

Molecular detection and phylogenetic analysis of *Leishmania major* in stray dogs in Riyadh Province, Saudi Arabia

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Abstract. Dogs can act as a reservoir of canine leishmaniasis disease, which is caused by *Leishmania* species. The study aimed to identify and document the genotype of cutaneous leishmaniasis (CL) in the stray dogs in Riyadh Province using kinetoplast DNA (kDNA) as a target gene by using *nested* polymerase chain reaction (*nPCR*). This cross-sectional investigation was conducted over the course of two years, from March 2016 to July 2018, in different districts of Riyadh Province, Saudi Arabia. A total of 237 dogs were examined, only 18 of the dogs were suspected clinically of cutaneous leishmaniasis due to the presence of cutaneous nodules and cutaneous lesion. Biopsy tissue collections were performed and DNA was extracted. CSB2XF and CSB1XR primers were used to amplify the *Leishmania* kDNA regions. The *Leishmania* species were detected by specific 13Z and LIR primers by applying nested PCR assay. Nine dogs were found to be positive for *Leishmania major*. The examined dogs were negative for other *Leishmania* spp. The phylogenetic analysis and blast results of kDNA showed that the 9 isolates *L. major* is closely related (99.9%) to the *L. major* isolate CMG_irfan5, accession number HQ727556.1 from human, Pakistan. This is the first molecular study on dog leishmaniasis from Saudi Arabia confirmed that dogs have a *L. major* infection. Further epidemiological and molecular investigations are required to study domestic and wild canine infections with *L. major* and other *Leishmania* spp in endemic and nonendemic areas of Saudi Arabia as part of leishmaniasis control.

INTRODUCTION

Cutaneous leishmaniasis (CL) is caused by infection with protozoan parasites belonging to the genus *Leishmania* (Desjeux 2004). It is transmitted through the bite of infected sand flies species of genus *Phlebotomus* or *Lutzomyia* (Alvar *et al.*, 2012). The

disease was widespread and it was reported as one of the nine parasitic infections by The World Health Organization (WHO) in 2013 and is commonly seen in tropical and subtropical countries (Gonzalez *et al.*, 2008; WHO 2013). In the Old World, cutaneous leishmaniasis (CL) disease primarily is caused by *Leishmania tropica* in urban

areas and *Leishmania major* in dry desert areas (WHO 2010). Molecular and epidemiological studies in the Middle East showed that cutaneous leishmaniasis caused by *Leishmania tropica* and *Leishmania major* occur in Saudi Arabia (Amin *et al.*, 2013), Iran (Shirzadi *et al.*, 2015), Syria (Salloum *et al.*, 2016) and Iraq (Al-Bajalan *et al.*, 2018a). In Saudi Arabia, cutaneous leishmaniasis (CL) is endemic mainly in Al-Hassa Oasis, Al-Qassim province and in the rural areas around Riyadh city (Al-Tawfiq *et al.*, 2004; Amin *et al.*, 2013; Alanazi *et al.*, 2016) and over the past 10 years (2006–2016), the number of leishmaniasis infection among patients was more than 26,300 cases (Abuzaid *et al.*, 2017).

Previous studies demonstrated that dogs could be a victim or potential primary reservoirs for cutaneous leishmaniasis (Dantas-Torres *et al.*, 2006; Baneth *et al.*, 2016). However, cases of cutaneous and visceral infections caused by *L. tropica* were reported in Iran and Israel (Bamorovat *et al.*, 2014; Baneth *et al.*, 2014). Likewise, cases of cutaneous infection in *L. major* infected dogs have been reported in Saudi Arabia, Egypt and Iraq (Elbihari *et al.*, 1984; Morsy *et al.*, 1987; Al-Bajalan *et al.*, 2018b). In Saudi Arabia, stray dogs have been shown to suffer from clinical disease associated with infection with these

Leishmania species (Peters *et al.*, 1985; Elbihari *et al.*, 1987), but there is a lack of information on cutaneous leishmaniasis particularly on molecular levels. Since dogs can be infected by sand flies in endemic areas, infected stray dogs may be a mobile risk for nonendemic areas (Elbihari *et al.*, 1987; Silva *et al.*, 2017). Therefore, the aim of this study was to identify the genotype of CL in the dogs in Riyadh Province using kinetoplast DNA (kDNA) gene amplification by nested polymerase chain reaction (nPCR) and sequencing.

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 under the number (SH 02-2016).

Study areas

This investigation was conducted over the course of two years, from March 2016 to July 2018 in the Riyadh Province, which is in the central part of Saudi Arabia between 24°38'N and 46°43'E (Fig. 1). Culling of stray dogs is common in Saudi Arabia, with health authorities euthanising over 13,000

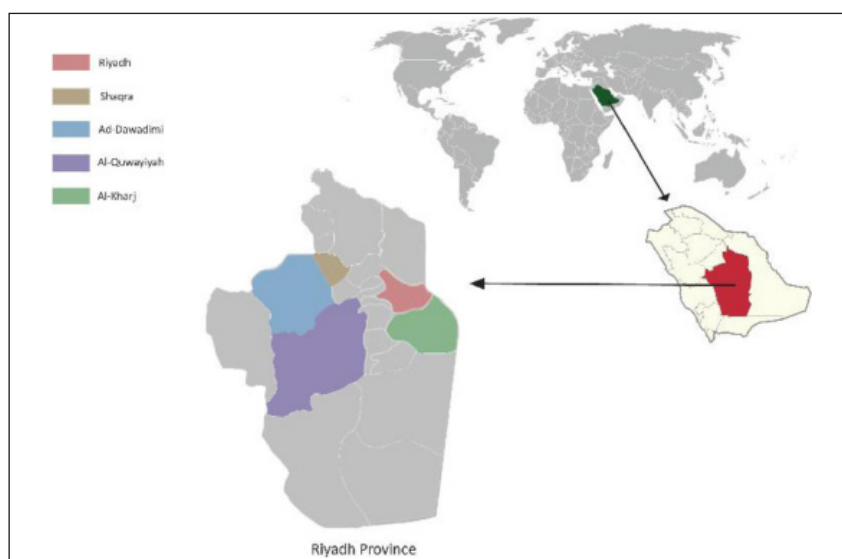


Figure 1. Map showing the study sites within Riyadh Province, Saudi Arabia.

dogs between 2010-2015, while in 2016 the Riyadh municipality alone euthanised 3,486 stray dogs (Al-Dossary, 2016). However, stray dog's population was trapped by live bait straps or by shooting (Bamorovat *et al.*, 2014). A field examination was set up at each location to examine the dogs. Additionally, dogs at veterinary clinics or pre-euthanasia at the municipal from those areas were included.

Biopsy tissue collections and parasites cultures

Out of 237 dogs, only 18 dogs were suspected for CL and diagnosed clinically by cutaneous nodules or ulcerated lesions present on skin. Skin biopsies of diameter 5 mm were taken under sterile conditions from the border of the ulcer, inoculated into medium M199 supplemented (Gibco, Life technologies, Germany) with 25 mmol/L HEPES (pH:7.5) and 20% fetal bovine serum (Gibco, Germany) followed by incubation (24°C). Ten days later, parasites were harvested, washed with ice-cold phosphate buffered saline (PBS, pH: 7.4) and stored in -20°C before DNA isolation.

Genomic DNA extraction

gDNA from parasite cultures and lesion biopsies was isolated by overnight lysis in NET buffer containing Proteinase K (Sigma) and 1% sodium dodecyl sulphate as described previously (Salotra *et al.*, 2001). After that, the extracted gDNA was checked by Nanodrop spectrophotometer (Thermo, USA), and then an aliquot (100 µl of gDNA from each sample) was stored at -20°C prior to sending to the Molecular laboratory, Faculty of Science, Waist University, Iraq, for nPCR amplification and analysis.

Nested PCR

Kinetoplast DNA (kDNA) of *Leishmania* was a target for Nested PCR using external primers CSB2XF (CGAGTAGCAGAACT CCCGTTCA) / CSB1XR (ATTTTTCGCGA TTTTCGAGAACG) and internal primers 13Z (ACTGGGGGTTGGTGAAAATAG) / LiR (TCGCAGAACGCC CT) for amplifying of 5750 bp PCR product for *L. tropica* and 5560 bp PCR product *L. major* respectively

(Noyes *et al.*, 1998). These primers were provided by Bioneer Company, Korea. The first round PCR master mix that include CSB2XF and CSB1XR were prepared by using AccuPower® PCR PreMix kit. (Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of Taq DNA polymerase 1U, dNTPs 250 µM, Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, MgCl₂ 1.5 mM, stabilizer, and tracking dye and the PCR master mix reaction was prepared according to kit instructions in 20 µl total volume by added 5 µl of purified genomic DNA and 1.5 µl of 10 pmole of forward primer and 1.5 µl of 10 pmole of reverse primer, then completed the PCR premix tube by deionizer PCR water into 20 µl and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Techne TC-3000. USA) by set up the following thermocycler 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 1 min, and extension 72°C for min and then final extension at 72°C for 7 min. The second round of nested PCR was including 13Z and LiR primers and the same PCR master mix except 3 µL of template PCR product. After that, PCR products were electrophoresed on a 1.5% agarose gel containing 1 µL/mL Syber safe (Thermo Scientific™, Nalgene, UK) in Tris-acetate-EDTA buffer at 100 V for 45 min and visualized under UV imaging system (Image Quant Laz4000, GE Healthcare Life Science, Hammersmith, UK). The size of each sample was estimated by comparison with (a 100 bp) DNA Ladder Marker (Bioneer company, Korea).

***Leishmania* kDNA sequencing and phylogenetic trees analysis**

To determine the *Leishmania* species, positive samples of *Leishmania* species were sent to Macrogen Europe (South Korea) for sequencing of the kDNA region, and the results were compared with the sequences available in GenBank database using BLAST (<http://blast.ncbi.nlm.nih.gov>). The phylogenetic analysis was performed based on NCBI-Blast alignment identification and maximum composite Likelihood

method by phylogenetic tree UPGMA method (MEGA 7.0 version). Bootstrap values were determined with 1,000 replicates of the data sets (Kumar *et al.*, 2016). The sequences were deposited in GenBank under accession numbers MH746268-MH746276.

RESULTS

Eighteen of 237 dogs (7.6%) had thick cutaneous lesions (1.5 × 3 centimeters) in areas such as mouth, nose, muzzle, abdomen

and between fingers. Ulceration in the left hind foot was noted in a two dogs. No other clinical sings were observed. The first PCR reaction using CSB2XF/CSB1XR primers showed that 9 of 18 samples were positive for genus *Leishmania* amplification bands at S750 bp (Fig. 2). Second PCR reaction product bands at S560 bp for the 9 samples confirmed that they were belong to *L. major* (Fig. 3). Analysis of the kDNA sequences further confirmed that they are all *L. major* and the sizes ranged from 467 bp to 475 bp. However, alignment revealed high level of conservation along with limited existence

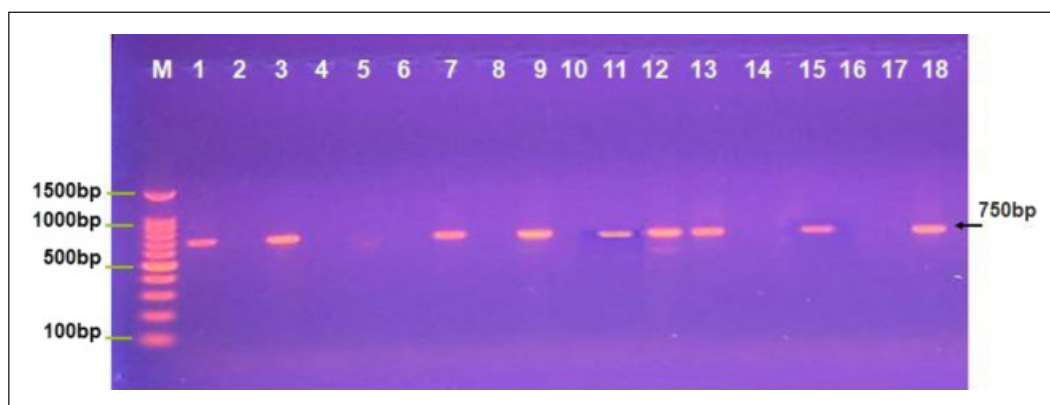


Figure 2. Agarose gel electrophoresis (1.5%) image that show the PCR product analysis of kDNA from dog samples using primers CSB2XF/CSB1XR, M: marker (100-1500 bp), Samples 1,3,7,9,11,12,13,15 and 18 showed positive with *Leishmania* spp. infection at (S750 bp) PCR product.

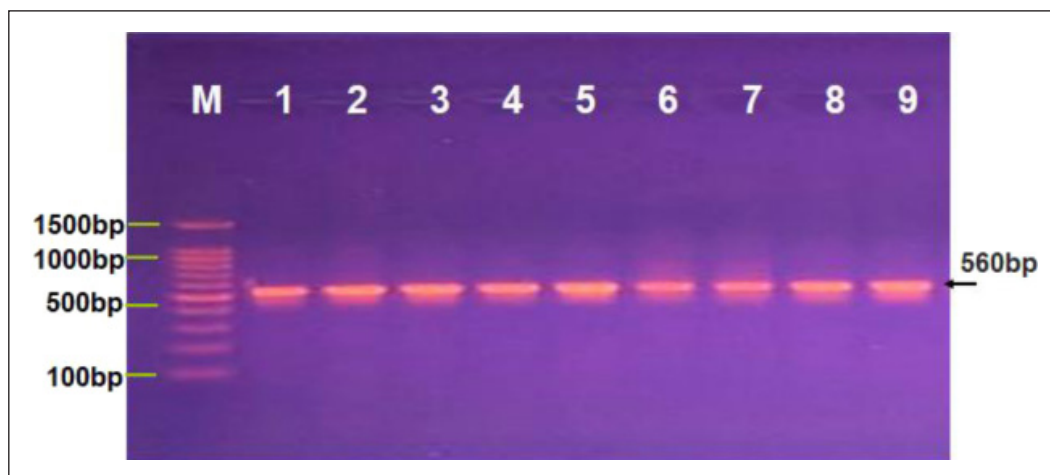


Figure 3. Agarose gel electrophoresis (1.5%) image that show the Nested PCR product analysis of kDNA from dog samples using primers 13Z/LiR, M: marker (100-1500bp), Lanes 1-9: positive infection with *L. major* at (S560 bp) PCR product.

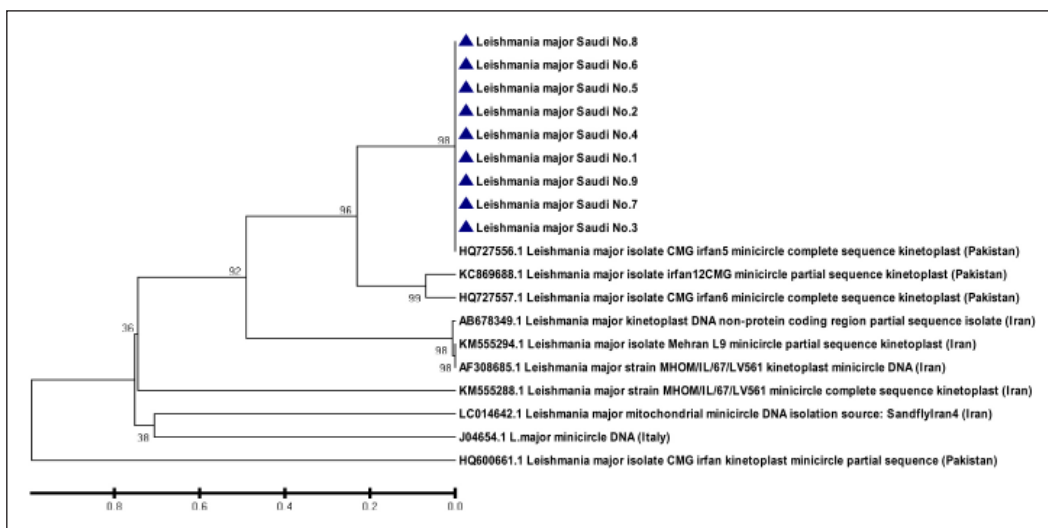


Figure 4. Phylogenetic tree analysis based on the partial sequence of mitochondrial kDNA in *L. major* Saudi dogs isolates that used genetic relationship analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method (MEGA 7.0 version). The local *L. major* isolates (No. 1 – No. 9) showed genetically closed related to NCBI-BLAST *L. major* Pakistan isolate (HQ727556.1), whereas other isolates showed different and out of tree.

of single point mutations. Phylogenetic analysis and blast results of kDNA showed that the 9 *L. major* isolates is closely related (99.9%) to the *L. major* isolate CMG_ irfan5, accession number HQ727556.1 from human, Pakistan (Fig. 4).

DISCUSSION

This study was the country's first extensive molecular and epidemiological study describing dog infections with *L. major* common agents of cutaneous leishmaniasis in Saudi Arabia and Middle Eastern countries (Shirzadi *et al.*, 2015; Al-Bajalan *et al.*, 2018a). However, the two previous Saudi studies that reported natural infections of *L. major* in dogs using enzymatic biochemical methods (Elbihari *et al.*, 1984; Peters *et al.*, 1985), but no clinical information was available, no serology was performed or molecular confirmation done as it was unavailable hence, no treatment has been reported.

The study showed cutaneous lesions are the common manifestation of

leishmaniasis in dogs, in agreement with previous studies in neighbouring countries (Morsy *et al.*, 1987; Tashakori *et al.*, 2003; Hajjaran *et al.*, 2013; Baneth *et al.*, 2017; Al-Bajalan *et al.*, 2018b). These dogs had cutaneous involvement in different areas of body, but the diagnosis of canine leishmaniasis based on clinical features is difficult, needing specific laboratory tests (Sabzevari *et al.*, 2016). In This study, we did not attempt histopathological and microscopic examinations due to the limitation of this study.

In the current study, electrophoresis data displayed a product size of approximately S750bp for CSB2XF/CSB1XR and S560 bp for 13Z/LiR primers pairs regions of the kDNA of *Leishmania* spp. and of the *L. tropica* and *L. major* respectively. These primers were used successfully in differential molecular diagnosis of *Leishmania* species causing cutaneous leishmaniasis (Noyes *et al.*, 1998; Anders *et al.*, 2002; Feizhaddad *et al.*, 2016). Targeting a region of kDNA mini circles appeared effective for detecting *Leishmania* spp. because of high copy

numbers and variability in amplicon size of *Leishmania* parasite DNA (Bensoussan *et al.*, 2006; Mohammadi *et al.*, 2011).

Our results showed that the adult dogs more infected more than younger dogs. Other studies reported dogs under a year old being infected with *L. major* (Baneth *et al.*, 2017; Al-Bajalan *et al.*, 2018b) and this could be attributed to several factors such as: dog migration or travelling to regions where flies are common, body condition leading to weak immunity and fly preference.

In Saudi Arabia, *Phlebotomus* flies have been considered as a vector of *L. major* (Killick-Kendrick, 1990; Mustafa *et al.*, 1994; El-Beshbishy *et al.*, 2013; Haouas *et al.*, 2017). Studies in neighboring countries showed that *L. major* spread through *Phlebotomus* species and dogs, acts as a reservoir (Sawalha *et al.*, 2003; Colacicco-Mayhugh *et al.*, 2011; Yaghoobi-Ershadi 2012; Sasani *et al.*, 2016). However, in Saudi Arabia, the role of other *Phlebotomus* species in CL transmission is still unknown and more molecular surveys are required to clarify their acts. Nevertheless, CL in dogs is veterinary importance, infected dogs serve as parasite reservoirs and contribute significantly to human transmission of CL in Middle East countries including Saudi Arabia. Therefore, it is necessary to prevent CL by controlling animal reservoirs and sand fly vectors and taking steps to avoid exposure to sandflies bites.

CONCLUSION

In this study, the cutaneous leishmaniasis in dogs caused by *L. major* was found in the rural areas of Riyadh province. The relationship between the disease vectors and reservoirs with transmission cycle in this area is still unknown. However, this kind of information is required for implementation of future cutaneous leishmaniasis control programs.

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AUTHORS' CONTRIBUTIONS

ADA and AAR designed the study. MSA coordinated the study design and the related activities. IOA, YAA, ASA and ADA collected dog samples from different districts of Riyadh Province, Saudi Arabia. ADA, ASA and IOA carried out the biopsy tissue collections and DNA extraction. MAA, AAR and ADA carried out the laboratory investigations of all the experiments and phylogenetic analysis. ADA, AAR, MSM and SA prepared and revised the manuscript. All authors read and approved the final manuscript.

DISCLOSURE

The authors declare that they have no competing interests.

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