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The First Record of Zoonotic Genes of Cutaneous Leishmaniasis among Human, Dogs, and Sandflies by Nested Polymerase Chain Reaction and Phylogenetic Analyses

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Abstract

BACKGROUND: Cutaneous leishmaniasis (CL) is one of the zoonotic diseases that is caused by protozoa of the genus Leishmania.

AIM: The study aimed to diagnosed CL in human, dogs, and sandflies by PCR, and identification the zoonotic gene of CL by the nested PCR technique.

METHODS: A total of 100 patients with CL, 237 of owned-dogs, and 147 females' sandflies collected.

RESULTS: Approximately, 88% of human samples, 95.77% skin biopsies and 20.69% of blood samples of dogs, and 40.58% of sandflies tissues were positive for L. major, while L. tropica infection was positive in 12% of human, in 4.23% symptomatic, and 6.89% asymptomatic of dogs, and in 27.54% of sandflies samples. The sequence ID of the local L. major in human were registered in NCBI as (MW421598.1, MW421599.1, MW421600.1), in dogs (MW421601.1, MW421602.1, MW421603.1), and sandflies (MW421604.1, MW421605.1, MW421606.1). While L. tropica in human were registered in NCBI as (MW421604.1, MW421605.1, MW421606.1), in dogs (MW421428.1, MW421429.1), and in sandflies (MW421430.1, MW421431.1).

CONCLUSION: To our knowledge, this is the first study that contributes to the diagnosis of CL spp. in three different hosts (human, dogs, and sandflies) at the same time, particularly in Iraq and in Middle East countries.

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 and hemoflagellate of the genus *Leishmania* [1], [2], [3], [4], [5]. Old World cutaneous leishmaniasis (CL) caused by *Leishmania tropica*, and a rural or wet type caused by *Leishmania major* [6]. Epidemiological studies in the Middle East have shown that anthroponotic CL caused by *L. tropica* and zoonotic CL caused by *L. major* in Saudi Arabia, Iraq, Iran, Afghanistan, Pakistan, and Yemen [7], [8]. It is endemic in Iraq, where both forms of the disease, cutaneous and visceral, are found [9]. Natural transmission of *Leishmania* parasites is carried out by sandflies of the genus Phlebotomus (Old World) [4]. Phlebotomine sandflies are responsible for the transmission of several human diseases, including bartonellosis, leishmaniasis, and sandfly fever viruses [10]. *L. major*-infected dogs have been reported in Saudi Arabia and Egypt, as are potential primary reservoirs host infected by sand flies [11], [12].

Recently, CL has increased and extensively spreading to new geographical zones as documented in neighboring countries. This unexpected rise of the patient's number in endemic regions is multifactorial and attributed to population movements and crowding, ecological disturbances, changing patterns of international trade and traveling, political unrest, natural disasters, and drug resistance of parasites [13].

For many years ago, several polymerase chain reaction (PCR)-based approaches have been employed for typing *Leishmania* spp. Multilocus Enzyme Electrophoresis is considered the gold standard for *Leishmania* identification [14]. DNA sequencing, PCR-restriction fragment length polymorphism (RFLP) [15], Nested PCR [16], and Multilocus Sequence Typing [17], [18] have all been employed for these objectives.

This work aimed to explore molecular properties and phylogenetic analyses by evaluating popular markers in SSU (18S) rRNA gene, from clinical samples with nested PCR and sequencing in Iraq. Moreover, this study is unique, and generally to

our knowledge, it is the first study conducting on three different hosts of *Leishmania* which differs from the studies previously performed in the regions concerning phylogenetic analyses of CL in Middle East countries.

Materials and Methods

Ethical consideration

The Medical Ethical Committee at the College of Veterinary Medicine, University of Baghdad approved this study (Ref. No. 2650, 17/12/2019). Written informed consent was obtained from each participant and personal data were kept confidential.

Patients

A total of 100 patients with CL were enrolled in this study. They attended the Dermatological and Venereology Department in Al-Sadder Teaching Hospital and public health laboratories in Misan Health Directorate, Misan Province, Iraq.

Dogs

A total of 237 (blood and skin lesions) from dogs were collected from patients with CL when visiting their homes of both sex and maturity. There were 74 dogs with clinical signs of CL, whiereas 163 were asymptomatic. The examination done by us, since we are veterinarian graduated from College of Medicine.

Pus and discharge aspiration

Each skin lesion was cleaned with iodine or ethanol, and then about 0.5 to 1 ml of Lock's solution was subcutaneously injected using a disposable insulin syringe at the peripheral edges of the lesion. The solutions were stored in Eppendorf tubes to be directly used for examination by direct smear and culture.

Swab collection

Some patients presented with ulcerative or cystic lesions and were eligible for applying a swab to collect pus or any discharge materials from the lesions. A cotton swab was used to swab overall lesion area and then placed in sample tubes containing 500 ml of phosphate-buffered saline solution.

Filter paper collection

Skin lesions were cleaned with 70% alcohol, and filter papers were gently touched on the lesion around the edges of the cuts and allowed to air dry thoroughly.

Blood samples collection

Blood samples from domestic dogs were collected. A sample of 2–5 ml was taken from the cephalic vein of each dog into EDTA tubes and transported to the laboratory.

Skin scraping

A blunt blade scrape in the direction of hair growth was used. Initially, we scraped the superficial layer and then subsequently deeply (enough to cause capillary ooze). Then, the scraped small pieces were transferred to a cup contain BPS (5 ml) and stored at −20°C before DNA isolation [32].

Sandflies

A total of 268 sandflies (engorged with blood from human and dogs) were collected from different areas near patients' homes in Misan province including indoors, outdoors, rat burrows, stables, residential clay houses, fields, and the edges of rivers. Light traps and sticky oil paper (insect glue snares) were used to collect whole insects; these were then put in cups containing sterile normal saline or petri dishes for examination and diagnosis. Identification and differentiation of sandflies from other insects depends on their shape, morphology, and size, according to Habeeb [10].

PCR

Molecular diagnosis depending on the three types of primers, which were 1st primer LEI-1 (Forward) and LEI-2 (Reverse) to diagnosed *Leishmania* spp.; 2nd primer LITSR (Forward) and L5.8S (Reverse) for genotyping of *L. major*; and 3rd primer Lmj4 (Forward) and Uni21 (Reverse) for genotyping of *L. tropica*. In addition, the SSU (18S) rRNA gene was amplified in a nested PCR reaction.

DNA extraction and purification

Genomic DNA was extracted from 100 human, 237 (74 biopsy, and 163 blood) dogs, and 147 female sandflies, diagnosed with CL using (MagPurix® Viral/ Pathogen Nucleic Acids Extraction Kit A/Zinexts Life Science Corp./Taiwan).

Agarose gel electrophoresis

DNA was visualized by (1%) agarose gel electrophoresis and stained with GelStain-GREEN (V2), according to Sambrook and Russell [19].

DNA concentration and purity measurement

All eluted DNA samples were measured using Nano-drop, by taking 0.5 μl of each eluted DNA sample using a micropipette, putting it in a well, closing the apparatus, then measuring the concentration of the eluted DNA. All concentrations were recorded in a Microsoft Excel 2010 spreadsheet labeled for each sample.

Primers preparation

The primers were supplied by Alpha-DNA Company (Canada) as lyophilized products of 100 picomole concentrations. Alpha-DNA's company protocol was adopted for primer use, by bringing the final concentration of the primers to 10 pmol/μl in nuclease-free water. The lyophilized primer was dissolved in 1 ml nuclease-free water and stored at −20°C. To avoid repeated freezing and thawing, small aliquots were prepared at suitable concentrations and stored at −20°C until use. The primers' names, sequences, and products size listed in Table 1.

Table 1: Names, sequences, and product size of the primers

Primers	Sequence	Size (bp)
SSU (18S) rRNA		
F		
LEI-1	5′-GGT TCC TTT CCT GAT TTA CG-3′	650
R		
LEI-2	5′-GGC CGG TAA AGG CCG AAT AG-3′	
F		
LITSR	5′-CTG GAT CAT TTT CCG ATG-3′	302–338
R		
L5.8S	5′-TGA TAC CAC TTA TCG CAC TT-3′	
F		
Lmj4	5′-CTA GTT TCC CGC CTC CGA G-3′	600

PCR amplification of SSU (18S) rRNA gene

Genomic DNA was amplified using the general primers LEI-1 (Forward) and LEI-2 (Reverse) for detection of *Leishmania* spp. and used to produce a 650 bp product. The lyophilized blue pellet was dissolved by vortexing and briefly spun down. Then, PCR was performed on the samples. After that, samples were loaded on agarose gel without adding a loading-dye mixture, and electrophoresis was performed. The PCR tubes were transferred to a preheated thermocycler, and the program was started as following steps: i. initial denaturation (at 96°C for 900 s), ii. annealing (at 60°C for 20 s in 40 cycles), and iii. final extension (at 72°C for 300 s in one cycle). A 5 μl sample of PCR product was added to each well of agarose gel. The molecular weight of PCR amplified product was determined according to 2000–100 bp ladder after 60 min at 70 V.

Nested PCR http://www.pcrstation.com/nestedpcr/2020

Detection of L. major

The PCR mix for the nested reaction consisted of new master mix tubes with the addition of the following: Forward primer (LITSR) (2.0 μl), reverse primer (L5.8S) (2.0 μl), 1st PCR product (5.0 μl), and nuclease free water (11.0 μl). As the previous protocol of amplificated PCR, tubes were transferred to preheated thermocycler and started the program, as following steps: i. Initial Denaturation (at 95°C for 300 s), ii. annealing (at 65°C for 40 s in 40 cycles), iii. final extension (at 72°C for 5 s in one cycle). The detection of the nested PCR amplified product of 302–338 bp was achieved by electrophoresis, as with the primary PCR.

Detection of L. tropica

The PCR mix for the nested reaction consisted of a new master mix tube with the addition of the following: Forward primer (Lmj4) (2.0 μl), reverse primer (Uni21) (2.0 μl), 1st PCR product (5.0 μl), and nuclease free water (11.0 μl). As with the previous protocol for PCR amplification, tubes were transferred to a preheated thermocycler, and the program was started, as following: i. Initial denaturation (at 95°C for 900 s), ii. Annealing (at 60°C for 20 s in 40 cycles), iii. Final extension (at 72°C for 300 s in one cycle). The detection of the nested PCR amplified product of 600 bp was achieved by electrophoresis, as with the primary PCR.

Sequence and phylogenetic analysis

The sequencing analysis of the desired gene as a method of choice for use in epidemiological, clinical, genetic, and taxonomic studies, and also for the management of CL patients with unknown origins, is highly emphasized [20], [21]. The *L. major* LITSR gene and *L. tropica* Lmj4 gene were registered after correspondence with the National Center for Biotechnology Information; accession numbers were obtained and became a reference for Iraq, the Middle East, and the world. A total of 15 sequence samples (three each for human, dogs, and sandflies for *L. major*; two each for human, dogs, and sandflies for *L. tropica*) were sent to the Macrogen Company, the Genomic Medicine Institute of the Seoul National University College of Medicine, Korea. Hence, all samples were analyzed by National Center for Biotechnology Information (NCBI-BLAST).

Statistical analysis

All data collected were entered for statistical analysis into the Statistical Package for the Social Sciences version 24 (SPSS v24) (SPSS Inc., Chicago, Illinois, USA). Differences between infection rates were described by Chi-square ($\chi 2$) for Fishers' exact test. A $p < 0.05$ was considered statistically significant.

Results

DNA extraction

Genomic DNA was extracted from samples from 100 patients (skin biopsies), 237 dog samples (74 skin biopsies and 163 blood samples), and 147 samples from female sandflies (tissue samples). DNA extraction and purification were successfully performed using Isolation Kit (MagPurix® Viral/Pathogen Nucleic Acids Extraction Kit A/Zinexts Life Science Corp./Taiwan). All samples contained whole eluted DNA, as shown in Figure 1. Each eluted DNA sample was measured using nanodrop.

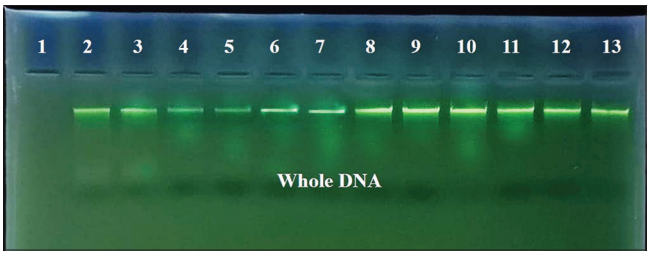

Figure 1: Gel electrophoresis of genomic DNA extracted from samples in 1% agarose gel at 70 V/cm² for 30 min. DNA was visualized under U.V. light after staining with GelStain-GREEN (V2)

PCR amplification of SSU (18S) rRNA genes

The genomic DNA was amplified using the general primers LEI-1 (Forward) and LEI-2 (Reverse) to detect *Leishmania* spp., which were used to produce a 650bp sequence. Product primers for the first amplification used 100 human skin biopsy samples, 74 skin biopsies and 163 blood samples from dogs, and 147 female's sandfly tissue samples.

The findings showed that all human samples (100, 100%), 71 (95.95%) skin biopsies from dogs, 58 (35.58%) blood samples from dogs, and 69 (46.94%) female sandfly tissues were positive for *Leishmania* spp., with a high statistically significant difference ($p < 0.01$), as shown in Table 2 and Figure 2.

Table 2: Results of molecular diagnosis of the initial PCR

Host	No. of samples	Positive for initial PCR *Leishmania* spp.	%	Negative for initial PCR *Leishmania* spp.	%
Human	100	100	100	0	0
Dogs					
Biopsies	74	71	95.95	3	4.05
Blood	163	58	35.58	105	64.42
Sandflies	147	69	46.94	78	53.06

Chi-Square ($\chi 2$)= 11.367, p<0.01. PCR: Polymerase chain reaction.

All positive PCR products based on the initial primers were examined in the next step using secondary primers for the amplification of nested PCR (LITSR, which generated a 302–338 bp sequence), used to diagnose *L. major*. The results revealed 88 (88%) human biopsies, 68 (95.77%) skin biopsies from dogs, 12 (20.69%) blood samples from dogs, and 28 (40.58%) females sandfly tissues were positive to

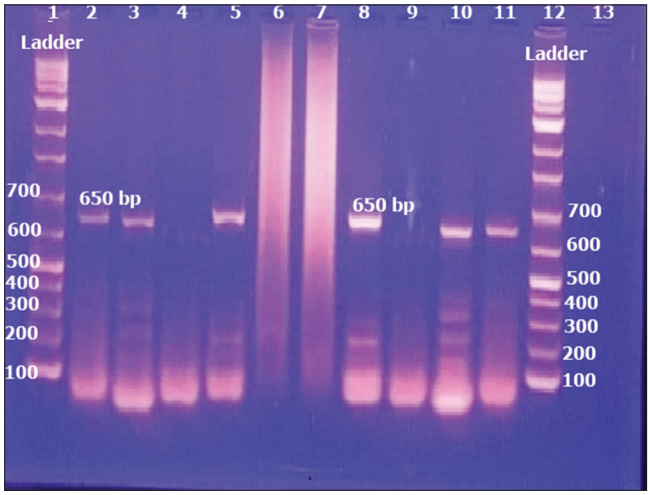

Figure 2: Gel electrophoresis of amplified DNA from Leishmania spp. by using the primer set LEI-1 and LEI-2 primers of SSU (18S) rRNA. Lane 0: DNA ladder. Lane 1, 3: Human DNA positive to 650 bp. Lane 4, 5: Dog DNA positive to 650 bp. Lane 6: Sandfly DNA positive to 650 bp. Lane 2: Water

L. major, with a high statistically significant difference ($p < 0.01$), as shown in Table 3 and Figure 3.

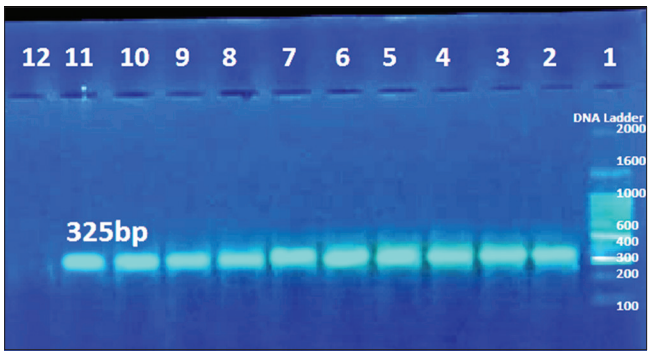

Figure 3: Gel electrophoresis of amplified DNA from Leishmania major using primer set LITSR and L5.8S primers of SSU (18S) rRNA. Lane 1: DNA ladder. Lane 2, 3, 4, 5: Human DNA positive to 302–338 bp. Lane 6, 7, 8, 9: Dog DNA positive to 302-338 bp. Lane 10, 11: sandfly DNA positive to 302–338 bp. Lane 12: Water

The negative PCR products using secondary primers were then examined using the third set of primers for the amplification of nested PCR (Lmj4, which generated a 600 bp sequence), used to diagnosed *L. tropica*. The results showed that 12 (12%) human biopsies, 3 (4.23%) dog skin biopsies, 4 (6.89%) dog blood samples, and 19 (27.54%) females sandfly tissue samples were positive to *L. tropica*, with a high statistically significant difference ($p < 0.01$), as shown in Table 3, and Figure 4.

Sequencing and phylogenetic analyses for LITSR gene

Sequencing of the LITSR gene was used DNA extracted from three lesions each from human, dogs, and sandflies) and was performed using phylogenetic tree analysis and compared with NCBI-BLAST L. major. The types of substitutions, locations, and nucleotides in all samples are shown in Table 4.

Table 3: Results of molecular diagnosis: Nested PCR (positive for secondary PCR *Leishmania major)* and (positive for third PCR Leishmania tropica)

Host	No. of samples infected with L. spp.	Positive for secondary PCR *Leishmania major*	%	Positive for 3rd PCR *Leishmania tropica*	%	Negative for 2nd and 3rd PCR	%
Human	100	88	88	12	12	-	-
Dogs							-
Biopsies	71	68	95.77	3	4.23	-	
Blood	58	12	20.69	4	6.89	42	
Sandflies	69	28	40.58	19	27.54	50	72.46
		Chi-square (χ^2)=13.78, p<0.01		Chi-square (χ^2)=17.92, p<0.01			

PCR: Polymerase chain reaction

Table 4: Type of polymorphism of *Leishmania major* of LITSR gene

S. No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Sequence ID with submission	Identities (%)
1	Transition	56	G\A	MN604128.1	MW421598.1	99
	Transition	69	A\G			
2	Transition	69	A\G	MN604128.1	MW421599.1	99
	Transversion	75	A\T			
	Transition	118	A\G			
3	Transition	69	A\G	MN604128.1	MW421600.1	99
	Transition	139	A\G			
	Transversion	141	C\G			
4	Transversion or Transition	193	N(A,C,G,T)\A	KU949582.1	MW421601.1	99
	Transversion or Transition	195	N(A,C,G,T)\G			
	Transversion or Transition	197	N(A,C,G,T)\G			
5	Transversion	151	A\T	KU949582.1	MW421602.1	99
	Transversion or Transition	193	N(A,C,G,T)\A			
	Transversion or Transition	195	N(A,C,G,T)\G			
	Transversion or Transition	197	N(A,C,G,T)\G			
6	Transversion or Transition	193	N(A,C,G,T)\A	KU949582.1	MW421603.1	99
	Transversion or Transition	195	N(A,C,G,T)\G			
	Transversion or Transition	197	N(A,C,G,T)\G			
	Transition	225	C\T			
7	Transition	264	T\C	KC880116.1	MW421604.1	99
8	Transition	242	G\A	KC880116.1	MW421605.1	99
	Transition	264	T\C			
9	Transition	248	T\C	KC880116.1	MW421606.1	99
	Transition	264	T\C			

Source: *Leishmania major* (Note :1, 2, 3 in human; 4, 5, 6 in dog ; 7, 8, 9 in sandflies).

Our sequence IDs of the local L. major in human, samples No.1, No.2, and No.3 are (MW421598.1, MW421599.1, and MW421600.1), recorded in NCBI-BLAST L. major. They are closely related, at 99%, to MN604128.1 in Jordan, MH347921.1 in Turkey: Sanliurfa, KY882278.1 in Iraq: Sulaimani, MF522259.1 in Iran, KF612022.1 in Thailand, KP874100.1 in Iran: Mashhad, HG512909.1 in Belgium, MW053321.1 in India: West Bengal, FR796423.1 in United Kingdom, FJ753394.1 in USA, AY550178.1 in Germany, AJ300482.1 in Kenya, KX821679.1 in Austria, and AJ300481.1 in Sudan.

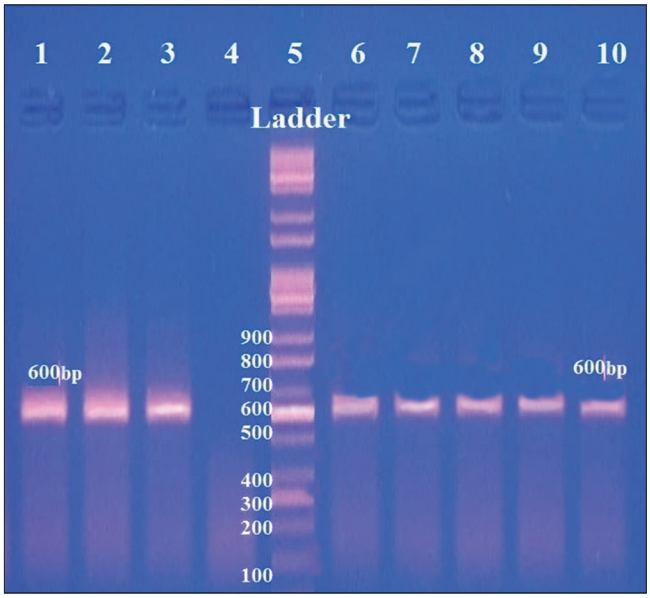

Figure 4: Gel electrophoresis of amplified DNA from Leishmania tropica using primer set Lmj4 and Uni21 primers of SSU (18S) rRNA. Lane 5: DNA ladder. Lane 1, 2, 3: Human DNA positive to 600 bp. Lane 6, 7, 8: Dog DNA positive to 600 bp. Lane 9, 10: Sandfly DNA positive to 600 bp. Lane 4: Water

The sequence IDs of the local L. major in dogs, samples No.4, No.5, and No.6, are MW421601.1, MW421602.1, MW421603.1, recorded in NCBI-BLAST L. major in dogs, which are closely related to EF413075.1 in Iran, KU949582.1 in Mediterranean Sea, and KU949581.1 in Mediterranean Sea, at 99%.

The sequence IDs of the local L. major samples in sandflies, No.7, No.8, and No.9 are MW421604.1, MW421605.1, MW421606.1, registered in NCBI-BLAST. L. major in sandflies is closely related to KC880116.1 in Iran: Ilam province, KC880115.1 in Iran: Ilam province, KC880114.1 in Iran: Ilam province, KC880113.1 in Iran: Ilam province, KC880112.1 in Iran: Ilam province, at 99%, and with MT966015.1 in West Bank, EF413078.1 in Iran, EF413077.1 in Iran at 98%, as shown in Tables 4 and 5, and Figure 5.

Sequencing and phylogenetic analyses for Lmj4 gene

Sequencing of the Lmj4 gene used DNA extracted from two lesions from each of human, dogs, and sandflies and was performed using phylogenetic tree analysis and compared with NCBI-BLAST *L. tropica*. The types of substitutions, locations, and nucleotides for all samples are shown in Table 6.

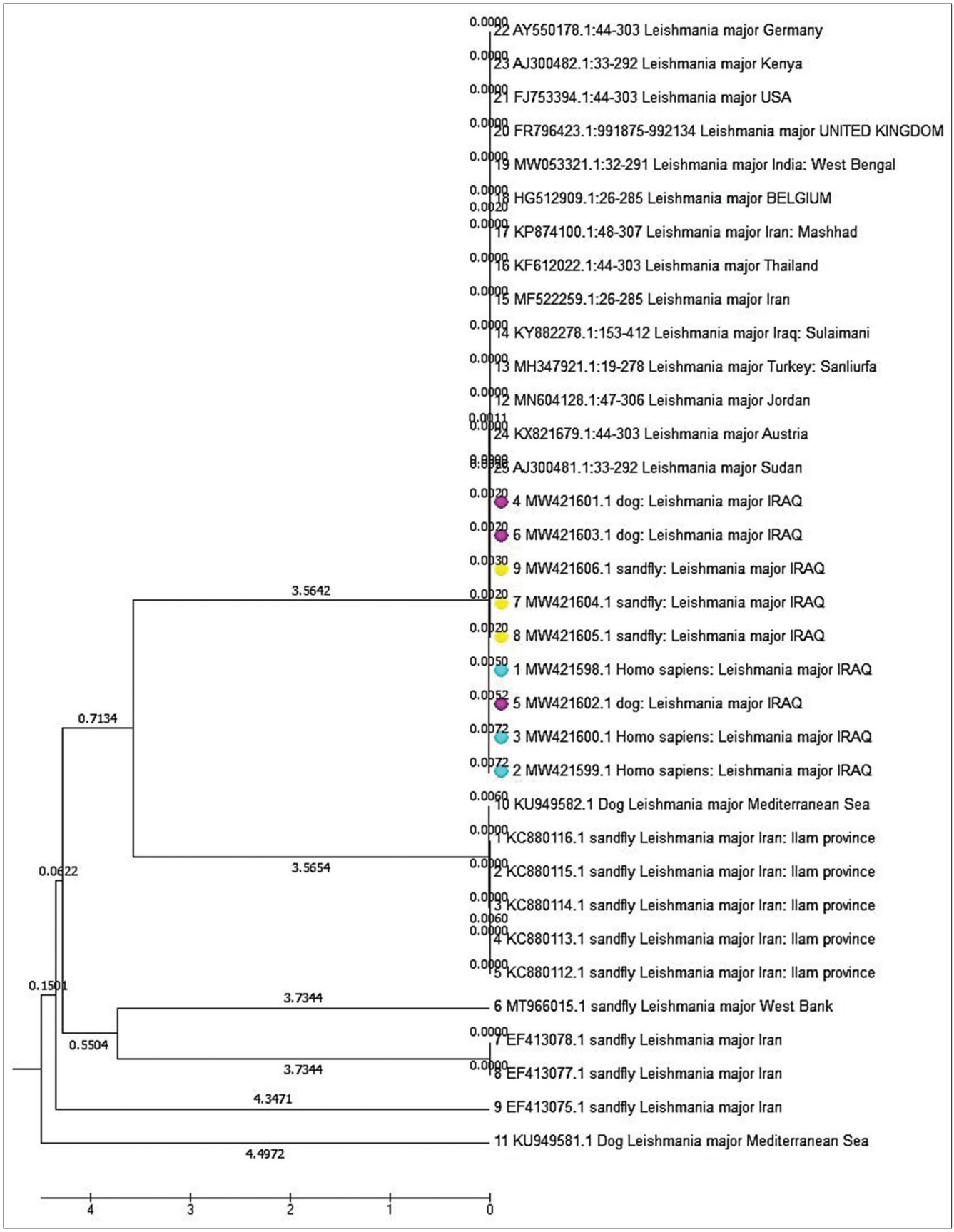

Figure 5: Neighbor-joining tree Leishmania major of LITSR gene. New sequences derived from this study are marked with blue for human, red for dogs, and yellow for sandflies. The GenBank accession numbers, species of infected host, and country of origin are included for each sequence

Table 5: Type of polymorphism of *Leishmania major* of LITSR gene

S. No	Accession No.	Country	Source	Host	Compatibility (%)
1.	KC880116.1	Iran: Ilam province	*Leishmania major*	Sandfly	99
2.	KC880115.1	Iran: Ilam province	*Leishmania major*	Sandfly	99
3.	KC880114.1	Iran: Ilam province	*Leishmania major*	Sandfly	99
4.	KC880113.1	Iran: Ilam province	*Leishmania major*	Sandfly	99
5.	KC880112.1	Iran: Ilam province	*Leishmania major*	Sandfly	99
6.	MT966015.1	West Bank	*Leishmania major*	Sandfly	98
7.	EF413078.1	Iran	*Leishmania major*	Sandfly	98
8.	EF413077.1	Iran	*Leishmania major*	Sandfly	98
9.	EF413075.1	Iran	*Leishmania major*	Dog	98
10.	KU949582.1	Mediterranean Sea	*Leishmania major*	Dog	99
11.	KU949581.1	Mediterranean Sea	*Leishmania major*	Dog	99
12.	MN604128.1	Jordan	*Leishmania major*	Homo sapiens	99
13.	MH347921.1	Turkey: Sanliurfa	*Leishmania major*	Homo sapiens	99
14.	KY882278.1	Iraq: Sulaimani	*Leishmania major*	Homo sapiens	99
15.	MF522259.1	Iran	*Leishmania major*	Homo sapiens	99
16.	KF612022.1	Thailand	*Leishmania major*	Homo sapiens	99
17.	KP874100.1	Iran: Mashhad	*Leishmania major*	Homo sapiens	99
18.	HG512909.1	Belgium	*Leishmania major*	Homo sapiens	99
19.	MW053321.1	India: West Bengal	*Leishmania major*	Homo sapiens	99
20.	FR796423.1	United Kingdom	*Leishmania major*	Homo sapiens	99
21.	FJ753394.1	USA	*Leishmania major*	Homo sapiens	99
22.	AY550178.1	Germany	*Leishmania major*	Homo sapiens	99
23.	AJ300482.1	Kenya	*Leishmania major*	Homo sapiens	99
24.	KX821679.1	Austria	*Leishmania major*	Homo sapiens	99
25.	AJ300481.1	Sudan	*Leishmania major*	Homo sapiens	99

In human, the sequence IDs of the local *L. tropica* samples No.1 and No.2 are MW421426.1 and MW421427.1, registered in NCBI-BLAST *L. tropica*. They are closely related to Z32843.1 in Russia and MH511158.1 in Iraq: Kute, at 99%.

Among dogs, the sequence IDs of the local *L. tropica* samples No.3 and No.4 are MW421428.1 and MW421429.1, recorded in NCBI-BLAST *L. tropica*. They are closely related to Z32843.1 in Russia, at 99%.

Table 6: Type of polymorphism of *Leishmania tropica* of Lmj4 gene

No. of sample	Type of substitution	Location	Nucleotide	Sequence ID with compare	Sequence ID with submission	Identity (%)
1	Transversion	118	T\G	Z32843.1	MW421426.1	99
2	Transition	189	T\C	Z32843.1	MW421427.1	99
3	Transversion	520	C\G	Z32843.1	MW421428.1	99
4	Transition	274	T\C	Z32843.1	MW421429.1	99
	Transition	463	A\G			
	Transition	464	A\G			
5	Transversion	208	A\T	Z32843.1	MW421430.1	99
6	Transversion	208	A\T	Z32843.1	MW421431.1	99
	Transition	307	A\G			

Source: *Leishmania tropica*. 1, 2 in human;: 3, 4 in dog ; 5, 6 in sandflies.

In sandflies, the sequence IDs of the local *L. tropica* samples No.5 and No.6 are MW421430.1 and MW421431.1, recorded in NCBI-BLAST *L. tropica*. They are closely related to Z32843.1 in Russia, at 99%, as shown in Tables 6 and 7 and Figure 6.

Table 7: Type of polymorphism of *Leishmania tropica* of Lmj4 gene

	Accession No.	Country	Source	Host	Compatibility (%)
26.	Z32843.1	Russia	*Leishmania tropica*	Homo sapiens	99
27.	MH51118.1	Iraq: Kute	*Leishmania tropica*	Homo sapiens	99

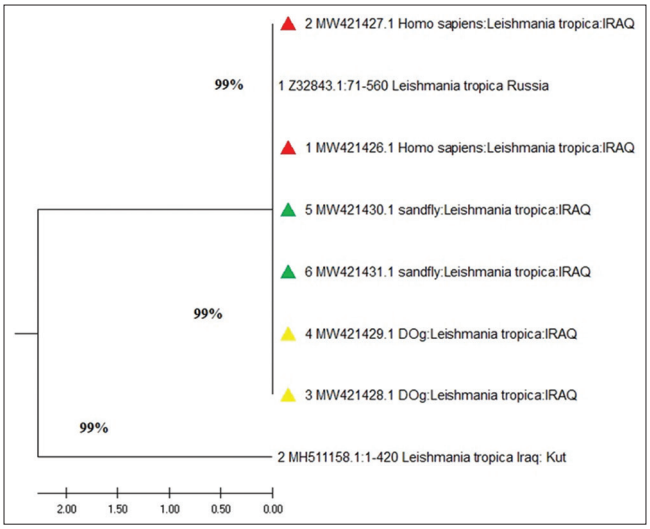

Figure 6: Neighbor-joining tree Leishmania tropica of Lmj4 gene. New sequences derived from this study are marked with green for sandflies, red for human, and yellow for dogs. The GenBank accession numbers, species of infected host, and country of origin are included for each sequence

Discussion

The genomic DNA from 100 skin biopsies from human, 237 dog samples (74 skin biopsies and 163 blood samples), and 147 samples of female sandfly tissues were extracted. DNA extraction and purification were successfully performed using Isolation Kit (MagPurix® Viral/Pathogen Nucleic Acids Extraction Kit A/Zinexts Life Science Corp./Taiwan).

Until now, the diagnosis of CL has been difficult in endemic regions where different species of *Leishmania* can present with very similar appearance and where different skin diseases with similar clinical symptoms occur. Both the gold standard parasitological techniques of the direct identification of amastigotes in microscopy samples and/or culture of promastigotes from infected tissues are highly specific but are not very sensitive [22]. Because of there are three different hosts (human, dogs, and sandflies) samples and to be more accurate as well as to reduce the effort, time, and cost. We contributed the diagnosis of CL spp. in combination particularly in Iraq and internationally in the Middle East countries by nested PCR, as the first study to our knowledge conducted in this way.

A search of the PubMed database revealed that, since 1989, more than 700 articles on PCR diagnosis of leishmaniases have been published, in which a multitude of gene targets, protocols, and applications have been described, including genus and/or species-specific PCR, ranging from low-tech to high-tech approaches [22].

We used the SSU (18S) rRNA gene to diagnose and confirm the diagnosis of leishmaniases in patients, their dogs, and sandflies caught from

the patient's environment. The PCR-based assays remain the main molecular diagnostic methods for the diagnosis of *Leishmania* parasites in clinical samples [23]. PCR primers targeting the kinetoplast and ribosomal DNA genes are among the most frequently utilized for the detection and/or identification of *Leishmania* spp. in the Old World and they reveal perfect findings [24], [25], [26], [27], [28], [29].

The PCR showed the rate of CL infection in the patients was 100%, and this is corresponding with the estimated CL infection rate using traditional and staining methods. These genes consider as an important virulent factor for *Leishmania* to make infection as reported by Mauricio *et al*. [30]. Mouttaki *et al*., Bensoussan *et al*., and Kumar *et al*. reported that LITSR/L5.8S and Lmj4/Uni21 primers are highly sensitive, sometimes reaching 100% in identifying CL by nested PCR and PCR-RFLP [22], [28], [29].

Patients with CL in Al-Diwanyah Teaching Hospital were diagnosed by real-time PCR and showed that 89.09% of the samples were positive, while only 10.9% were negative [31].

The infection rate was 95.95% in dogs that showed clinical signs on their skin and 35.58% in asymptomatic dogs. This is compatible with what researcher found in Saudi Arabia, showed 31 (5.9%) of 526 samples were positive for genus *Leishmania*. Two of 198 Riyadh dogs identified as infected with *Leishmania* spp., with 18 of 175 and 11 of 153 found positive in Al-Ahsa Oasis and Al-Qaseem by kDNA-PCR, respectively [32].

In this study, nested PCR of the SSU (18S) rRNA gene proved to be more sensitive for *Leishmania* species identification. This was consistent with several previous studies that used nested-PCR [33], [34], [35], [36], [37], [38], [39].

The difference in the ratios between this study and comparative studies may be that domestic dogs returning to infected patients were the target while stray dogs were the target in Saudi Arabia.

The rate of infection in engorged sandflies was 46.94%, because the parasite does not stay long in the insect when the final host is present. This was indicated by a study revealed that 16 pools (100%) were positive after PCR amplification, while none of the specimens were negative for the parasite [40]. Azizi *et al*., reported the natural infection of *Phlebotomus papatasi* with *L. major* in this endemic focus of ZCL by nested PCR [41]. Recently, molecular studies have shown that *P. papatasi* has a key role in *L. major* transmission by nested and real-time PCR [38], [42], [43].

The results of the molecular study were probably based on sampling location, primer and DNA extraction. The PCR procedure offers several advantages, including faster processing time, higher sensitivity, and decreased contamination risk, and seems to be a suitable tool for the direct diagnosis and characterization of *Leishmania* species compared with other methods reported by Seray *et al*. [44].

The nested PCR results showed that 88% of human and 95.77% of symptomatic dogs and 20.69% of asymptomatic dogs, and 40.58% of sandflies samples were positive for *L. major*. These high percentages of *L. major*, explained by its more prevalent in central and southern Iraq, and are represent the zoonotic type among the three hosts, while the rate of *L. tropica* infection was positive in 12% of human, in dogs the percent were 4.23% (symptomatic), and 6.89% (asymptomatic), and 27.54% of sandflies samples, and this similar to other studies in Al-Qadisiyah province [31]. The nested PCR method was used to determine the *Leishmania* parasite and its species, the results for positive samples showed that the proportion 95.91% were *L. major* and just 2% were *L. tropica* [31]. These findings, especially for human, are disagreement with [45], [46], who found *L. tropica* was the main species, comprising 88.5% of CL cases, while *L. major* was found in 11.5% of the detected cases in Kerman, Iran and they used nested PCR. These findings are also unlike the conclusions of many researchers used molecular methods of nested PCR in Yemen, who mentioned that CL is most commonly caused by *L. tropica*, suggesting anthroponotic transmission of CL [47].

Mohebali *et al*., supporting our results of secondary set of primers amplification by nested PCR, they concluded that domestic and wild canine infections with *L. major* may be more prevalent in areas of endemic human *L. major* CL than currently recognized, and canines should be evaluated as possible additional reservoirs for human infection [48]. Also, Solano-Gallego *et al*., studied different clinical and parasitological findings in canine *L. major* and *L. tropica* infections and indicated that PCR with DNA sequencing of the affected tissues or blood should be used in dogs to discriminate between infections with these three Old World *Leishmania* spp. [49].

Bamorovat *et al*., found *L. tropica* infection was more prevalent in stray dogs in Iran by PCR amplification of kDNA, which is different from our findings [50].

Abdulwahab, reported that from 55 positive samples, 33 samples were identified as *L. major* (60%) with amplicon molecular weighted 430 bp, and 22 samples were identified as *L. tropica* [51].

Other studies in nearby countries, such as Iran Saki *et al*. documented that the high frequency of *L. major* may be due to the presence of large numbers of reservoir animals, especially rodents and dogs (reservoir of *L. major*) by used RFLP-PCR [52]. Obviously, dense populations of natural hosts of *L. major*, together with abundant vector sandflies, are the key elements responsible for the high rate of human infection [52].

The prevalence of *Leishmania* species infecting stray dogs in Saudi Arabia was 4.0% *L. major* and 1.9%

L. tropica [32]. This variation in the rate of infection in dogs is due to the fact that the dogs targeted in this study are owned-dogs for patients with CL, whereas the target dogs in Saudi Arabia were stray dogs, as well as the use of different special primers, in addition to the sensitivity, which have a fundamental role in the appearance of the results more accurate and clear [32].

In the last two decades, PCR-based procedures targeting different genes have been successfully used to directly detect and characterize *Leishmania* spp. in clinical specimens and used for taxonomic, epidemiologic, population genetic, and phylogenetic analyses. In addition, the refined features of a gene are carried out by sequencing for spatial distribution and molecular identification [20], [53], [54].

However, *Leishmania* species are associated with certain clinical features of the disease, and the parasite identification should not be diminished at species level detections, for that reasons typing techniques are required. The nucleotide sequencing analysis process is a popular and effective method for *Leishmania* species typing [53]. This kind of data can help in solution strain heterogeneity link to geographical origin, and this technique has the benefit of identifying genetic variability directly from samples without having to culture the causative organism [55].

The phylogenetic tree of this study showed more heterogeneity in *L. major* compared with *L. tropica*, possibly due to circulation of different clones within the study area in Misan province. This finding is in disagreement with [45].

The phylogenetic analysis of Iraqi strains of local *L. major* organisms in human samples is closely related to those from Jordan, Turkey: Sanliurfa, Iraq (Sulaimani), Iran, Thailand, Iran (Mashhad), Belgium, India (West Bengal), UK, USA, Germany, Kenya, Austria, and Sudan at 99%. The phylogenetic analysis of dog strains of the local *L. major* samples shows they are closely related to strains from Iran, Mediterranean Sea at 99%. Regarding sandfly sequences, those of the local *L. major* are closely related to those from Iran, Ilam province, at 99%, and with West Bank and Iran at 98%.

In human, the sequences of the local *L. tropica* samples are closely related to those from Russia and Iraq (Kute) at 99%. Among dogs, the sequences are closely related to those from Russia, at 99%. In sandflies, the sequences are closely related to those from Russia at 99%.

These variations may be due to multifactorial reasons and patients factors, for example, patients' immunity, medication, habits, lifestyle, social factors, and economic factors, or may be due to environmental reasons such as a region's demography, climate factors, and change in metrological factors. There may also be factors related to human such as migrations from rural to urban areas, traveling to endemic areas, and use of insecticides. Genotyping at the species level is essential for monitoring the relative frequency of CL in the Iraq area that is correlated to two different *Leishmania* species (*L. major* and *L. tropica*), each characterized by distinct epidemiological features. The obtained results highlight the need to find a universally accepted diagnostic tool for *Leishmania* typing [31].

Many observations have led to the hypothesis that *Leishmania* spp. rapidly adapts to individual hosts, sparking intensive research into the underlying mechanisms of genetic variation [56]. Isolation in Iraqi showed the similarity with the strains of the Western type as the role of human migration may be ruled out since *L. tropica* and *L. major* seemed to have been spread in Africa and Middle East, besides European colonial expansion for 500 years [56], [57]. Most probably, strains from the Middle East arrived in Europe and took the Western type as its genotype [58].

Finally, genomic detection not only has a role in the diagnosis of *Leishmania* species responsible for disease but also for the postulation of targeted therapies, management, favorable identification, effective control measures and prevention strategies, and the study of the epidemiology and dynamics of the disease.

Conclusion

To our knowledge, this is the first study that contributes to a diagnosis of CL spp. in combination with three hosts (human, dogs, and sandflies), particularly in Iraq and generally in Middle East countries by nested PCR. Nested-PCR has high sensitivity, specificity, and accuracy powerful, and is a perfect approach for the identification of *Leishmania* species extracted from clinical samples (i.e., aspiration fluids and culturing specimens). The phylogenetic nucleotides sequencing analyses targeting SSU (18S) rRNA gene are valuable tools in genetic analytical characterization and management of CL patients where the disease is highly endemic. In addition, the study indicated that *L. major* has a high degree of intraspecific diversity relatively over the diversity of *Leishmania tropica.*

Authors' Contribution

Study design: ARKA, KMH. Main manuscript preparation: ARKA. An important revision of the manuscript: ARKA. All authors read and approved the final manuscript.

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