different species were hunted in Ahar Province. In microscopic examination no Leishman bodies were observed in contact smears. Four (6%) cat had positive titer, 14 (22%) cat titers of 1:80 and were suspect and 47 (72%) had seronegative. *Leishmania* was not isolated from tissue after culturing of parasite in the NNN and RPMI 1 640 media. To verify the results, DNA was extracted from spleen and PCR was performed. Bands related to *Leishmania* genus were not observed (Figure 1).

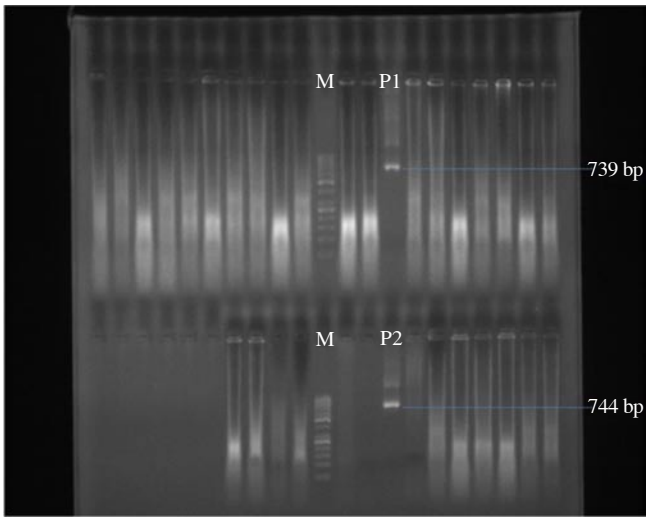

Figure 1. Gel electrophoresis of PCR samples of different products. M: Marker; P1: Positive control for *L. infantum*; P2: Positive control for *L. tropica.*

4. Discussion

Canine leishmaniasis caused by *L. infantum* is a severe zoonotic public health and veterinary problem in the Mediterranean countries. Dogs are commonly considered as the domestic reservoirs host of zoonotic VL caused by *L. infantum* because of certain epidemiological and biological parameter. Various studies were conducted on prevalence of VL in different countries. The zoonotic form of VL caused by *L. infantum* occurs sporadically in all geographical zones of Iran except northwestern and southern parts of the country and the disease is endemic. In addition to the definite role of dogs as the main reservoirs, high percentages of rodents in Meshkinshar, Azarshahr and northwest of Iran were seropositive in cases of VL[9]. A study on rodents leishmaniasis conducted by Fallah *et al*. showed *L. infantum* infection in rodents from northwest of Iran[17]. In recent years, cases of feline visceral leishmaniasis (FVL) have been described in different countries and seldom conveyed. In some areas, domestic cats are proposed as probable alternative reservoir of the parasite. Thus, a growing trend with regard to cats as a possible domestic reservoir host of *L. infantum* exists. Therefore, considering that Ahar is one of the endemic areas for *L. infantum* both in dogs and rodents[18], this study was carried out to perform on feline *Leishmania* infection, which is the first epidemiological investigation in this area. PCR has also been used in combination with serological tests to assess the prevalence of feline *Leishmania* infection. In our study of 65 hunted cats, 4 cats had positive titer, 14 cats were suspect and 47 cats were seronegative. *Leishmania* was not isolated from tissues after culturing in NNN and RPMI 1640 media. DNA was extracted from the spleen of cats and PCR was performed. Bands related to *Leishmania* genus were not observed. Leishman bodies were not found in the tissue contact smears. Culture results were negative for *Leishmania* parasite. The 4 serologically positive samples, which are PCR-negative, can be interpreted as cross reactions that is possible in all serological tests. In a similar study that was conducted on 40 cats, *Leishmania* parasites were detected in 4 cats (10%)[21]. A study on cats with dermatologic lesions conducted by Vides *et al*. showed that specific FVL antibodies were found in 6 (10.9%) of 55 cats, of which 5 (83.3%) of 6 had leishmaniasis and real time PCR successfully confirmed *L. chagasi* infection[22]. In another study conducted for the detection of *Leishmania* spp. in cats by molecular methods, the infection was detected in 5.76% (3/52) of the examined cats and two had amastigote forms of *Leishmania* spp. PCR of kinetoplast minicircle DNA indicated positive results and sequencing resulted in 97% similarity with *L. chagasi*[23]. The study of Cardoso *et al*. showed a low seroprevalence of *L. infantum* infection in cats, so the seroprevalence of *Leishmania* infection was 2.8%, based on ELISA and DAT[24]. Savani *et al.* reported the first case of feline cutaneous leishmaniasis in a domestic cat and nucleotide sequence was identical to that of *L. infantum chagasi*[25]. In the study of Poli *et al*., one case of leishmaniasis in domestic cats (*Felis catus* domesticus) was described[26]. The diagnosis was achieved by serological, parasitological, and light and electron microscopic investigation[25]. By molecular techniques, the etiological agent was identified as *L. infantum*. Despite many studies were conducted by using serological and microscopic tests, molecular methods are better for detection compared to other methods. The isolation of *Leishmania* and its DNA from infected tissues present better evidence that a species can act as a reservoir host and this technique allows to identify the responsible *Leishmania* species. Our study in one of the endemic areas showed no infected cat, and molecular methods verified the results. Information on the prevalence of *L. infantum* infection is necessary to define control measures for zoonotic leishmaniasis. Although dogs are the main reservoir of the parasite in the Mediterranean countries including Iran, the role of cats in the epidemiology of *Leishmania* needs further attention, so *Leishmania* infection must not be underestimated. Although we presented no case of FVL, we consider that our results are important, because to our knowledge, this is the first time that a study has been conducted in this area. In order to confirm diagnosis, other methods such as fast agglutination screening test might be very suitable. We propose that more extensive studies on a larger number of cats should be done. Also studies on vectors will definitely help to firmly assess leishmaniasis in this area.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

This is an interesting study on *Leishmania* infection in cats came from Iran. This is the epidemiology report from an area with extremely limited data.

Research frontiers

It is an interesting report on parasitosis. The epidemiological data from the area without previous report can be seen in the present article.

Related reports

There is no previous report on the epidemiological data in the study area. This is a classical investigation technique and the derived data is useful.

Innovations and breakthroughs

The standard technique, not an innovation, is used. However, the report contains very new and useful information from the area with lack for scientific data. The report can be linked to the human problem in the area and the epidemiological data can be the piece of jigsaw to complete the overall view of the leishmaniasis in the Middle East.

Applications

The work can be further applied in relating researches. The data can

be useful in epidemiological data for future followers. The reported data can be a good record that can be further referenced.

Peer review

This is a good and interesting report on tropical veterinarian science. The work reports the data from the area without previous report. The report can be linked to the human problem in the area and the epidemiological data can fulfill the presently absent knowledge of leishmaniasis in the Middle East. The data can be useful in epidemiological study for future followers.

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