Molecular characterization of *Leishmania* species from stray dogs and patients in Saudi Arabia

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Research

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Abstract

Background: *Leishmania major* and *Leishmania tropica* cause cutaneous leishmaniasis in humans and dogs in several parts of the world, with a large number of cases recorded in the Middle East. However, when occurring in sympatry, the role of each species of *Leishmania* in the epidemiology of cutaneous leishmaniasis (CL) is not clear.

Methods: To determine the prevalence and to identify the species of *Leishmania* that infects humans and stray dogs in Riyadh and Al-Qassim (Saudi Arabia), 311 stray dogs and 27 human patients, suspected for *Leishmania,* were examined for CL by a nested PCR (nPCR).

Results: Nested PCR (nPCR) detected seven patients (25.9%) positive for cutaneous leishmaniasis. Five patients from Riyadh were infected by *L. major* and two from Al-Qassim by *L. tropica.* In addition, five dogs (1.6%) were infected by *L. tropica*.

Conclusions: This is one of the first molecular studies of leishmaniasis from Saudi Arabia. The relationship between the sand fly vectors and the reservoirs of both *Leishmania* spp. is still scarcely known in this region, and further epidemiological investigations of domestic and wild canids infected with *L. major* and *L. tropica* are needed towards a control and prevention of the infection in canine and human populations.

Background

Leishmaniases are a complex of sand fly transmitted protozoa diseases, listed amongst the neglected tropical diseases affecting millions of people, mainly the world's most vulnerable populations [1]. These diseases are transmitted by phlebotomine sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Leishmaniases include cutaneous (CL), visceral (VL) and mucocutaneous leishmaniasis (MCL) forms, all of which have been reported in Saudi Arabia [2-4]. Moreover, in that Country CL by *Leishmania major* has the highest prevalence mainly in Riyadh, Qassim, Al-Madinah, Al-Hassa, Hail and Asir [5-7] with an estimated number of more than 26,300 cases [2] over the past 10 years (2006–2016). In addition, in Saudi Arabia there are several reports of leishmaniasis by *Leishmania infantum* (*L. infantum*), *Leishmania major* (*L. major*), and *Leishmania tropica* (*L. tropica*) among humans and wild animals [8-12]. In spite of the number of molecular studies available to diagnose and identify *Leishmania* species worldwide [13-16], there is a lack of information on human CL patients as well as dog populations from endemic areas of Saudi Arabia. El-Beshbishy et al., [17] reported the molecular characterization of CL in patients from Al-Madinah Al-Munawarah province, western Saudi Arabia by internal transcribed spacer 1 (ITS-1) PCR-restriction fragment length polymorphism (RFLP) and kinetoplast DNA (kDNA) PCR established *L. major* and *L. tropica* as the causative organisms, with a kDNA PCR sensitivity of 90.7%, whereas ITS-1 PCR had a sensitivity of 70.1%. Rasheed et al., [18] reported the prevalence of *Leishmania* species among patients with CL in Qassim province, central Saudi Arabia. They recorded that out of total 206 CL biopsies, 49.5% biopsies were found to be positive for *L.*

major, 28.6% biopsies were positive for *L. tropica*, 3.9% were found to be positive for *L. infantum/donovani*. In addition, all tested CL biopsies showed negative results for *Leishmania mexicana (L. mexicana)* and *Leishmania viannia* (*L. viannia*). Therefore, the aims of the current study were to use molecular tools to detect and identify the *Leishmania* species infecting humans and stray dogs in Al-Qassim province and Riyadh city, Saudi Arabia in order to better understand the epidemiology of the infection.

Materials And Methods

Ethical approval

This study was reviewed and approved by the Ethics Committee of the Department of Biological Science at Shaqra University, according to the ethical principles of animal research (protocol SH 2-2017).

Study areas

The investigation was conducted from January 2018 to May 2019 in Al-Qassim province and Riyadh city, Saudi Arabia. Al-Qassim province is located at the central part of Saudi Arabia (latitude 25°–23° N and longitude 42°–24° E). It has an area of about 58.046 km^2 with a population of 1,423.000 people in 2017 [19]. Al-Qassim province is known as an agricultural region and it has a typical desert climate, with an average temperature of 13˚C and hot summer (an average temperature of 35˚C), with few annual rainfalls (214 mm) and low humidity ranging from 25% to 76% (http://www.pme.gov.sa). Conversely, Riyadh city is the capital of Saudi Arabia (latitude 24°–08°N and longitude 47°–18° E), with an area of about 1798 km^2 and inhabited by approximately seven million people in 2017 [19]. Riyadh is characterized by very hot summers with an average temperature of 45˚C in July, whereas winters are cold. The overall climate is arid, with scarce annual precipitations (21.4 mm), with a relative humidity ranging from 10% to 47% throughout the year. Riyadh is also known to have many dust storms (http://www.pme.gov.sa) (**Figure 1**).

Patients Biopsy tissue collections and DNA extraction

A total of 27 suspected patients were attended in both King Saud Medical City in Riyadh city (n =16) and *Buraydah Central Hospital* (n =11) in Al-Qassim province. All samples were diagnosed after clinical and microscopy examination [20]. Briefly, skin biopsies (i.e., 5-10 mm of diameter) were taken under sterile conditions from the border of the ulcer and cutaneous lesions and DNA samples were extracted from all biopsies by MagNaA pure DNA extraction Pure LC DNA Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions and the extracted DNA was quantified by Nanodrop spectrophotometer (Thermo, USA), DNA concentration differed from sample to another, but

it ranged from 18ng/ul to 33ng/ul, and an aliquot (100 µl of DNA from each sample) was stored at -20 °C prior to nPCR amplification and analysis.

Sampling of stray dogs

From January 2018 to May 2019, 311 stray dogs were trapped in Al-Qassim province by bait traps (Havahart®) (**Figure 1**).Dogs were examined physically for canine leishmaniasis skin lesions in the field. Seven dogs were suspected for canine leishmaniasis due to the presence of cutaneous nodules or ulcerated lesions on the skin (**Table 1**). Skin biopsies (5 mm in diameter) were taken under sterile conditions from the border of the ulcer and inoculated into medium M199 supplemented (Gibco, Life technologies, Germany) with 25 mmol/L HEPES (pH:7.5) and 20% fetal bovine serum (Gibco, Life technologies, Germany) followed by incubation at 24 °C. Ten days later parasites were harvested and washed with ice-cold phosphate buffered saline (PBS, pH: 7.4) and stored in -20 °C before DNA isolation. DNA from parasites cultures was isolated by using the ReliaPrep™ gDNA Tissue Miniprep System Kit (Promega, Madison, United States), following the manufacturer's instructions.

Leishmania Nested PCR (nPCR)

The specific external CSB2XF primers (5′-ATTTTTCGCGATTTTCGCAGAAACG-3′) and CSB1XR (5′-CGAGTAGCAGAAACTCCCGTTCA-3′) were used initially. In the second step, specific internal 13Z primers (5′-ACTGGGGGTTGGTGTAAAATAG-3′) and LiR (5′-TCGCAGAACGCCCCT-3′) were applied [21]. The specificity and sensitivity of this primers is reported to be 92% and 100%, respectively [21]. In addition, these primers were able to track and multiply the variable part of all forms of the *Leishmania* kDNA. Amplified fragments of *L. infantum* were 680 bp in length and fragments of *L. tropica* and *L. major* were 750 and 560 bp in length, respectively [21]. The first step of PCR master mix that included CSB2XF and CSB1XR were applied using AccuPower® PCR PreMix kit (Bioneer, Daejeon, Korea). The prepared PCR pre-mix volumes contained KCl 30 mM, $MgCl_2$ 1.5mM, Tris-HCL (pH 9.0) 10mM, Taq DNA polymerase, and dNTP were adjusted to 2 µl. In addition, 1 µl of the first step of each initial CSB1XR and CSB2XF primers at concentrations of 10 pmol/uL (Bioneer, Daejeon, Korea) and 3 µl of DNA were added to the complex. Finally, 13 µl of deionized water (ddH2O) were added for a total volume of 20 µl for reaction. The reaction was performed in a thermal cycler (Techne TC-3000, USA) by set up the following conditions; initial denaturation temperature of 94 °C for 5 min; followed by 30 cycles at denaturation 94 °C for 30 s, annealing 55 °C for 60 s, extension 72 °C for 60 s, final extension at 72 °C for 7 min and then the reaction was held at 4 °C. The second step of PCR included 13Z and LiR primers and the same PCR master mix except 3µL of template PCR product. After that, PCR products were electrophoresed on a 1.5% agarose gel containing 1 µL Syber safe (Thermo Scientific™, Nalgene, UK) in Tris-acetate–EDTA buffer at 100 V for 45 min and visualized under UV imaging system (ImageQuant Laz4000, GE Healthcare Life Science,

Hammersmith, UK). The size of each sample was estimated by comparison with a 100 bp DNA Ladder Marker (Solis BioDyne OU, Estonia).

Leishmania kDNA sequencing and BLAST analysis

Positive amplified products of *Leishmania* species were sent to **Macrogen (South Korea)** for sequencing, and the results were compared with the sequences available in GenBank database using BLAST (http://blast.ncbi.nlm. nih.gov/). The BLAST analysis was performed based on NCBI-BLAST alignment identification and an UPGMA tree performed based on distances calculated using a composite likelihood model (MEGA 7.0 version). Bootstrap values were determined with 1,000 replicates of the data sets [22].

Results

Of the 27 human patients examined, five from Riyadh and two from Al-Qassim were positive to *L. major* and *L. tropica* respectively (**Figure 2 and 3)**. Of 311 dogs, seven (2.3%) presented with thick cutaneous lesions (i.e., 1.5 × 5 centimeters) in different anatomical sites (e.g., nose, muzzle, abdomen and between fingers) and five of them were positive to *L. tropica.* Sequencing analysis of *Leishmania* kDNA confirmed that the five positive samples (nos. H1-H5) of the human patients from Riyadh were all *L. major* with a size ranging from 441 bp to 451 bp yielding a nucleotide identity ranging from 99.34% to 100% with query cover 100% with previous *L. major* sequences from Iraq (MN313423). *Leishmania* sequences from two human patients (no. H1 and H3) from Al-Qassim were most similar 99.66% to 100% with query cover 100%, respectively to *L. tropica* from Iraq (MF166799) **(Figure 4**). Sequences of *Leishmania* kDNA from stray dogs (no. D5, D6 and D7) were closely related (i.e., 99.33% to 99.80%) to kDNA of *L. tropica* from Iraq (MF166799), whereas two sequences (no. D2 and D4) displayed a close nucleotide identity (i.e., 99.35% and 99,80) to *L. tropica* kDNA from Iraq (MN334665) and UK (AF308689), respectively (**Figure 4)**. Phylogenetic tree clustered *L. major* (no. H1-H5) and *L. tropica* (no H1 and H3) sequences from human samples to those from Iraq (accession numbers MN313423 and MF166799, respectively), and the phylogenetic tree clustered *L. tropica* (no. D2 and D4) from Iraq and UK (accession numbers MN334665 and AF308689, respectively) , while (no. D5, D6 and D7) sequences from dog's samples to those from Iraq isolate (accession number MF166799) **Figure 4**. In addition, the isolates of *L. tropica* from human and dogs in present study were closely related (i.e., 98.60% to 99.65% with query cover ranged 98.20% - 99.50%) to kDNA of *L. tropica* (Saudi strain, MHOM/SA/91/WR1063) that recorded on GenBank (accession number X84845.1).

Discussion

This study provides molecular evidence of the circulation of *L. major* and *L. tropica* in human and dog populations from the investigated areas. The above *Leishmania* spp. have already been recorded as

agents of cutaneous leishmaniases in Saudi Arabia and Middle Eastern countries [23-26]. However, *L. tropica* infection has been herein molecularly diagnosed for the first time in humans and dogs in the central part of the Saudi Arabia since it was previously reported in the west and southern west of Saudi Arabia in association with the distribution of the sand fly species (i.e., *Phlebotomus sergenti*), which is a proper vector for that species [27]. Conversely, *L. major* is more prevalent throughout the Country and can be found in the open deserts regions of Saudi Arabia [2, 28]. Previous studies in Saudi Arabia have reported the natural infection of *L. major* in dogs using enzymatic biochemical methods [29, 30], though no clinical information was available, nor serology or molecular confirmation were performed. The high nucleotide identity of human *L. major* and *L. tropica* isolates with those of Iraq (accession number MN313423 and MF166799) as well as of dog *L. tropica* isolates with Iraq and UK (accession number MN334665, MF166799 and AF308689, respectively) was also confirmed by the phylogenetic dendrogram herein presented. Moreover, this study showed that the isolates of *L. tropica* from human and dogs were closely related to kDNA of *L. tropica* (Saudi strain, MHOM/SA/91/WR1063) that recorded on GenBank (accession number X84845.1). This might be due to the distribution of similar sand flies species in the different parts of Saudi Arabia and Middle East, which may act as proper vectors of both *Leishmania* spp. [24, 31]. However, the phylogenetic analysis in this study was limited and performed based on the available sequences on GenBank based on the sequences of *L. tropica* and *L. major* in the Middle East countries particularly those from Iraq.

Of the 25 species of *Phlebotomus* reported in Saudi Arabia only five (i.e., *Phlebotomus papatasi, Phlebotomus sergenti, Phlebotomus bergeroti, Phlebotomus kazeruni,* and *Phlebotomus arabicus*) have been incriminated as vectors of CL [27, 28, 32, 33]. Of these, *P. papatasi* is the main and most predominant vector species for *L. major* [28, 32], while *P. sergenti* is the natural vector species for *L. tropica* [27]. Presence of *P. papatasi* and *P. sergenti* in Al-Qassim province suggests that they could have a potential role in the transmission of human and canine leishmaniasis. Nonetheless, more studies are required to elucidate the role of *Phlebotomus* spp. in CL disease transmission in Saudi Arabia.

Stray dogs have been often diagnosed in Saudi Arabia, with clinical disease associated with *Leishmania* species, however previous studies focused on the epidemiology, clinical, histopathological and biochemical aspects [11, 29, 30]. Conversely, molecular studies have reported the occurrence of dog infection by *Leishmania* spp. in Qatar [34], *L. tropica* in Iran [26, 35] and Israel [36, 37] and by *L. major* in Iraq [23] and Israel [25], which are in agreement with the current study. Though CL is endemic in many parts of Saudi Arabia, the paucity of data concerning the relationship between the disease, the vectors and reservoirs is a major hindrance to understand the transmission cycles in endemic areas, considering that the distribution patterns can easly change through the years in specific geographic areas [38]. Data herein provided contribute to fill existing gaps in order to increase the alert by the Ministry of Health in Saudi Arabia in preventing outbreaks and the spread of CL.

Conclusion

This was the first study that detected and identified the causative agent of CL in stray dogs and patients from Saudi Arabia thus confirming that *L. major* and *L. tropica* are endemic in Al-Qassim province and Riyadh City. However, it is still unclear the relationship between the sand fly vectors and reservoirs and their specific role in the transmission cycles in endemic areas of Saudi Arabia. Further epidemiological and molecular studies focusing on CL these areas are advocated also in order to elaborate better strategic control plans and assess the risk for human health.

Declarations

Ethics approval and consent to participate

Blood and tissue sampling for this study was approved by the Ethical Research Committee, Shaqra University and complied with relevant guidelines for animal handling and welfare. (Approval no. SH 02-2017)

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

ADA , ASA and AAR conceived the study. MSA, ASA performed field works. ASA collected patient's and dog's samples. ASA, AAR and MAA carried out the biopsy tissue collections and DNA extraction. MAA, AAR and FAR performed laboratory works and analyzed data. ADA, MSA and FAR wrote the first draft of the manuscript. JM-R, DO, RD and ADA reviewed and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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Table

Table 1: Data of suspect dogs by canine leishmaniasis that trapped in Al-Qassim province.

Dog ID	Site of trapped dogs	Gender	Age (years)	Location of cutaneous lesions
Dog No.1	Unayzah	Male	2	left ear
Dog No.2	Al-Bukkayriah	Male	1	nose
Dog No.3	*Buraydah*	Male	4	abdomen
Dog No.4	Al-Bukkayriah	Female	2	under mouth lips
Dog No.5	*Buraydah*	Male	3	muzzle
Dog No.6	Ar Rass	Female	3	nose
Dog No.7	*Buraydah*	Male	2	upper right leg

Figures

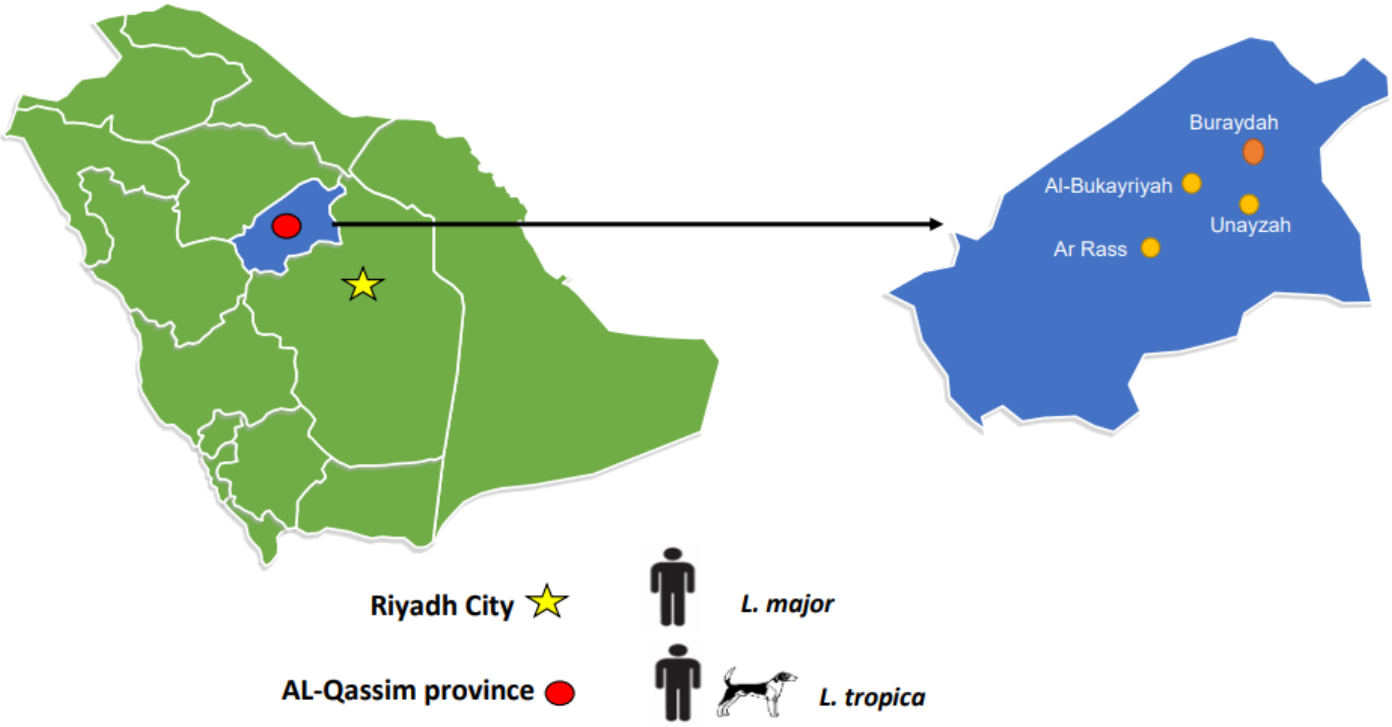

Figure 1

Map showing the location of the study areas in Saudi Arabia.

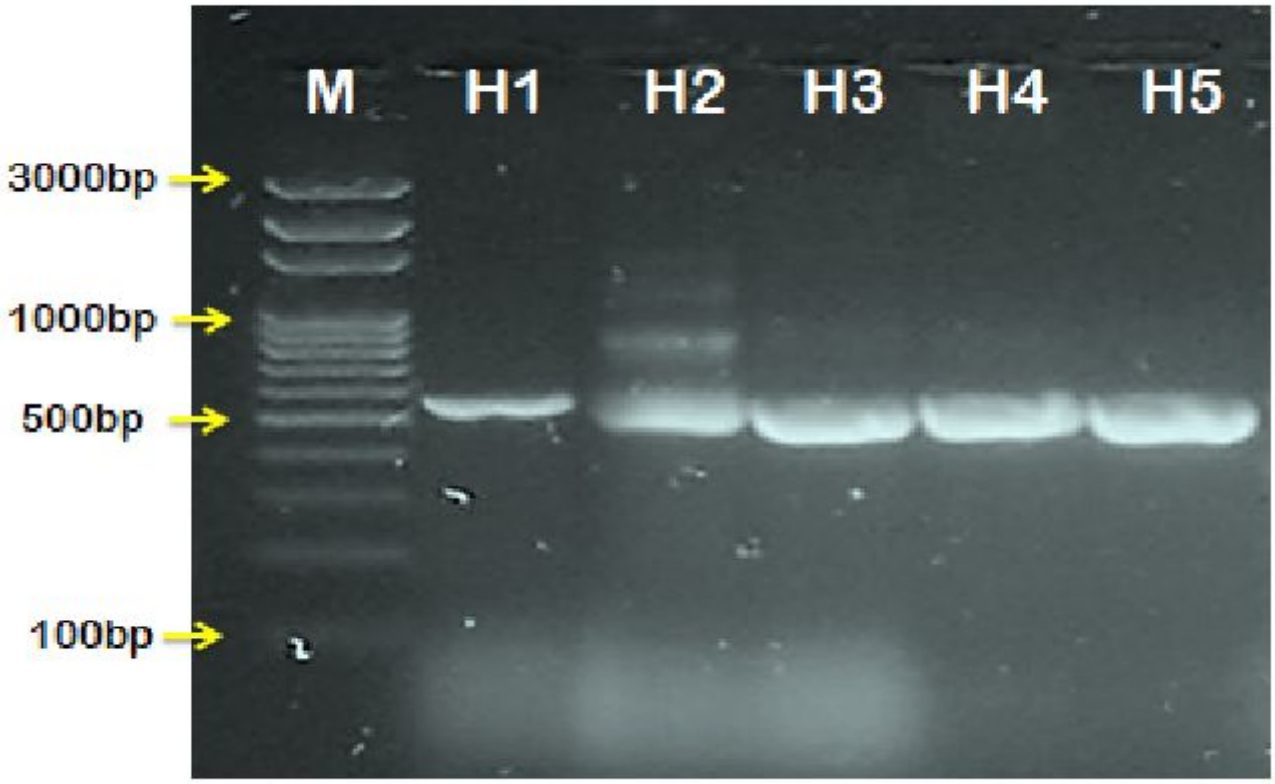

Figure 2

Agarose gel electrophoresis (1.7%) image that show the Nested PCR product analysis of kDNA in Cutaneous L. major from human skin lesion samples from Riyadh city. Where M: marker (3000-100bp) and lane (H1 – H5) positive Human L. major at (560bp) nPCR product.

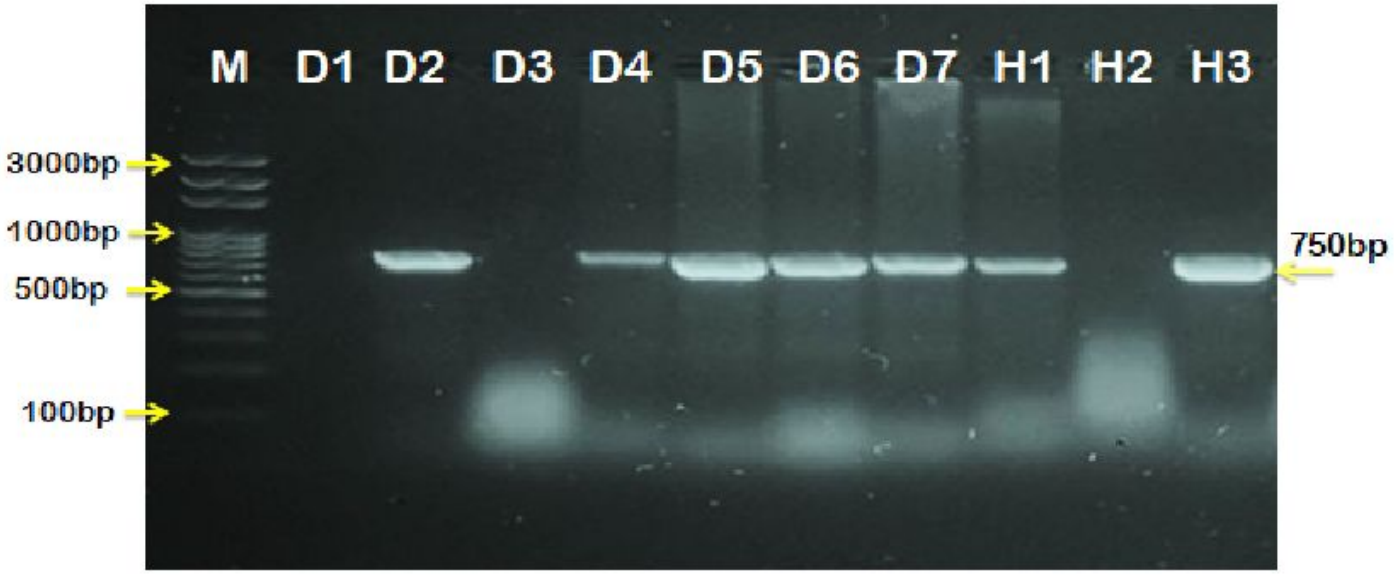

Figure 3

Agarose gel electrophoresis (1.7%) image that show the Nested PCR product analysis of kDNA in Cutaneous L. tropica from dogs and human skin lesion samples from Al-Qassim province. Where M: marker (3000-100bp), lane (D2 and D4-D7) positive dogs L. tropica and lane (H1 and H3) positive Human L. tropica at (750bp) nPCR product.

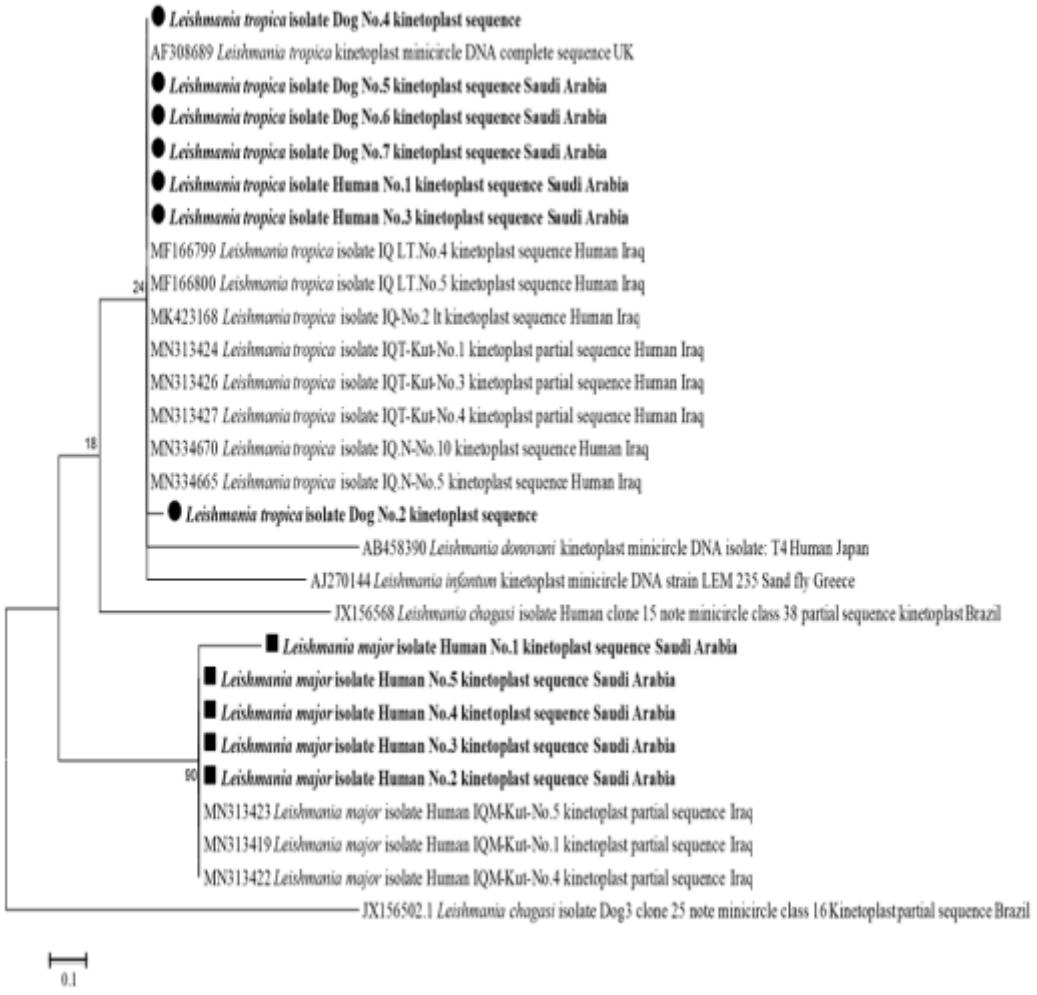

Figure 4

Phylogenetic tree analysis based on the partial sequence of kinetoplast DNA, non-protein coding region in local L.major and L. tropica human and dogs isolates that used for confirmative genetic detection and genetic relationship analysis. The evolutionary distances were computed using phylogenetic UPGMA tree type (MEGA 7.0 version).

Supplementary Files

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