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In vitro Cultivation of *Leishmania* spp. using Blood from Human and Dog in Novy-MacNeal-Nicolle (N.N.N.) Modified Medium

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ABSTRACT

Leishmaniasis is one of the major vector-borne communicable diseases in the world, and it is widely distribution in endemic regions including Iraq. N.N.N. modified medium is used for cultivation of *Leishmania*. It was utilized for initial isolation of the parasite suspected from aspirate fluids and for sub culturing the parasite. Blood from human and dogs were used in the modified medium. The microscopic examination was performed under oil immersion light microscopy (100X) after staining of samples collected from 436 skin biopsies of suspected cases from humans and 74 skin biopsies of suspected symptomatic owned-dogs. Fifty aspirate samples from humans (25) and owned-dogs (25) were cultured on modified N.N.N. medium. All human samples from suspected cases (436, 100%) were positive, whereas 71 (95.95%) samples from owned dogs were positive. 5/25 (20%) of human samples showed growth, whereas 20/25 (80%) of samples were contaminated. Of the dog samples, 14/25 (56%) showed growth, whereas 11/25 (44%) of the samples were contaminated. The samples from dogs had a significantly higher growth rate than samples aspirated from humans (P<0.05). 10/25 (40%) human samples showed growth, whereas 15/25 (60%) of samples were contaminated. Of the dog samples, 18/25 (72%) showed growth, whereas 7/25 (28%) samples were contaminated. The samples from dogs had a significantly higher growth rate than samples aspirated from humans (P<0.05). To our knowledge this is the first study contribute to diagnosis of *L.* spp. using *in vitro* cultivation by N.N.N. modified medium with blood from human and dog. The cultivation in modified N.N.N medium showed a high growth rate of samples from owned-dogs were than human samples. Blood of dogs seems to be a very good media for growing of this microorganism *in vitro* and may be also *in vivo*.

Keywords
In vitro Cultivation; *Leishmania*; Novy-MacNeal-Nicolle (N.N.N.) Modified Medium; Blood of dogs

Introduction

Leishmaniasis is one of the major vector-borne communicable diseases in the world. It is a zoonotic infection that is caused by obligate intracellular protozoa of the genus *Leishmania* (Mandell *et al.*, 2005; Kumar and Samant, 2016). Haemo-flagellate *Leishmania* cause widespread disease leading to serious health problems in communities throughout the Mediterranean regions and the Middle East, including Iraq (Hepburn, 2003; CDC, 2004; Markle and Makhoul, 2004). Old world cutaneous leishmaniasis (CL) has three distinctive varieties in the Eastern Hemisphere: urban or dry, caused by *L. tropica*; a rural or wet type, caused by *L. major*; and a diffuse cutaneous type, caused by *L. aethiopica*. Clinical, epidemiological, immunological, and biochemical differences among these varieties indicate that each is a distinct entity (Filho and Brazil, 2003). Epidemiological studies in the Middle East have shown that anthroponotic CL caused by *L. tropica* and zoonotic CL caused by *L. major* occur in Saudi Arabia, Iraq, Iran, Afghanistan, Pakistan, and Yemen (Gonzalez, *et al*., 2008; WHO, 2007). Cases of cutaneous and visceral infections caused by *L. tropica* have been reported in Iran and Iraq (Mohebali, *et al.*, 2011). The World Health Organization (WHO) considered CL one of the six most important

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infectious diseases due to its high incidence rates and the potential to cause deformities in patients (WHO, 2010). CL is endemic in large areas of the tropics, subtropics, and the Mediterranean basin (Madeira *et al.*, 2006).
Leishmaniasis was called "Balkhsore" in north Afghanistan, "Delhi boil" in India and "Baghdad boil" in Iraq (Khan and Muneeb, 2005). It is endemic in Iraq, where both forms of the disease, cutaneous and visceral, are found (Al-Samarai and Al-Obaidi, 2009).

Methods

N.N.N. modified medium is used for cultivation of *Leishmania*. It was utilized for initial isolation of the parasite suspected from aspirate fluids and for sub culturing the parasite. This medium was first used in 1929, and then the pH was adjusted to (7.4±0.1) (Colle*et al*., 1996). Table 1 showed the N. N. N. media components.
Initially, we suspended 31 grams of Part A components in 1000 ml D.W and heated the mixture to boiling point to dissolve it completely. It was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After that, it was cooled to 45-50°C; we then added 10% of sterile defibrinated human blood after inactivation at 56°C for 30 min. This was mixed then dispensed in 5 ml amounts into test tubes or 25 ml amounts into flasks .Finally, we allowed the media to cool with the tubes in a slanted position (Colle*et al*., 1996), as shown in Figure 1A.
Defibrinated human blood is whole blood from which fibrin has been separated. It refers to a method to prevent blood from clotting after it has been withdrawn from a donor, without the addition of any chemical additives to the blood (http://ecoursesonline.iasri.res.in/ mod/page/view.php?id=126063), as shown in Figure 2 A. Glass containers were used to collect blood. Then, a magnetic stirrer was placed in the containers. The containers were shaken continuously in a manner to agitate the stirrer through the blood for a specified time sufficient to remove the fibrin.
We suspended 11.2 grams of Part B in 1000 ml D.W., and then heated the mixture to boiling point to dissolve it completely. It was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. Finally, we cooled it and added approximately 2 ml to tubes or 10-15 ml to flasks on top of the solidified Part A medium (Colle*et al*., 1996), as shown in Figure 1 B. The tubes/flasks were incubated at 37ºC for 24 hours to ensure sterility, and were then stored in a refrigerator until use, as shown in Figure 2.

Table 1. N. N. N. media components.

Contents		Weight (g)/ Volume (ml)
Part A Final pH (7.3±0.2) at 25°C	Meat extract	3 g
	Peptone	5 g
	NaCl	8 g
	Agar	15 g
Part B Final pH (7±0.2) at 25°C	NaCl	8 g
	KCl	0.2 g
	$CaCl_2$	0.2 g
	KH_2PO_4	0.3 g

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Dextrose	2.5

Figure 1. NNN media: A. Part A and defibrinated blood, B. Part B.

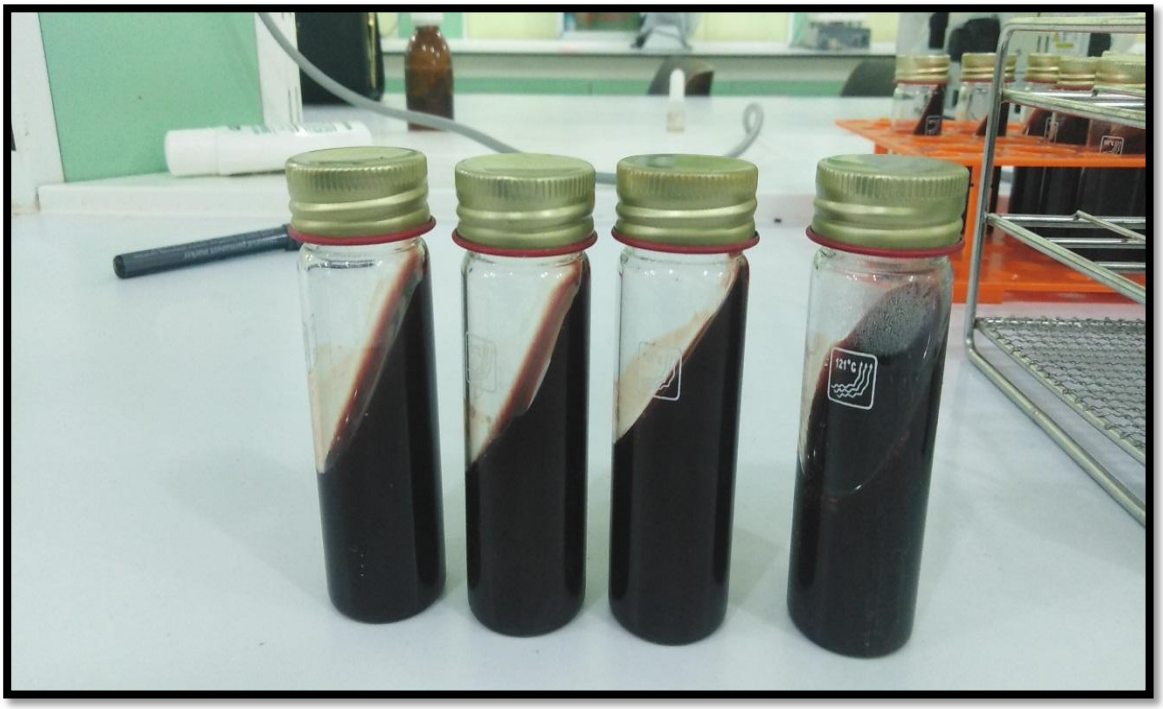

Figure 2. Tubes containing N. N. N. medium.

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Results

The microscopic examination was performed under oil immersion light microscopy (100X) after staining of samples collected from 436 skin biopsies of suspected cases from humans and 74 skin biopsies of suspected symptomatic owned-dogs. Smears stained with Giemsa stain are shown in Figure 3 and with Leishman stain are shown in Figure 4. All human samples from suspected cases (436, 100%) were positive, whereas 71 (95.95%) samples from owned dogs were positive, (Table 2).

Table 2. Rate of infection according to suspected cases of CL in humans and owned dogs.

Sample	Samples collected	Positive	Negative
	No. (%)		
Suspected human cases	436	436 (100)	0
Suspected owned-dog cases	74	71 (95.95)	3 (4.05)

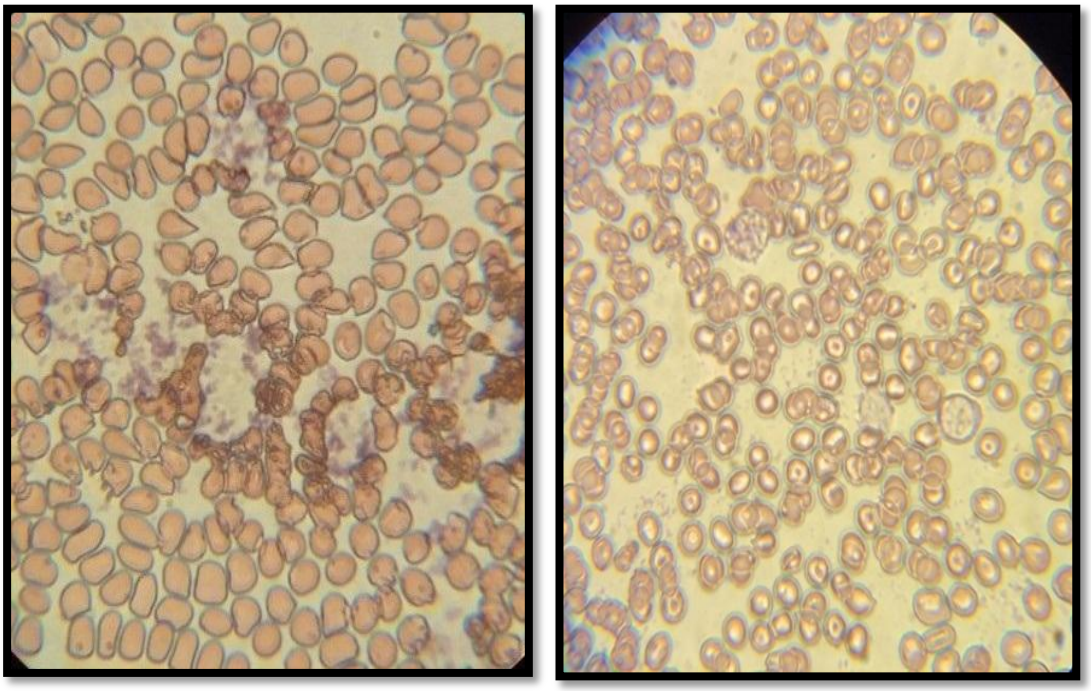

Figure 3. Smears from lesions stained with Giemsa stain show amastigotes in macrophages under oil immersion (100X).

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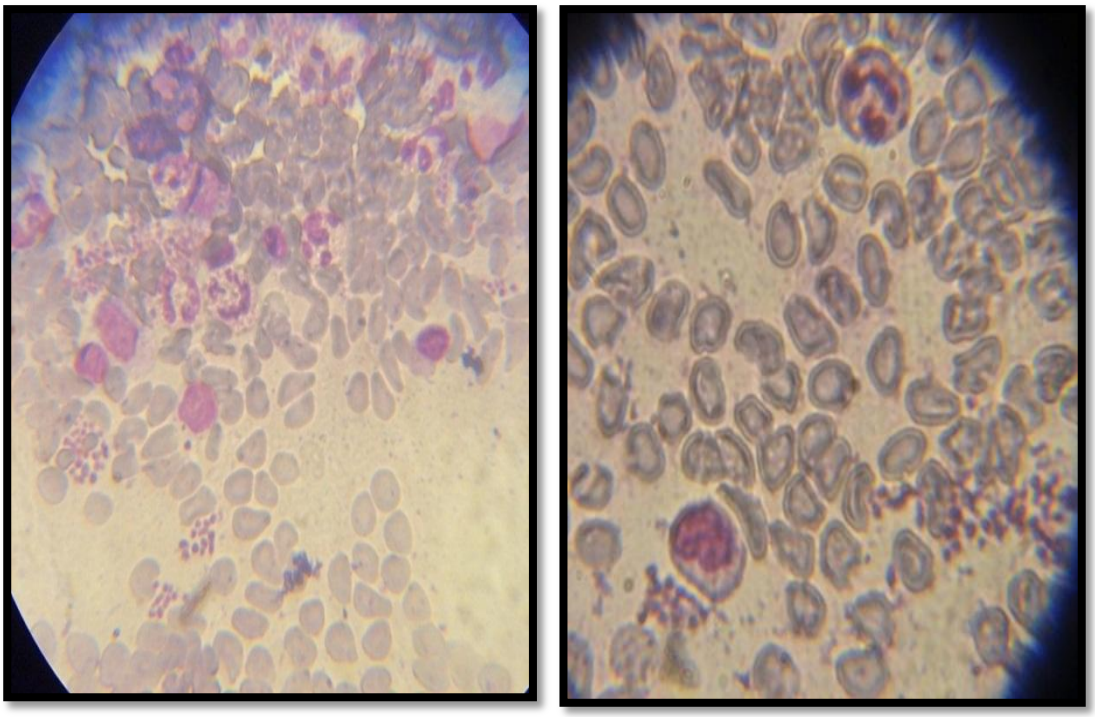

Figure 4. Smears from lesions stained with Leishman stain show amastigotes in macrophages under oil immersion (100X).

Fifty aspirate samples from humans (25) and owned-dogs (25) were cultured on modified Novy-MacNeal-Nicolle medium, (Figure 5), with findings observed as follows:
5/25 (20%) of human samples showed growth, whereas 20/25 (80%) of samples were contaminated. Of the dog samples, 14/25 (56%) showed growth, whereas 11/25 (44%) of the samples were contaminated. The samples from dogs had a significantly higher growth rate than samples aspirated from humans (P<0.05), (Table 3).
10/25 (40%) human samples showed growth, whereas 15/25 (60%) of samples were contaminated. Of the dog samples, 18/25 (72%) showed growth, whereas 7/25 (28%) samples were contaminated. The samples from dogs had a significantly higher growth rate than samples aspirated from humans (P<0.05), (Table 4).

Table 3.*Leishmania* growth on modified N.N.N. with human blood.

Host	No. of samples cultivated with N.N.N. + human blood	%	No. of samples positive	%	No. of contaminated samples	%
Human	25	50	5	20	20	80
Dog	25	50	14	56	11	44
Chi-square = 6.876, *P*<0.05						

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Table 4.*Leishmania* growth on modified N.N.N. with dog blood.

Host	No. of samples cultivated with N.N.N. + dog blood	%	No. of samples positive	%	No. of contaminated samples	%
Human	25	50	10	40	15	60
Dog	25	50	18	72	7	28
Chi-square = 5.195, *P*<0.05						

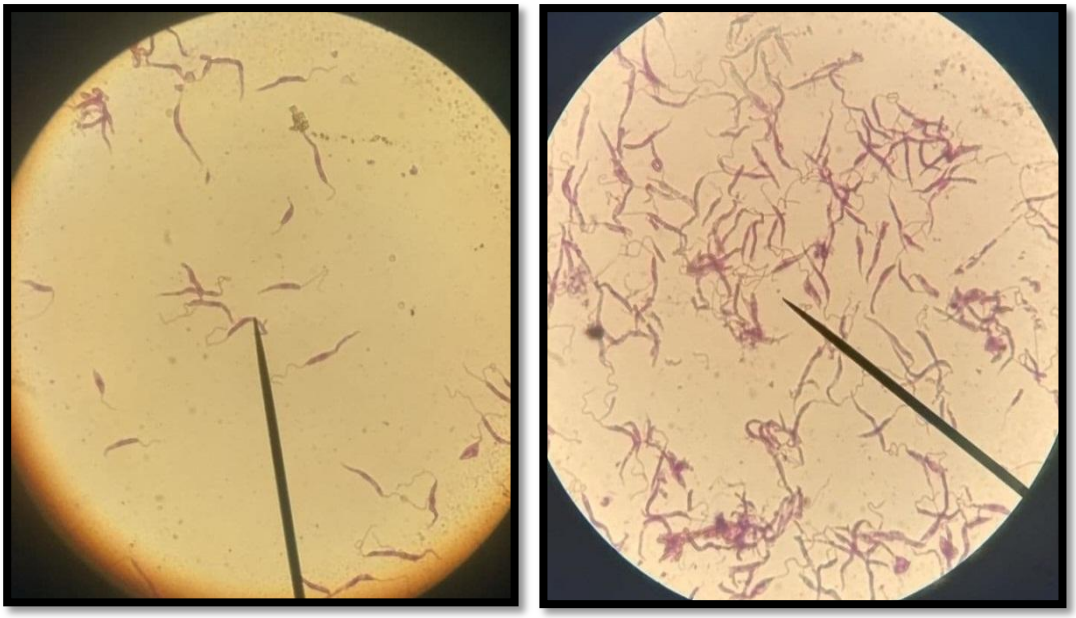

Figure 5. Smears from modified N.N.N. media stained with Giemsa show promastigotes under oil immersion (100X).

Discussions

In Misan government, CL was the most common endemic disease, with a high prevalence. During this study, 436 patients were detected and diagnosed clinically and in the laboratory to be infected with CL.

The cultivation of 25 pre-diagnosed samples from human and owned-dogs in modified N.N.N medium showed the growth rate of samples from owned-dogs were higher than the growth rate of human samples, in both N.N.N medium with human and dog blood, (56% versus 20% and 72% versus 40%), respectively.

The samples contaminated under conditions beyond our control, such as power off during the cultivation period inside the incubator, and fluctuation in the temperature.

A study conducted in Baghdad by Younis (2018) found that 12/75 sample with pure growth on (NNN) medium, and 38/75 samples contaminated, whereas the remaining 25/75 samples did not show any growth. Other study done by Abdulwahab, (2013), documented (75%) of samples cultured on N.N.N. were positive, and (25%) were negative.

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Leishmaniasis is a zoonotic disease, involving different canine species, including domestic dogs and two fox species. The disease in dogs is characterized by local, self-healing ulcerative lesions on the ears, scrotum, feet, nipples, and muzzle (Mehregan *et al.*, 1999; Madeira *et al.*, 2006). However, canine CL is a neglected disease, mainly because most of the cases occur far away from cities, that is, far from veterinary services. Dogs have been identified to be an important host for the parasite. Infected dogs normally develop both visceral and cutaneous lesions, whereas restriction to the skin occurs in some forms of human leishmaniasis (oriental sores) (Mehregan *et al.*, 1999; Perez-Molina *et al.*, 1999).

There are many methods for diagnosing the parasite. They include microscopic examination of direct smears using Giemsa stain or Leishman stain, culturing on Novy–MacNeal–Nicolle (NNN) medium, histopathology, serology, and specific molecular techniques based on detecting the parasite DNA using polymerase chain reaction (PCR) to amplify the *Leishmania* genome(Fahriye *et al*., 2017).

Leishmaniasis is divided into three general clinical patterns according to the form of the disease; visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL) (WHO, 2007; Igbineweka *et al.*, 2012). There are over 20 species of *Leishmania* that have been recorded as causative infectious agents in humans; the distribution of each is determined by the distribution of vectors, reservoir hosts, or both (Ashford, 2000).

Several cases of cutaneous infection in *L. major*-infected dogs have been reported in Saudi Arabia, Egypt, and Iraq. Infected dogs are a potential primary reservoir host infected by sandflies in endemic areas (Dantas-Torres, *et al.*, 2006).

Conclusion

To our knowledge this is the first study contribute to diagnosis of *L*. spp. using *in vitro* cultivation by N.N.N. modified medium with blood from human and dog. The cultivation in modified N.N.N medium showed a high growth rate of samples from owned-dogs were than human samples. Blood of dogs seems to be a very good media for growing of this microorganism *in vitro* and may be also *in vivo*.

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