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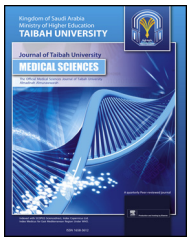

Original Article

Molecular identification of *Leishmania major* species in phlebotomine sand flies from Al Ahsa, Eastern KSA

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الملخص

أهداف البحث: يعتبر داء الليشمانيات الجلدي من المخاطر التي تهدد الصحة العامة في المملكة العربية السعودية. العديد من المناطق السعودية مستوطنة للغاية بما في ذلك مدينة الأحساء، شرق المملكة العربية السعودية. لا يوجد توثيق جزيئي سابق لطفيل الليشمانيا في النواقل في الأحساء. هدفت هذه الدراسة إلى التعرف الجزيئي على أنواع الليشمانيا من ذباب الرمل الذي تم جمعه من الأحساء.

طريقة البحث: أجريت الدراسة المقطعية الحالية في الأحساء من يوليو 2020 إلى مايو 2021. تم جمع ذبابة الرمل من 3 مناطق بها أعلى نسبة إصابة بداء الليشمانيات الجلدي وذباب الرمل وفقا لبيانات مركز الوقاية من الأمراض المنقولة بالنواقل في الأحساء. تم استخدام مصيدة الضوء المصغرة سي دي سي والمصائد اللاصقة لجمع ذباب الرمل. تم فرز إناث ذباب الرمل الفليبوتومين وفصلها لمزيد من التحليل. تم استخدام أحواض من 3-10 إناث من ذباب الرمل لاستخراج الحمض النووي الجيني. تم تضخيم الحمض النووي المستخرج باستخدام بروتوكول يستهدف جنس الليشمانيا والبادئات الخاصة بالأنواع. بالنسبة لانتواع الليشمانيا، تم استخدام طريقة تعدد الأشكال بعد تفاعل البوليميراز المتسلسل.

النتائج: تم تضمين 113 بركة من ذباب الرمل في الدراسة. من بين 113 مجموعة من العينات، كانت 10 منها إيجابية لجنس الليشمانيا بعد بروتوكولنا التجريبي. أدى توصيف أنواع الليشمانيا عن طريق تعدد الأشكال في تفاعل البوليميراز المتسلسل، وتعدد الأشكال، إلى جعل الليشمانيا الرئيسية هي النوع الوحيد الموجود في ذباب الرمل الذي تم جمعه.

الاستنتاجات: هذا هو أول توثيق جزيئي لأنواع الليشمانيا في ذباب الرمل الفليبوتومين في منطقة الأحساء. كانت الليشمانيا الكبرى هي النوع الوحيد الموجود في دراستنا. مزيد من البحث الشامل حول الناقل والخزانات أمر حيوي لإنشاء ديناميكيات انتقال الليشمانيا في الأحساء. تساعد هذه المعلومات في التخطيط الاستراتيجي لأساليب الوقاية والسيطرة على أنواع الليشمانيا في المنطقة.

الكلمات المفتاحية: الأحساء؛ تفاعل البوليميراز المتسلسل فاصل نسخ داخلي 1؛ الليشمانيا الكبرى؛ الجزيئية؛ ذبابة الرمل الفليبوتومين؛ المملكة العربية السعودية

Abstract

Objectives: Cutaneous leishmaniasis (CL) is considered an overlooked public health threat in KSA. CL is endemic to several Saudi regions, including Al Ahsa City, Eastern KSA. To our knowledge, no prior molecular identification of *Leishmania* parasites in vectors in Al Ahsa has been published. The aim of this study was to perform molecular identification of *Leishmania* species in sand flies collected from Al Ahsa.

Methods: This cross-sectional study was conducted in Al Ahsa from July 2020 to May 2021. Sand flies were collected from the three areas with the highest rates of CL cases and sand flies, according to data from the Vector Borne Diseases Prevention Center in Al Ahsa. CDC miniature light traps and sticky traps were used to collect sand flies. Pools of 3–10 female sand flies were subjected to genomic DNA extraction. The extracted DNA was then amplified with a protocol targeting the *Leishmania* genus and using species-specific primers. For *Leishmania* species identification, a PCR-restriction fragment length polymorphism (PCR-RFLP) method was used.

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Results: Ten of 113 pools of samples tested positive for the *Leishmania* genus, according to our experimental protocol. Characterization of *Leishmania* species by PCR-RFLP established *Leishmania major* as the only species found in the collected sand flies.

Conclusion: This is the first documentation of molecular identification of *Leishmania* species in phlebotomine sand flies in the Al Ahsa region. *L. major* was the only species identified in our study. Further comprehensive research investigating the vectors and reservoirs will be crucial to establish the dynamics of transmission of *Leishmania* in Al Ahsa.

Keywords: Al Ahsa; ITS1 PCR; KSA; *Leishmania major*; Molecular; Phlebotomine sand fly

Introduction

Leishmaniasis is a protozoan disease borne by flies.[1] Phlebotomine species (*Diptera*, Psychodidae) are the principal vectors of old world cutaneous leishmaniasis worldwide.[1,2] These hematophagous vectors are involved in the transmission of multiple microorganisms, such as protozoa (*Leishmania* species), bacteria (*Bartonella bacilliformis*), and viruses (Rhabdoviridae, Bunyaviridae, and Reoviridae). Phlebotomine sand flies have a broad host range, including humans and animals, and are of critical importance for the health of both civilians and military personnel.[3]

According to clinical presentation, leishmaniasis disease is classified into cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML), and visceral leishmaniasis (VL).[4] More than 20 *Leishmania* species have been reported as infectious for humans.[5] Nine countries have reported more than 5000 CL cases.[6] In the Middle East, KSA is the country with the fourth highest number of endemic CL cases.[7] Several Saudi provinces are considered CL endemic areas, including Al-Madinah Al-Munawwarah, Al Qassim, and Al-Ahsa oasis.[8] Multiple surveys in KSA have defined the prevalence and geographical distribution of phlebotomine species.[9–13] A total of 25 species have been reported in KSA, and *Phlebotomus papatasi* has been found to predominate in all explored regions of the country.[9–13]

Despite the endemicity and prevalence of leishmaniasis in KSA, the molecular profiles of *Leishmania* species in vectors have remained inadequately studied. Determining the genetic diversity of *Leishmania* species in vectors would aid in understanding *Leishmania* transmission dynamics in KSA and developing high-quality preventative measures. To our knowledge, no previous structured molecular studies have investigated *Leishmania* species in vectors in Al Ahsa.

The objective of this study was to molecularly determine the *Leishmania* species in potential sand fly vectors.

Materials and Methods

Study design

This cross-sectional study was performed in collaboration with the Vector Borne Diseases Prevention Center in Al Ahsa (coordinates, 25°2300″N 49°36′00″E), in the Eastern Province of KSA.

Sand fly collection

Sand flies were collected from July 2020 to May 2021. Eastern and southern towns of Al Ahsa (South Hofuf, Al Jarn, and Al Munaizelah) were the targeted collection areas. These areas are considered to have the highest rate of sand flies and cases of CL, according to data from the Vector Borne Diseases Prevention Center in Al Ahsa.

CDC miniature light traps (John W. Hock Company, Florida, USA) and sticky traps were used to collect sand fly vectors from vector habitats, both indoors (such as animal houses and farms) and outdoors (such as yards, rodent burrows, stones, and wall crevices).

Processing of collected sand flies

Collected sand flies were sorted from mosquitoes and identified at the genus level.[14] The head, posterior abdominal part, and wings of female sand flies were separated, and the rest of the bodies were kept frozen at −20 °C for molecular studies.[11,13,14]

DNA extraction and PCR-based genotyping assays

DNA extraction

Commercial genomic DNA extraction tissue kits (QIAamp Fast DNA Tissue Kit, Qiagen, Germany) were used to extract genomic DNA from collected female sand flies, according to the manufacturer's instructions. Pools of 3–10 female sand flies were used for DNA extraction, according to the number of sand flies collected from each region.

DNA amplification and post-PCR assays

Extracted DNA was amplified with PCR targeting the *Leishmania* genus and species-specific primers, with Internal Transcribed Spacer 1 (ITS1) PCR assays and ITS1 nested PCR (ITS1 nPCR) assays.[14–17] ITS1 PCR was conducted with one set of primer pairs comprising LITS and L5.8S targeting ITS1. The ITS1 nPCR was conducted with two sets of two pairs of primers: the primer pair R221 and R332 targeting small subunit ribosomal RNA (SSU rRNA) in the first PCR reaction, and the nested primer pair LITS and L5.8S targeting ITS1 in the second reaction (Table 1).[8,16] Figure 1 shows the PCR protocol.

Genotyping of Leishmania *species*

The PCR-restriction fragment length polymorphism (PCR-RFLP) method was used for *Leishmania* species identification.[16] A 10 μL volume of each positive ITS PCR/n PCR amplified product was digested with 1 U of the restriction enzyme *HaeIII* (Molequle-On, New Zealand) at

Table 1: Primers, primer targets, names, amplicon sizes, and thermal cycling conditions for the PCR reactions.

PCR target	Primer (sequence)	Size of amplicon	Thermal conditions	Reference
ITS1	LITSR: 5′- CTGGATCATTTTCCGATG-3′ L5.8S: 5′ TGATACCACTTATCGCACTT-3′	320 BP	95 °C (2 min), [95 °C (30 s), 53 °C (40 s),72 °C (1 min) for 34 cycles], 72 °C (7 min)	[16]
SSU rRNA	R221: 5′- GGTTCCTTTCCTGATTTACG-3′ R332: 5′-GGCCGGTAAAGGCCGAATAG-3′	603 BP	94 °C (3 min), [94 °C (1 min), 56 °C (1 min s),72 °C (2 min) for 37 cycles], 72 °C (6 min)	[16]

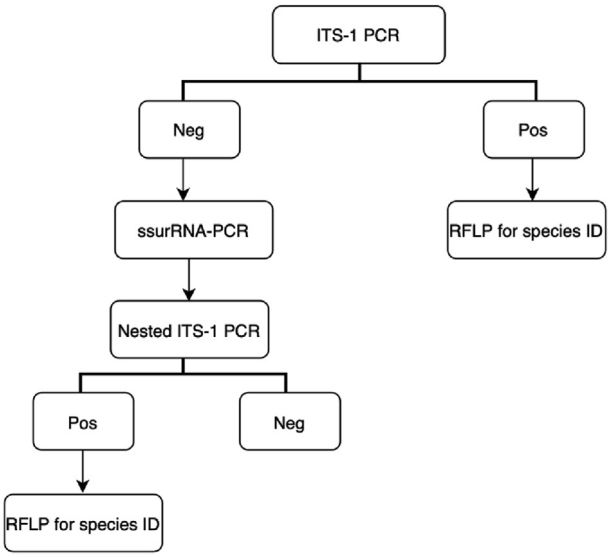

Figure 1: PCR/nPCR protocol for detection and characterization of *Leishmania* species from sand flies.

37 °C for 15 min, according to the manufacturer's recommendations. The RFLP products were examined on 4% (MetaPhor) agarose gel and visualized under ultraviolet light.

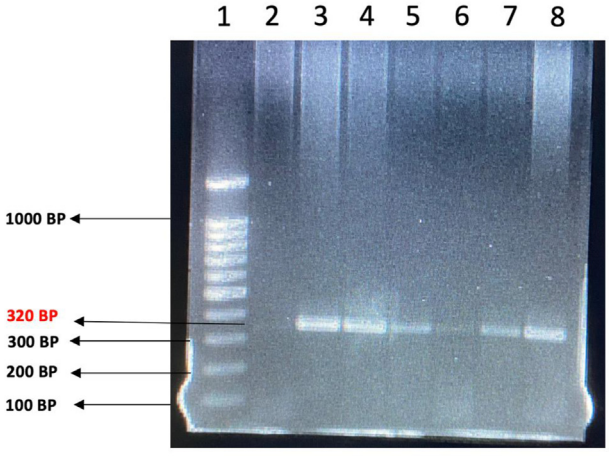

Figure 2: Agarose gel (1.5%) electrophoresis of the ITS1-PCR amplified products of the *Leishmania* genus in collected phlebotomine sand flies. Lane 1: 100 bp ladder. Lane 2: negative control. Lane 3: positive control for ITS1 PCR for the *Leishmania* genus (320 bp). Lanes 4–8: positive samples for ITS1 PCR for the *Leishmania* genus (320 bp).

Results

Sand fly characteristics

Phlebotomus was the only documented phlebotomine sand fly genus in the studied areas of Al Ahsa. A total of 1117 female phlebotomine sand flies were collected from three main towns in the province of Al Ahsa and were grouped into 113 pools. The positive detection rates of *Leishmania* species from the sand flies collected from the three areas was as follows: Al Jarn (5 positive pools out of 10) > Al Munaizelah (3 positive pools out of 10) > South Hofuf (2 positive pools out of 10).

Of the 113 female *Phlebotomus* pools, 10 were positive for the *Leishmania* genus, on the basis of ITS1 PCR/or nPCR with our experimental protocol (Figure 1). Figure 2 shows the PCR amplified products of ITS1 PCR/nPCR for the *Leishmania* genus in the collected phlebotomine sand flies.

Identification of Leishmania *species through RFLP analysis*

L. major was the only *Leishmania* species identified in all 10 samples PCR positive for the genus *Leishmania* (Figure 3).

Discussion

More CL cases have been suggested to be found in rural rather than urban areas,[18] owing to the vector abundance in the former. In our study, we collected sand flies from three main areas comprising two rural areas (Al Jarn and Al Munaizelah) and one urban area (South Hofuf). Sand fly densities were higher in the rural areas. *Phlebotomus* was the only detected phlebotomine sand fly genus in the surveyed areas in Al Ahsa and was the only vector responsible for cases of CL in these three areas of the Al Ahsa region.

Leishmania species identification in sand flies has historically been performed through microscopic examination of freshly collected sand flies. This approach is time consuming, and is limited by a very low rate of identification of *Leishmania* species in examined sand flies (0.01–1%), and low sensitivity and specificity, even in endemic areas.[19] Furthermore, examining sand flies requires expertise and considerable skill.[13] Highly sensitive molecular identification was used to replace the entomological protocol.

P. papatasi is the only reported species transmitting *L. major* in KSA including the Al Ahsa region.[14,20] In our

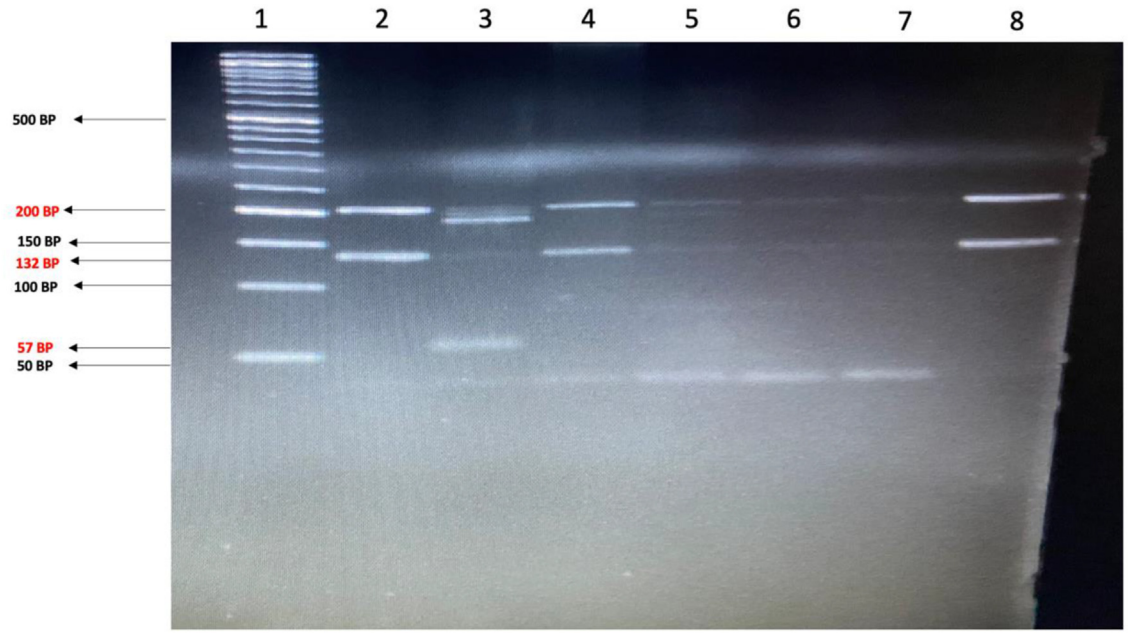

Figure 3: MetaPhor gel (4%) electrophoresis characterization of *Leishmania* species in sand flies with ITS1 PCR and restriction enzyme (*HaeIII*) analysis. Lane 1: 50 bp DNA ladder. Lane 2: *L. major* reference strain (203 and 132 bp). Lane 3: *L. tropica* reference strain (200 and 57 bp). Lanes 4–8: *L. major* samples.

study, we applied a protocol combining ITS1 PCR and ITS1 nPCR for samples with initial negative results for the collected female *Phlebotomus* sand flies (Figure 1).[8,16] Other protocols have been used for identification of *Leishmania* species in collected sand flies. El-Beshbishy et al. have used a semi-nested kinetoplast DNA PCR followed by ITS1 PCR.[14] Another study has reported the use of small subunit ribosomal DNA (SSU rDNA) gene analysis followed by the use of primers targeting the coding DNA sequences of the putative translation initiation factor for the alpha subunit gene in positive samples.[21]

L. major was the only species identified in our samples through RFLP analysis. To our knowledge, this is the first study using molecular analysis to investigate the *Leishmania* species in sand flies in the Al Ahsa region. Different distributions of *Leishmania* species have been documented in other areas and regions. El-Beshbishy et al. reported both *Leishmania tropica* and *L. major* in freshly collected sand fly vectors in western KSA (Al-Madinah Al-Munawarah).[14] Moreover, Haouas et al. reported *Leishmania* DNA in sand flies in Northwestern KSA but did not identify the species.[21]

This work is a continuation of our recently published study on identifying *Leishmania* species from CL skin lesions in Al Ahsa.[8] We have reported identification of *L. tropica* in three patients with CL lesions without any travel history outside the Al Ahsa region in the 6 months before clinical presentation.[8] In the current study, we did not detect *L. tropica* in any collected sand flies from Al-Ahsa, possibly because we included only a portion of the areas (three towns) where CL cases are prevalent. This main limitation of our study was due to limited financial resources and personnel available to collect sand flies from remote areas. On the basis of prior epidemiological investigation, we concluded that all *L. tropica* patients were from remote rural areas from which we did not collect sand fly samples. Our work thus provides a first step toward a comprehensive investigations to identify the genotypes of *Leishmania* in the Al Ahsa region.

The density of *P. papatasi*, a proven *L. major* transmitter in the Eastern Province, was studied sporadically in central, southern, and western areas of KSA. *P. papatasi* abundance has been observed during the summer and found to peak in August to September; *P. papatasi* occurrence was higher in intra-domiciliary than extra-domiciliary locations.[22–24]

Conclusion

To our knowledge, this is the first molecular detection of *Leishmania* species in phlebotomine sand flies in the Al Ahsa region. *L. major* was the only species identified in our study. Further comprehensive research on vectors and reservoirs is crucial for establishing the dynamics of transmission of *Leishmania* in Al Ahsa.

Source of funding

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The study was ethically approved by the Institutional Review Board (IRB) Committee of the Deanship of Scientific

Research at Imam Abdulrahman Bin Faisal University (IRB number: PGS-2020−01-427: 30-12-2020 and PGS-2020−01-186: 24-06-2020) and IRB Committee of the Ministry of Health, KSA (KFHH RCA number 08−25-2020: 25-08-2020).

Authors contributions

AAR, RA, and AAE conceived and designed the study, and wrote the first manuscript. AA collected sand flies, and AAR, AA, and AAE examined and reviewed the collected sand flies. SA, AAR, and AAE conducted the molecular assays and provided research materials. AAR, SA, RA, and AAE collected, organized, analyzed, and interpreted the data and reported the results. AAE and RA reviewed the manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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