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Molecular detection of *Leishmania* species in Sand Flies by PCR-RFLP technique in refugee camps

[*Detecção molecular de espécies de Leishmania em flebotomíneos por meio da técnica PCR-RFLP em campos de refugiados*]

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ABSTRACT

Leishmaniasis is one of the most important health dilemmas facing the World Health Organization (WHO), due to it being widespread and the great diversity of sand flies that transmit it. This study aimed to detect the presence of *Leishmania* parasites in the sand flies spread in Refugee camps by PCR- RLFP technique. A total of 437 sandflies were collected and classified into two species *Phlebotomus papatasi* and *Phlebotomus sergenti*. DNA was extracted from the female fly species, then the PCR reaction was amplified by two primers (LITSR, L5.8S) that transcribed a partial internal transcribed spacer (ITS)-1 gene for *Leishmania* parasite with a length of 320 bp. PCR showed the presence of *Leishmania* DNA in females of both *P. papatasi* (10%) and *P. sergenti* (20%). To determine *Leishmania* species transmitted by the two previous fly species, the RFLP-PCR technique was performed by the HaeIII enzyme for *Leishmania* DNA extracted from them. RFLP-PCR showed that *P. papatasi* females transmitted *Leishmania major* and *P. sergenti* females transmitted *Leishmania tropica* in Refugee camps. It could be concluded that leishmaniasis is widely distributed in Refugee camps due to the presence of its vector.

Keywords: HaeIII enzyme, *P. papatasi*, *P. sergentii*, RFLP-PCR, *Leishmania*

RESUMO

A leishmaniose é um dos mais importantes dilemas de saúde enfrentados pela Organização Mundial da Saúde (OMS) devido à sua ampla disseminação e à grande diversidade de flebotomíneos que a transmitem. Este estudo teve como objetivo detectar a presença de parasitas de Leishmania nos flebotomíneos espalhados em campos de refugiados por meio da técnica PCR-RLFP. Um total de 437 flebotomíneos foi coletado e classificado em duas espécies: Phlebotomus papatasi *e* Phlebotomus sergenti. *O DNA foi extraído das espécies de flebotomíneos das fêmeas e, em seguida, a reação de PCR foi amplificada por dois primers (LITSR, L5.8S) que transcreveram um gene parcial do espaçador transcrito interno (ITS)-1 para o parasita* Leishmania *com um comprimento de 320 pb. A PCR mostrou a presença de DNA de Leishmania em fêmeas de* P. papatasi *(10%) e* P. sergenti *(20%). Para determinar as espécies de* Leishmania *transmitidas pelas duas espécies de moscas anteriores, a técnica de RFLP-PCR foi realizada pela enzima HaeIII para o DNA de* Leishmania *extraído delas. A RFLP-PCR mostrou que as fêmeas de* P. papatasi *transmitiam* Leishmania *major e as fêmeas de* P. sergenti *transmitiam* Leishmania *tropica nos campos de refugiados. Pode-se concluir que a leishmaniose está amplamente distribuída nos campos de refugiados devido à presença de seu vetor.*

Palavras-chave: enzima HaeIII, P. papatasi, P. sergentii, *RFLP-PCR,* Leishmania

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INTRODUCTION

Phlebotomy belonging to family (Psychodidae) is of the order Diptera. It is one of the most important medical insects, as its females form a group of vectors of many pathogens such as protozoa, bacteria, and viruses (Defuentes *et al*., 2005; Mariwan *et al*., 2021; Manseur *et al*., 2022). *Leishmania* parasites that cause leishmaniasis are transmitted by phlebotomy (Cecílio *et al*., 2022; Usman *et al*., 2023). Leishmaniasis is one of the most common infectious diseases globally and exists in multiple forms, including cutaneous leishmaniasis (Al-Rashed *et al*., 2022; Vries and Schallig, 2022), visceral leishmaniasis (Saini *et al*., 2020; Scarpini *et al*., 2022) and mucocutaneous leishmaniasis (Handler *et al*., 2015; Severino *et al*., 2022). This disease spreads in four continents Asia, Africa, Europe, and America. The World Health Organization (WHO) estimated that the number of new infections annually ranges between 700,000 to one million infections globally (Leishmaniose, 2022) due to the great diversity of vectors that transmit its parasites. Many methods have been used to detect the presence of *Leishmania* in living organisms, whether hosts or vectors. The most accurate techniques are polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) (Monroy-Ostria *et al*., 2014; Echchakery *et al*., 2017). So, this study aimed to classify *Phlebotomus* species that spread in refugee camps in Raqqa (Syria). Molecular identification of *Leishmania* species was also detected in female *Phlebotomus* species by PCR-RFLP technique.

MATERIALS AND METHODS

Insect collection. Sand flies were collected in the refugee camps in Raqqa (geographical location E ºº35.9399, Nº38.5887) using sticky paper traps (white papers A4 size greased with castor oil). Flies were identified at both morphological and morphometric levels according to the local taxonomic key of Lewis (1982).

DNA extraction. Total DNA (*Phlebotomus* DNA and DNA organisms inside them) was extracted from 100 female insects. The 50 females of *P. sergenti* were divided into 10 groups, each group included 5 females, and similarly, 50 females of *P. papatasi* were divided into 10 groups, each group included 5 females. Each group was placed in an Ependorf tube, then frozen, and extracted using the QIAamp DNA Mini Kit (Qiagen, Germany).

Polymerase chain reaction (PCR). The PCR mixture was prepared within a volume of 25µL, including 12.5µL reaction mixture (0.2mm dNTPs and 1.5Mm $MgCl_2$ 1.25U Taq Polymerase, 1× PCR Buffer), 1µL of the forward primer (L5.8S: 5´-TGA TAC CAC TTA TCG CAC TT-3´), 1µL of the reverse primer (LITSR: 5´-CTG GAT CAT TTT TCC GAT G-3´), 4µL of the DNA sample, and then complete the mixture with 6.5.µL distilled water for the final volume of 25µL. The amplification reaction was carried out in the PEQ Lab thermal circulator according to the following program: initial denaturation at 94°C for 5 min, then 30 cycles consisting of 94 °C for 30 sec, primers coalesced at 53°C for 40 sec and copied at 72 °C for 1 min. Finally, a copy was performed at 72°C for 10 min (Schonian *et al*., 2003).

Gel electrophoresis. The PCR products were separated by electrophoresis on the Agarose gel at a concentration of 2% by a Mupid-One electrophoresis device (Takara company). Gel was prepared by dissolving 2gm of Agarose powder in 100mL electrophoresis solution 1×TBE (Tris-Borate-EDTA buffer) and warmed using a microwave, then cooled to about 50°C and added 5µL of ethidium bromide at a concentration of 0.5mg/mL, after that poured into gel composition mold until solidification, where placed in an electrophoresis tank containing 1000mL of 1×TBE electrophoresis solution. Loading the samples: First, 3µL of Loading buffer solution was mixed with 7 µL of PCR products, and then loaded into the gel wells. Similarly, 5µL of the marker was mixed with Loading buffer solution and loaded in the 1st well. Finally, electrophoresis was performed at a potential difference of 100 volts for 30 minutes. Gel was examined by UV in a gel documentation device, a digital image was taken for the gel to analyze the results.

Restriction fragment length polymorphism (RFLP). Enzymatic restriction of PCR products was carried out according to the following steps: (1) 15µL of DNA amplified by PCR technique was placed in the Eppendorf tube. (2) 10 units of the restriction enzyme (HaeIII) were added. (3)

5µL of the restriction enzyme protective solution. (4) Incubate previous mixture at a temperature of 37°C for 2 hr. (5) Transfer the mixture to the electrophoresis device to show the results.

RESULTS

Identification of sand flies collected from refugee camps. A total of 437 sand flies were classified according to local taxonomic key in two species, *Phlebotomus papatasi* and *Phlebotomus sergenti*. *P. papatasi* was more prevalent than *P. sergenti* (Table 1).

Phlebotomus were characterized by their small size, as length of its members ranged between (2-4) mm and their yellow-brown color. Its body was covered with dense hairs. The wings are placed during rest diagonally at an angle of 45 degrees upwards, thus taking the form of a butterfly, and the head is located perpendicular to the axis of the body, elongated in shape, and covered with dense hairs (Figure 1 A, B). The most important taxonomic characteristic of males *P. papatasi* is back end, which was characterized by the presence of the distinctive cylindrical shape and equipped with five spoon-shaped spines, three of which are final (located at the end of the pen) and two basal (Figure 1 C). The most important feature that distinguishes females of *P. papatasi* is the sperm preservative, which consisted of ten rings, the last of which was the largest and carried many poems (Figure 1 D).

Table 1. Species of sand flies collected from refugee camps

Species	Sandflies		Total	Percentage
	Males	Females		
P. papatasi	218	61	279	63.84 %
P. sergenti	103	55	158	36.16 %

The most important taxonomic characteristic of males *P. sergenti* is the posterior end, which contains an oval-shaped reproductive pen, with two long spines at the end, one thick and another thin near its base (Figure 2 A). Most important feature of females *P. sergenti* is the presence of a sperm capsule: it consists of (4-6) rings, and the last ring is larger than the others (Figure 2 B).

Detection of Leishmania DNA in P. papatasi by PCR. About 50 females of *P. papatasi* were divided into 10 groups (each group included 5 females) and then were examined. The DNA was amplified by PCR with the primers (L5.8S and LITSR) for the partial ITS-1 gene region. PCR showed that the results of 10% (1/10 groups) were positive only for *Leishmania* DNA and the length of the amplified fragment was 320 bp (Figure 3).

Detection of Leishmania DNA in P. sergenti by PCR. About 50 females of *P. sergenti* were divided into 10 groups (each group included 5 females) then were examined. The DNA was amplified by PCR with the two primers (L5.8S, LITSR) for the partial ITS-1 gene region. PCR showed that the results of 20% (2/10 groups) were positive only for *Leishmania* DNA and the length of the amplified fragment was 320 bp (Figure 4).

PCR-RFLP. It was performed using HaeIII enzyme and the results of electrophoresis were as follows: The results of PCR-RFLP on DNA extracted from *Leishmania* present in *P. papatasi* showed two bands, the first with a length of approximately 200 bp, and the second with a length of approximately 120 bp. This corresponds to the DNA of *Leishmania major* parasite (Figure 5). The results of PCR-RFLP on DNA extracted from *Leishmania* that is present in *P. sergenti* showed two bands, the first with a length of 190 bp, and the second with a length of 60 bp, and this corresponds to the DNA of the *Leishmania tropica* parasite.

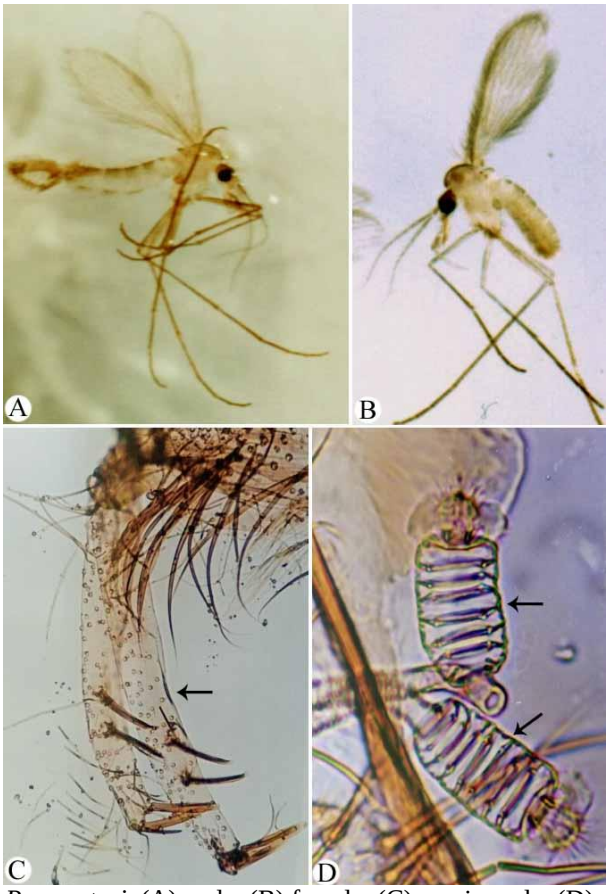

Figure 1. Sand fly of *P. papatasi.* (A) male, (B) female, (C) pen in male, (D) sperm preservative in female.

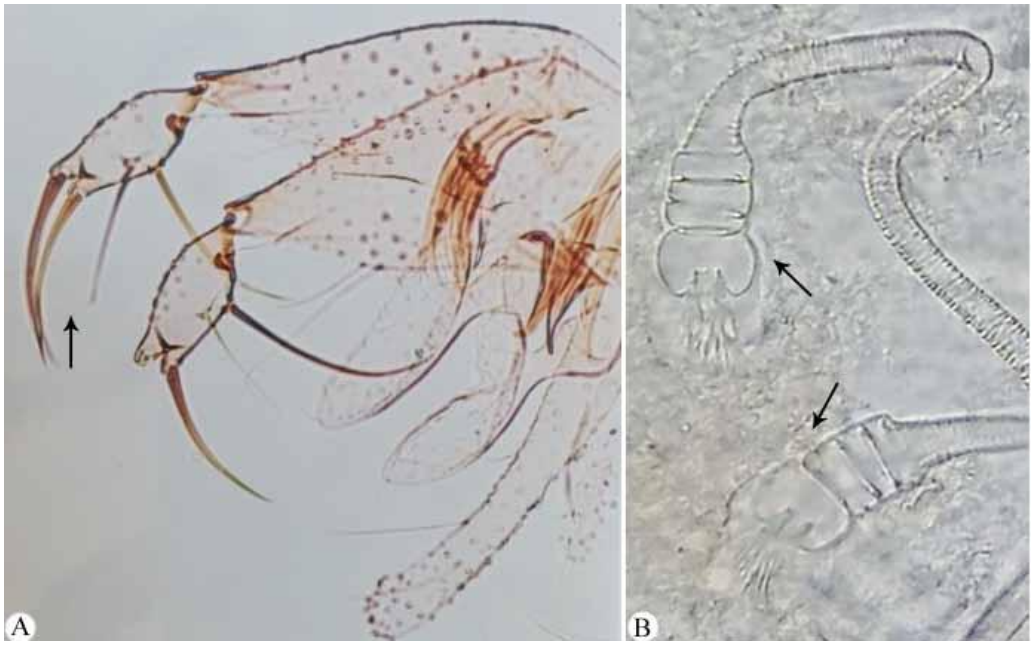

Figure 2. Sand fly of *P. sergenti*. (A) male reproductive pen, (B) sperm preservative in females.

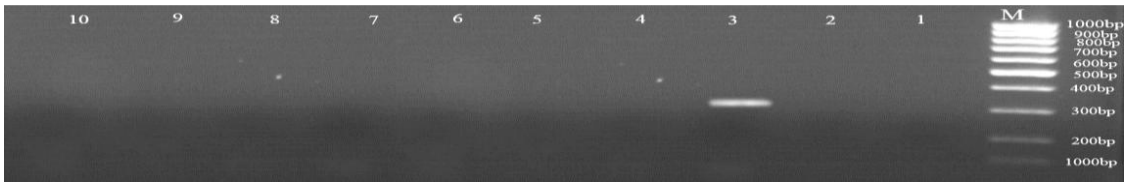

Figure 3. Electrophoresis of PCR products by two Primers (L5.8S, LITSR). M: marker, Numbers groups were detected of *Leishmania* DNA in *P. papatasi*

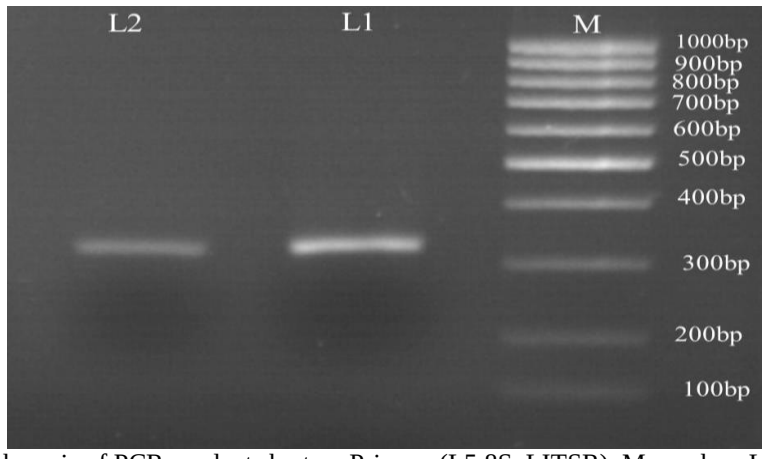

Figure 4. Electrophoresis of PCR products by two Primers (L5.8S, LITSR). M, marker; L1, L2 two groups contain *Leishmania* DNA in *P. sergenti*

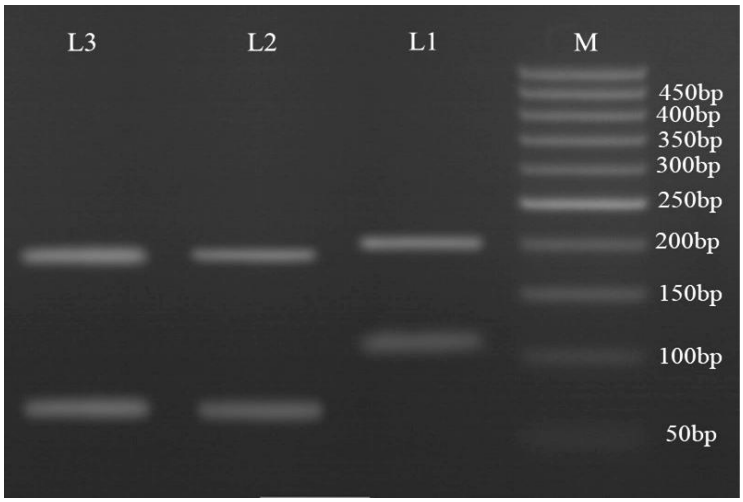

Figure 5. PCR-RFLP products by HaeIII (M, marker; L1, DNA *Leishmania major* in female *P. papatasi*; L2, L3, DNA *Leishmania tropica* in *P. sergenti*

DISCUSSION

In this study, the PCR-RFLP technique of the partial ITS-1 gene region was used to identify species of *Leishmania* transmitted by (sand flies) *Phlebotomus* that spread in refugee camps. PCR-RFLP is an important and widely used technique to identify *Leishmania* species (Gadisa *et al*., 2007; Mouttaki *et al*., 2014; Mosleh *et al*., 2015) and compare those species.

In recent years, cases of cutaneous leishmaniasis in refugee camps have increased. In the past, it was widely believed that all *Phlebotomus* species are transmitted *Leishmania* species without specific, but according to reference studies, there is a variation between *Phlebotomus* species in

ability to transport *Leishmania* species, due to geographically related and varied from one country to another, so this research came to detect *Leishmania* in *Phlebotomus* species that spread in refugee camps and to identify species of *Leishmania* transmitted by each *Phlebotomus* species.

A total of 437 sand flies were classified into two species *P. papatasi* and *P. sergenti*. *P. papatasi* (63.84%) was more prevalent than *P. sergenti* (36.16%). A possible hypothesis for this issue is that females *P. papatasi* feed on rodent blood in addition to human blood, while females of *P. sergenti* prefer to feed on human blood only (Akhoundi *et al*., 2013) and this is consistent with taxonomic studies of *Phlebotomus* in the world that confirm the dominance of the spread *P. papatasi* on the rest of the species of sand flies (Sofizadeh *et al*., 2017; Zivdari *et al*., 2018; Karmaoui, 2020).

The results of the PCR showed the presence of *Leishmania* DNA in females *P. papatasi* (10%) and *P. sergenti* (20%). This is consistent with other studies that reported *P. papatasi* (Parvizi *et al*., 2005; Parvizi and Ready, 2006) and *P. sergenti* (Es-Sette *et al*., 2014; Ajaoud *et al*., 2013) could transmit *Leishmania* parasites.

PCR-RFLP with HaeIII restriction enzyme for PCR products on the DNA of *Leishmania* parasites extracted from females *P. papatasi* showed the presence of two bands, 200 bp and 120 bp. When comparing this result with the relevant global studies, it was found that the DNA extracted from *P. papatasi* belongs to *Leishmania major*, and this proves that *P. papatasi* is a vector of the *Leishmania major* parasites in refugee camps. This corresponds to previous studies (Parvizi and Ready, 2008; Dabirzadeh *et al*., 2016; Kykalová *et al*., 2021) reported that the main vector of *Leishmania major* is *P. papatasi* in the Middle East (Knight *et al.*, 2023) and North Africa (Karmaoui *et al*., 2022) and several countries as Sudan (Soltani *et al*., 2015).

The results of PCR-RFLP performed on the DNA of *Leishmania* parasites extracted from female *P. sergenti* showed the presence of two bands, 190 bp and 60 bp, it belongs to *Leishmania topica* parasites and this is confirmed by global studies (Dweik *et al*., 2013; Mosleh *et al*., 2015; Es-Sette *et al*., 2014; Koohsar *et al*., 2020), and this proves that *P. sergenti* is a vector of the *Leishmania tropica* parasite in refugee camps, and this is consistent with global studies (Mosleh *et al*., 2015; Karmaoui *et al*., 2022) reported that in Jordan, *P. sergenti* is the primary vector of *Leishmania tropica* (Janini *et al*., 1995), as well as, in Saudi Arabia (Karmaoui *et al*., 2022) and Turkey (Demir and Karakuş, 2015).

By comparing the percentage of prevalent *L. major* and *L. tropica* in our study (10% to 20%) with another study (Paiva *et al*., 2006), it was the highest in refugee camps, and due to the poor living, the proximity of refugee tents to each other and low level of health. *L. tropica* is more prevalent than *L. major* because the former which is transmitted by females of *P. sergenti* does not require a stored host and is transmitted between humans only, while the latter, which is transmitted by *P. papatasi* females, requires a stored host from rodents (Mirzaei *et al*., 2014; Karakuş *et al*., 2019).

CONCLUSION

It could be concluded that leishmaniasis is widely distributed in refugee camps (Syria) because of the natural occurrence of its vector. Further studies should be done to control sand flies to reduce the spread of leishmaniasis.

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