Molecular Characterisation of Leishmania Species from Stray Dogs and Patients in Saudi Arabia

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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 they occur in sympatry, the role of each species of *Leishmania* in the epidemiology of cutaneous leishmaniasis (CL) is not clear.

Methods: To determine the frequency of occurrence and to identify the species of *Leishmania* that infect humans and stray dogs in Riyadh and Al-Qaseem (Saudi Arabia), 311 stray dogs and 27 human patients who were suspected for *Leishmania* were examined for CL by a nested polymerase chain reaction (nPCR).

Results: The use of nPCR detected seven patients (25.9%) who were positive for cutaneous leishmaniasis. Five patients from Riyadh were infected by *L. major* and two from Al-Qaseem 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 to be conducted in Saudi Arabia. The relationship between the sand-fly vectors and the reservoirs of both *Leishmania* spp. is still scarcely known in this region, and epidemiological investigations are required in order to progress towards control and prevention of the infection in canine and human populations.

Background

Leishmaniases are a complex group of protozoa diseases that are transmitted by sand flies. They are listed among a group of neglected tropical diseases that affect 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 the forms known as cutaneous (CL), visceral (VL) and mucocutaneous leishmaniasis (MCL), all of which have been reported in Saudi Arabia [2–4]. Moreover, in that country, CL that is caused by *Leishmania major* has the highest prevalence in the Riyadh, Qassim, Al-Madinah, Al-Hassa, Hail and Asir regions [5–7], where more than 26,300 cases are estimated to have occurred [2] over a 10 years period (from 2006 to 2016). In addition, in Saudi Arabia there are several reports of leishmaniasis by *Leishmania infantum*, *Leishmania major* and *Leishmania tropica* in humans and wild animals [8–12].

In spite of the availability of several molecular studies that report the diagnosis and identification of *Leishmania* species worldwide [13–16], there is a lack of information on human CL patients or on dog populations from endemic areas of Saudi Arabia.

In western Saudi Arabia (Al-Madinah Al-Munawarah province) CL was diagnosed in human patients by internal transcribed spacer 1 (ITS-1) polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) and kinetoplast DNA (kDNA) amplification [17]. PCR established *L. major* and *L. tropica* as the causative organisms for the above infection, 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 Al-Qaseem province, central Saudi Arabia. They recorded that, of a total of 206 CL biopsies, 49.5% were found to be positive for *L. major*, 28.6% for *L. tropica*, and 3.9% for *L. infantum/donovani*, respectively. Therefore, the aims of the current study were to use molecular tools to detect and identify the *Leishmania* spp. that infected humans and stray dogs in Al-Qaseem province and in Riyadh city, Saudi Arabia, in order to understand better the epidemiology of the infection.

Methods

Ethical approval

This study was reviewed and approved by the Ethics Committee of the Department of Biological Sciences 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-Qaseem province and Riyadh city, Saudi Arabia. Al-Qassim province is located in 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 and in 2017, it had a population of 1,423,000 people [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 a hot summer (an average temperature of 35°C). It has low annual rainfall (214 mm) and low humidity that ranges 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). It covers an area of about 1,798 km^2 and in 2017, was inhabited by approximately seven million people [19]. Riyadh is characterised 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 rainfall (21.4 mm) and a relative humidity that ranges from 10% to 47% throughout the year. Riyadh also has many dust storms (http://www.pme.gov.sa) (Fig. 1).

Patient biopsy tissue collection and DNA extraction

A total of 27 patients who were suspected to be infected by *Leishmania* species were seen in either King Saud Medical City in Riyadh city (n =16) or *Buraydah Central Hospital* (n =11) in Al-Qaseem province. The presence of *Leishmania* was investigated in all samples, which were collected after clinical and microscopy examination [20]. Briefly, skin biopsies (i.e., 5-10 mm in diameter) were taken under sterile conditions from the border of the ulcerous and cutaneous lesions and DNA samples were extracted from all biopsies by MagNaA pure DNA extraction through use of a Pure LC DNA Isolation Kit (Roche Applied Science, Germany) according to the manufacturer's instructions. The extracted DNA was quantified by Nanodrop spectrophotometer (Thermo, USA). The DNA concentration differed from sample to sample, but it ranged from 18ng/ul to 33ng/ul. An aliquot (100μl 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-Qaseem province by bait traps (Havahart®) (Fig. 1) and were examined physically for canine leishmaniasis skin lesions in the field. Seven dogs were suspected of infection with canine leishmaniasis due to the presence of cutaneous nodules or ulcerated lesions on the skin (Table 1). Skin biopsies (5mm in diameter) were collected under sterile conditions from the borders of the ulcers and were inoculated into M199 medium (Gibco, Life Technologies, Germany), which was supplemented with 25 mmol/l of 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH:7.5) and 20% fetal bovine serum (Gibco, Life Technologies, Germany). These samples were then incubated at 24 °C. Ten days after sample incubation, parasites were harvested and washed with ice-cold phosphate-buffered saline (10X PBS, pH: 7.4) and stored at -20°C before DNA isolation. DNA from parasite cultures was isolated by use of the ReliaPrep™ gDNA Tissue Miniprep System Kit (Promega, Madison, United States), following the manufacturer's instructions.

Leishmania nested PCR

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 these primers are reported to be 92% and 100%, respectively [21]. These primers were able to track and multiply the variable part of all forms of the *Leishmania* kDNA. Amplified fragments of *L. infantum* were 680bp in length and fragments of *L. tropica* and *L. major* were 750bp and 560bp in length, respectively [21].

The first step of the addition of the PCR master mix, which included CSB2XF and CSB1XR, was performed using the AccuPower® PCR PreMix kit (Bioneer, Daejeon, Korea). The prepared PCR pre-mix volumes contained potassium chloride (KCl) at a concentration of 30mM, magnesium chloride (MgCl$_2$) at 1.5mM, tris (hydroxymethyl) aminomethane *hydrochloride (*Tris-HCL at pH 9.0) at 10mM, Taq DNA polymerase, and deoxynucleoside triphosphate (dNTP) were adjusted to 2µl. In addition, 1µl of each initial CSB1XR and CSB2XF primer at concentrations of 10pmol/ul (Bioneer, Daejeon, Korea) and 3µl of DNA were added to the reaction mixture. Finally, 13µl of deionised water (ddH$_2$O) were added up to a total volume of 20µl for reaction. Negative control was included in the final nPCR. The reaction was performed in a thermal cycler (Techne TC-3000, US) according to the following conditions: initial denaturation temperature of 94°C for 5min; 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 60s and extension at 72°C for 60s; final extension at 72°C for 7min; and then the reaction was held at 4°C. The second step of PCR involved 13Z and LiR primers and the same PCR master mix except that 3µl of template PCR product were used. The second round, PCR products that were obtained were electrophoresed on a 1.5% agarose gel that contained 1µl Syber safe (Thermo Scientific™, Nalgene, UK) in tris-acetate−ethylenediaminetetraacetic acid (EDTA) buffer (50X) at 100V for 45min and visualised under a UV imaging system (ImageQuant Laz4000, GE Healthcare Life Science, Hammersmith, UK). The size of each sample was estimated by comparison with a 100bp 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 that were available in the GenBank database through use of BLAST (http://blast.ncbi.nlm. nih.gov/). The obtained sequences were aligned with a set of reference sequences that were available in GenBank using CLUSTALW in MEGA software version 7.0 [22]. The phylogenetic tree was constructed using the maximum-likelihood method and with the Hasegawa-Kishino-Yano (HKY) model with 2000 bootstrap replicates in MEGA 7.0 software, using *Trypanosoma cruzi* (AJ748063) as outgroup [22, 23].

Results

Of the 27 human patients who were examined, five from Riyadh and two from Al-Qaseem were positive for *L. major* and *L. tropica*, respectively (Figs. 2 and 3). Of 311 dogs, seven (2.3%) presented with thick cutaneous lesions (i.e., 1.5 × 5 cm) in different anatomical sites (e.g., nose, muzzle, abdomen and interdigital spaces) and five of them were positive for *L. tropica.* Sequencing analysis of the *Leishmania* kDNA confirmed that the five positive samples (nos. H1-H5) that were taken from the human patients from Riyadh were all *L. major* of sizes that ranged from 441 bp to 451 bp. These yielded nucleotide identities that ranged from 99.34% to 100% with query cover of 100% with previous *L. major* sequences from Iraq (MN313423). The *Leishmania* sequences that were collected from two human patients (nos. H1 and H3) from Al-Qaseem were most similar - 99.66% to 100% - with query cover 100%, respectively, to *L. tropica* from Iraq (MF166799) (Fig.4). The sequences for *Leishmania* kDNA that were obtained from stray dogs (nos. D4, D5, D6 and D7) were closely related (i.e., 99.33% to 99.80%) to kDNA of *L. tropica* from Iraq (MF166800), whereas one sequence (nos. D2) displayed close nucleotide identity (i.e., 99.35% and 99.80%) with *L. tropica* kDNA from Iraq (MN334661) and with that from the UK (AF308689), respectively (Fig. 4). The phylogenetic tree for *L. major* sequences (nos. H1-H5) and *L. tropica* sequences (nos. H1 and H3) from human samples clustered these with those from Iraq (accession numbers MN313423 and MF166799, respectively), and the phylogenetic tree for *L. tropica* (nos. D2 and D4) clustered these with samples from Iraq and UK (accession numbers MN334665 and AF308689, respectively). The sequences from dogs (nos. D5, D6 and D7) matched with those from Iraq isolate (accession number MF166799) (Fig. 4). In addition, the isolates of *L. tropica* that were taken from humans and dogs in the present study were closely related (i.e., 98.60% to 99.65% with query cover that ranged from 98.20% - 99.50%) to kDNA of *L. tropica* (Saudi strain, MHOM/SA/91/WR1063) that were recorded on GenBank (accession number X84845.1). Representative sequences of *L. major* and *L. tropica* that were retrieved in the present study were deposited in the GenBank database under the accession numbers MT787488 - MT787499.

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, in this

study *L. tropica* infection has been molecularly diagnosed for the first time in humans and dogs in the central part of Saudi Arabia. This infection has been previously reported in the west and south west of Saudi Arabia in association with the distribution of *Phlebotomus sergenti*, a proper vector for that species [27]. Conversely, *L. major* is more prevalent throughout the country and can be found in the open desert regions of Saudi Arabia [2, 28]. Previous studies that have been performed in Saudi Arabia have reported the natural infection by *L. major* in dogs through use of enzymatic biochemical methods [29, 30], though in these studies no clinical information was available, nor were serology or molecular confirmation studies performed.

The high similarities of the nucleotides of human *L. major* and *L. tropica* isolates with those of Iraq (accession number MN313423 and MF166799) and of the dog *L. tropica* isolates of Iraq and UK (accession number MN334665, MF166799 and AF308689, respectively) were confirmed by the phylogenetic analysis. Moreover, this study showed that *L. tropica* from humans and dogs was closely related with the kDNA of *L. tropica*. This might be due to the distribution of similar sand fly species in the different parts of Saudi Arabia and the Middle East, which may act as proper vectors of both *Leishmania* spp. [24, 31]. However, the phylogenetic analysis in this study was performed based on the sequences available on GenBank, which were based on the sequences of *L. tropica* and *L. major* from Middle East countries, particularly Iraq. In the phylogenetic tree that was inferred, *L. tropica* and *L. major* clustered in separate clades, distinct from the *L. donovani* complex (i.e., *L. infantum* and *L. donovani*). Moreover, *L. tropica* sequences presented very limited intra-specific genetic diversity, unlike the sequences that were classified as belonging to the *L. donovani* complex [32].

Of the 25 species of *Phlebotomus* (*P*) reported in Saudi Arabia, only five (i.e., *P. papatasi, P. sergenti,P. bergeroti, P. kazeruni* and *P. arabicus*) have been incriminated as vectors of CL [27, 28, 33, 34] with *P. papatasi* as the major and most predominant vector species for *L. major* [28, 33], and *P. sergenti* for *L. tropica* [27]. The presence of *P. papatasi* and *P. sergenti* in Al-Qaseem 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* spp. infection. However, previous studies have 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 [35], by *L. tropica* in Iran [26, 36] and Israel [37, 38] and by *L. major* in Iraq [23] and Israel [25], and these findings 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 the comprehension of the transmission cycles, particularly given that the distribution patterns can easily change through the years in specific geographic areas [39]. Data that have been provided in this study contribute to the filling of existing gaps in knowledge in order to increase the awareness of the Ministry of Health in Saudi Arabia to prevent outbreaks and the spread of CL.

Conclusion

This is the first study that has detected and identified the causative agent of CL in stray dogs and patients in Saudi Arabia and has confirmed that *L. major* and *L. tropica* are endemic in Al-Qaseem province and Riyadh City. However, the relationship between sand fly vectors and reservoirs of disease remains unclear and their specific role in transmission cycles in endemic areas of Saudi Arabia is unknown. Further epidemiological and molecular studies that focus on CL in these areas are advocated in order to draw up better strategic control plans and assess the risk for human health.

Declarations

Acknowledgements

Ethics approval and consent to participate

Blood and tissue sampling for this study was approved by the Ethical Research Committee, Shaqra University. The work 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 that were generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Authors' contributions

ADA, ASA and AAR conceived the study. MSA and ASA performed field work. ASA collected patient and dog samples. ASA, and ADA carried out the biopsy tissue collections and DNA extractions. MAA, AAR and FAB performed laboratory work and analysed data. HHAMA and ADA and JM-R performed phylogenetic analysis. ADA, MSA and FAB wrote the first draft of the manuscript. JM-R, DO and ADA reviewed and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1: Data of dogs that were suspected to carry canine leishmaniasis and that were trapped in Al-Qaseem 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
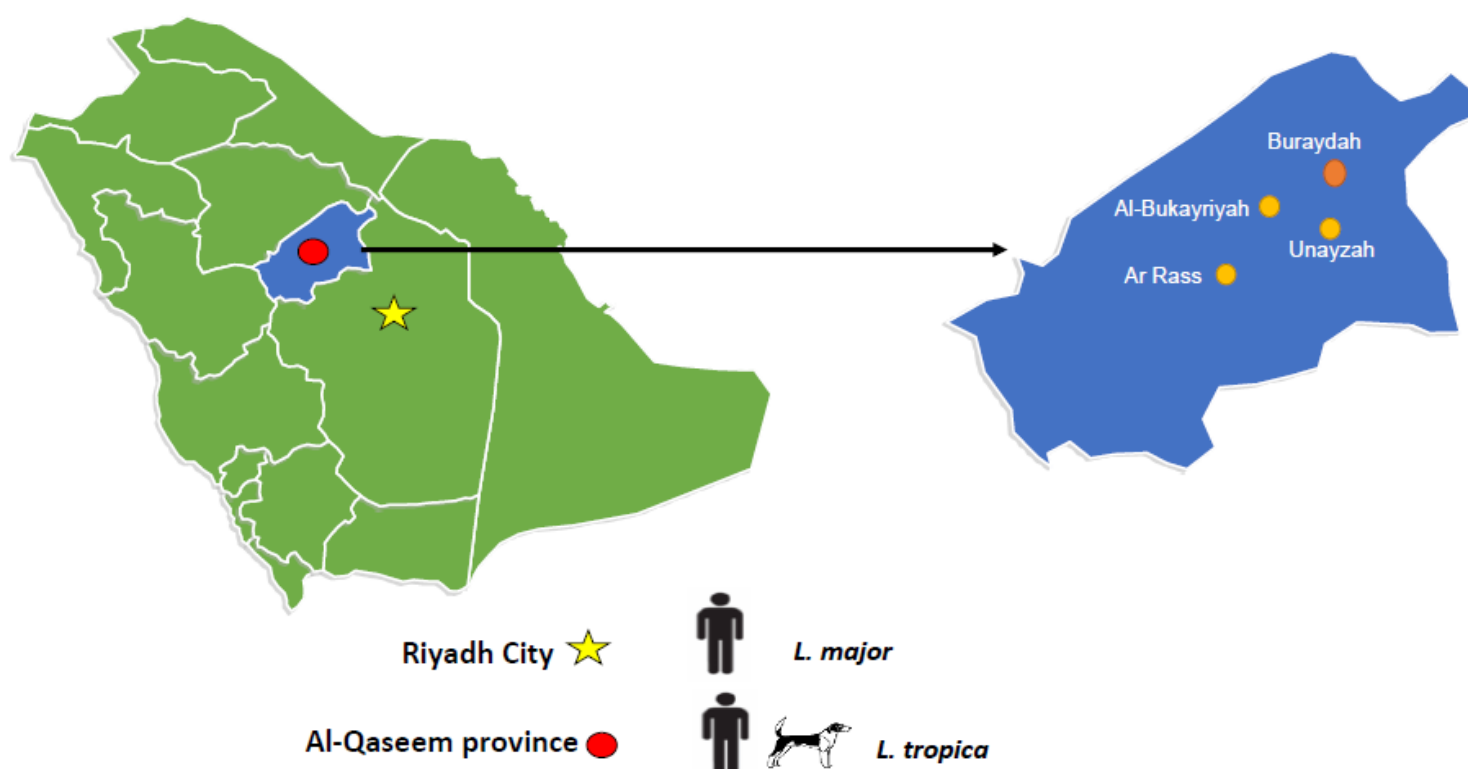
Figure 1

Map showing the location of the study areas in Saudi Arabia. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
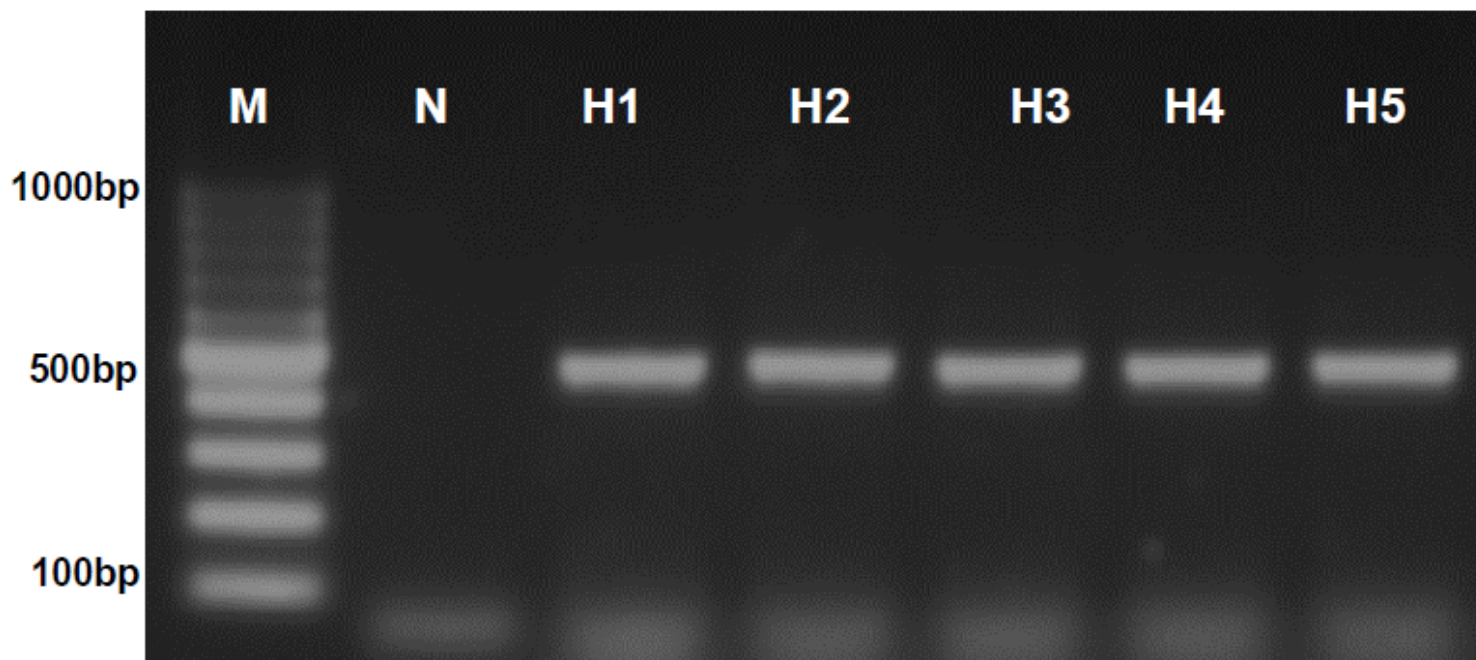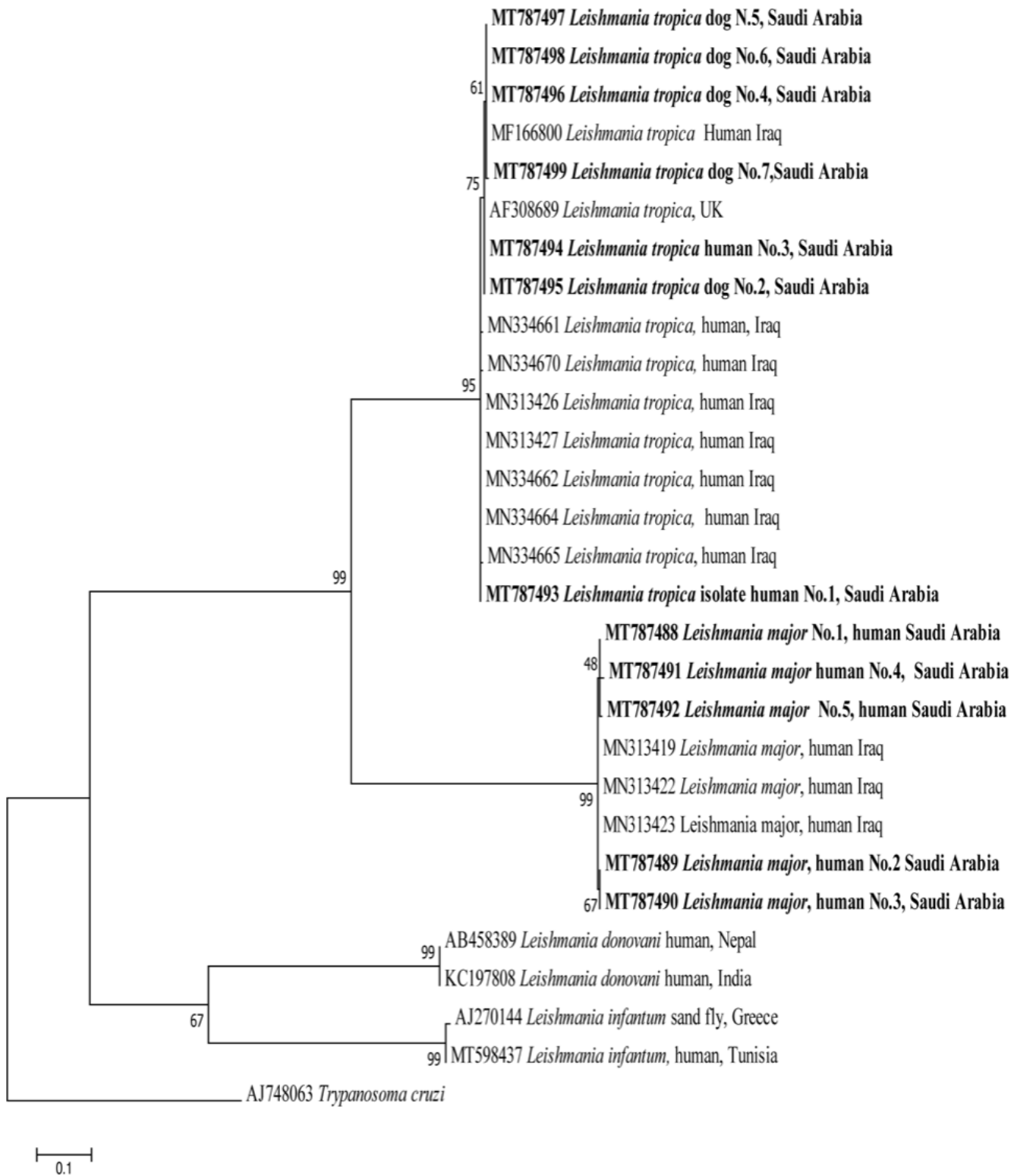
Figure 2

Agarose gel electrophoresis (1.5%) image shows the nPCR product analysis of kDNA in cutaneous L. major from human skin lesion samples from Riyadh city. Where M: marker (1000-100bp); lanes (H1 - H5): nPCR products (560bp) amplified from human samples
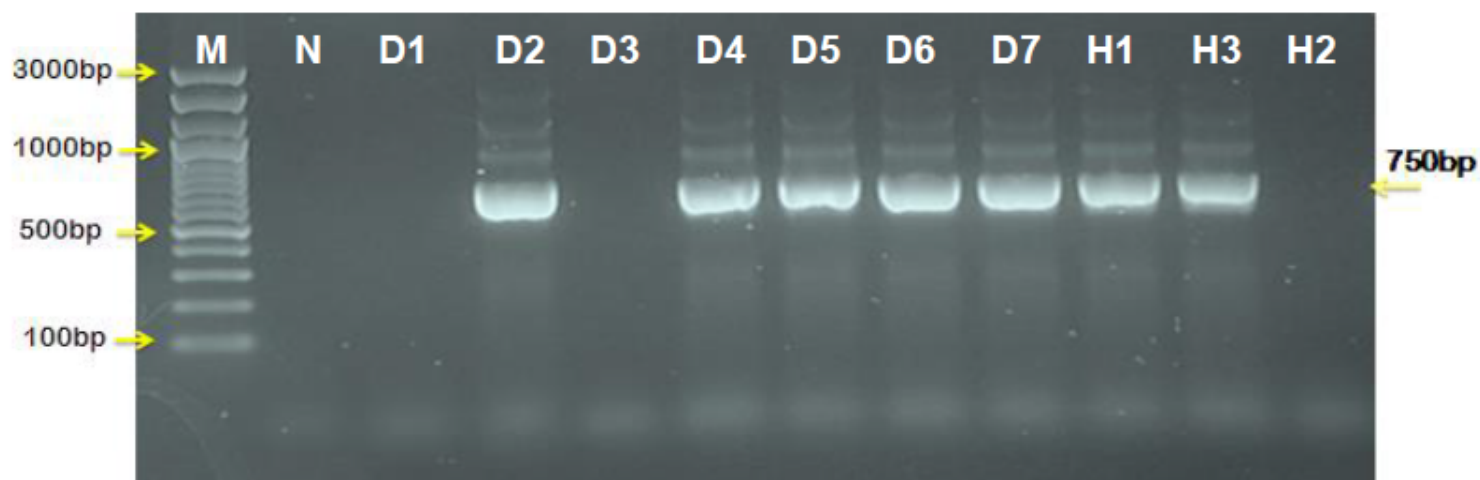
Figure 3

Agarose gel electrophoresis (1.5%) image shows the nPCR product analysis of kDNA in cutaneous L. tropica from dogs and human skin lesion samples from Al-Qaseem province. Where M: marker (3000-100bp); lanes (D2 and D4-D7) positive for dogs and lanes (H1 and H3) positive for human; nPCR products (750bp) amplified from human and dogs samples.

Figure 4

Maximum likelihood Kinetoplast DNA-based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlights the position of Leishmania spp. of the present study (bold) using 2000 bootstrap replicates and Trypanosoma cruzi as outgroup.

Supplementary Files

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- GraphicalAbstract.pdf