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Research Article

Molecular characterization of *Leishmania* species infecting sandflies in the centre of Iran

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Abstract. Cutaneous leishmaniasis, a zoonotic disease caused by protozoan *Leishmania*, is prevalent worldwide, including Iran. This study aimed to assess the sandfly species composition and their *Leishmania* infection in the Kavirat district of Aran and Bidgol County (Isfahan Province). Sandflies were trapped using sticky traps once every two weeks from March to October 2020. In each district, 3 residential houses were chosen, with 30 indoor and 30 outdoor sticky traps placed near rodent colonies within a 300-meter radius of the last house. Specimens were identified using standard keys, and the sandfly bodies were examined for *Leishmania* using PCR and sequencing techniques. A total of 2050 sandflies were captured, with 774 (58.14%) *Phlebotomus* and 1276 (43.96%) *Sergentomyia* identified. *Leishmania major* and *L. turanica* were each detected in a single sandfly, marking the first record of *L. turanica* in Aran and Bidgol County. The predominant sandfly species was identified as *Ph. papatasi*. *Ph. papatasi* activity was observed from May to October. To combat cutaneous leishmaniasis effectively, particularly during July to August, the implementation of a robust health program is imperative.

Keywords: *Phlebotomus papatasi*, *Phlebotomus sergenti*, *Leishmania major*, *L.turanica*

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Introduction

Leishmaniasis is a zoonotic vector-borne disease, caused by the protozoan *Leishmania*, and occurs worldwide in the forms of cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL). These conditions stem from over 20 *Leishmania* species transmitted to humans through the bites of infected female phlebotomine sandflies. Approximately 70 to 75% of CL instances are documented in ten nations, including Iran (WHO, 2024). Currently, CL stands as the primary vector-borne disease in Iran (Yaghoobi-Ershadi, 2012), manifesting in two forms: rural Zoonotic Cutaneous Leishmaniasis (ZCL) and urban Anthroponotic Cutaneous Leishmaniasis (ACL). Iran reports over 20,000 CL cases annually, with approximately 80% being ZCL instances prevalent in 18 provinces (Yaghoobi-Ershadi, 2012; Nadim *et al.*, 1968; Baghaei *et al.*, 1996). Despite worldwide annual reports of 0.2 to 0.4 million new VL cases and 0.7 to 1.2 million new CL cases, leishmaniasis remains categorized as a neglected tropical ailment (Nadim *et al.*, 1968).

In Iran, ZCL arises from *Leismania major*, with *Phlebotomus papatasi* acting as the primary vector. Additionally, *Rhombomys opimus* (great gerbil) serves as the central and northeastern Iranian reservoir for *L. major* (Yaghoobi-Ershadi & Javadian, 1997; Yaghoobi-Ershadi *et al.*, 1998). The causative agent of ACL is L. tropica, humans are the primary host while dogs act as a secondary reservoir for the disease (Eshghi, 1998). In endemic areas, the abundance of sandflies causes disease transmission, long-term treatments, high costs, and physical and psychological harm to people.

Isfahan province is a key endemic area for leishmaniasis. This province is the sixth largest and third most populous province in Iran, and its area is about 106,786 square kilometers. Various studies have been carried out to determine the sandfly species composition and leishmaniasis status in different regions of Isfahan province,

particularly Kashan city and neighboring areas. Nadim *et al.* (1968) were among the first to research the sandfly fauna in regions such as Isfahan, Faridan, Ardestan, Nayin, Shahreza, and Najafabad (Nadim *et al.*, 1968). Several other studies followed, including in Rudasht region of Isfahan (Beghaei *et al.*, 1996), in Barkhar region of Isfahan (Yaghoobi & Javadian, 1997), in Ardestan region (Yaghoobi *et al.*, 1998), in Shahreza region (Eshghi, 1998), in Natanz (Yaghoobi & Akhavan, 1999), in Jarghoye region of Isfahan (Zahraei-Ramazani & Sedaghat, 2016), in Isfahan city (Abdoli *et al.*, 2006), in Kohpayeh region (Emami & Yazdi, 2008) and in Mobareke city (Emami *et al.*, 2009), in Natanz city (Zahraei Ramezani *et al.*, 2015) were conducted in Isfahan province. In addition, two other studies were conducted in Kashan city (Dourodgar *et al.*, 1999; Hesam Mohammadi *et al.*, 2014). In all these studies, *Ph. papatasi* was reported as the dominant sandfly species.

Identifying the sandflies' composition and understanding the peak leishmaniasis infection in different months are crucial for implementing control measures. Detecting *Leishmania* parasite infection in sandflies is vital for recognizing vector species and potential disease transmission in local areas. This study aimed to investigate sandfly species composition and their *Leishmania* infection using PCR for the first time in Kavirat district of Aran and Bidgol County.

Materials and methods

Area of study

Aran and Bidgol County is located in the southwestern margin of the central desert of Iran in Isfahan Province (Fig. 1). The county is subdivided into two districts, Central and Kavirat Districts and has four cities, including Aran and Bidgol, Abuzeydabad, Nushabad, and Sefidshahr. Aran and Bidgol County is surrounded by desert from the north and east, and thus has a typical climate of hot and dry in summer, and cold and dry in winter. The annual rainfall is 100-150 mm. The minimum and maximum temperatures overall recorded were –5 °C in winter and 48 °C in summer. Kavirat District with the center of Abuzeydabad city, is located in 30 km east (southeast) of Kashan and Aran and Bidgol County, in a desert area (sand dunes) and 35 km from Badrud city (Wikipedia, 2024).

Sampling

Adult sandflies were trapped fortnightly between March and November 2020 using sticky traps. Six points were chosen in Abuzeydabad city (Ao (outdoor) and Ai (indoor)), the villages of Mohammadabad (Bo (outdoor) and Bi (indoor)), and Kaghazi (Co (outdoor) and Ci (indoor)). (Fig. 1).

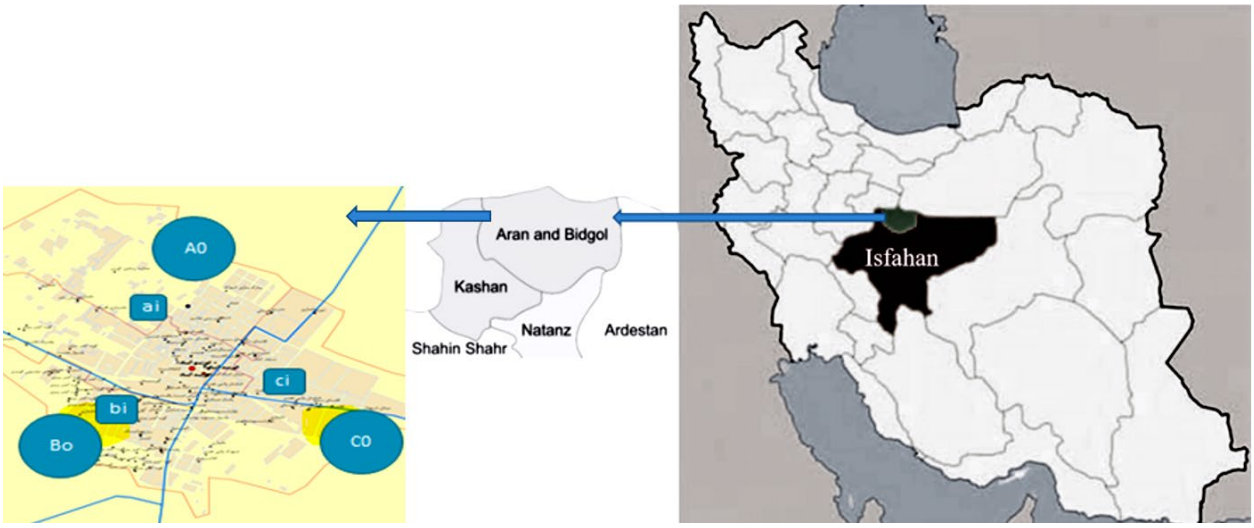

Fig. 1. The geographical map of sampling place of sandflies and sampling points Abuzeydabad city (Ao (outdoor) and Ai (indoor)), the villages of Mohammadabad (Bo (outdoor) and Bi (indoor)), and Kaghazi (Co (outdoor) and Ci (indoor)) in Kavirat District, Aran and Bidgol County, Isfahan Province.

Each night, 3 residential houses were selected from each points, and a total of 30 sticky traps were installed in the rooms and courtyards of each house (indoor), and 30 traps were installed outside the houses, near the rodent colony, within a radius of 300 meters of the last residential house (outdoor).

Traps were set before sunset and retrieved the next morning before sunrise once every two weeks from March to December. Collected sandflies were removed from sticky papers using entomological needles or fine brushes, washed several times with 75% ethanol to remove oil, preserved in 70% ethanol, and kept in micro tubes before identification.

Microscopic study

To identify sandfly species, the head and last two abdominal segments of female sandflies were detached. These parts were mounted in Puri's medium and identified with a recognized morphological key for adult sandflies (Theodor & Mesghali, 1964; Rassi &Hanafi Bojd, 2006). To assess sandfly infection with *Leishmania* parasite, the remaining body parts were preserved in 96% Ethanol for DNA extraction.

Molecular study

Phenol-chloroform methods were used to extract DNA from *Phlebotomus* body. The PCR test was performed for detection and identification of *Leishmania* species. The ITS1 region of *Leishmania* parasite was amplified, using the following primers (Forward: 5'- CTGGATCATTTTCCGATG-3' Reverse:5' – TGATACCACTTATCGCACTT-3).

In the PCR test, according to the gene amplification program, one cycle of primary denaturation at 94 C for 60s was performed, then 35 cycles of denaturation at 94 C for 30 s, annealing at 52 C for 30 s, elongation at 72 C for 30 s with extension at 72 °C for 30 s. The final extension at 72 °C for 10 minutes was followed by cooling to 4 °C. Then the final product from each reaction was subjected to electrophoresis and analysis on a 1% agarose gel with safe stain.

Sequencing

20µl of PCR products, 10µl forward primer and 10µl reverse primer were sent to Niagen Noor Company (Iran). Sequences were compared to homologous sequences in GenBank to the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST:

www.ncbi.nlm.nih.gov/BLAST). The parasite species were identified based on their sequence compare to the sequences deposited in GenBank.

Statistical analysis

SPSS software version 16 was used for the statistical analysis of the variables. Data were evaluated using descriptive statistics and calculating ratios.

Results

Morphology study

In total, 2050 sandflies were captured, with 774 (58.14%) identified as *Phlebotomus* and 1276 (43.96%) as *Sergentomyia*. Among these, 41.86% of *Phlebotomus* and 56.03% of *Sergentemyia* were females; notably, 7.49% of female *Phlebotomus* had blood meals (Table 1). The percentages of female sandflies by species were as follows: *Ph. papatasi* (28.39%), *Ph. sergenti* (9.36%), *S. sintoni* (27.02%), *S. tiberiadis* (20%), *S. sumbarica* (9.07%), and *S. clydei* (6.15%). Consequently, *Ph. papatasi* and *S. sintoni* emerged as the predominant species in the Aran and Bidgol regions based on frequency. Details regarding the total number of sandflies captured in the Kavirat area of Aran and Bidgol County, categorized by gender and blood feeding of female sandflies, are presented in Table 1.

The peak sandflies activity was observed in July, with activity tapering off by the end of December. During the months of July and August when sandflies are most prevalent (Fig. 2), morning temperatures ranged from 31.6 to 33°C with relative humidity at 13 to 28%, while evening temperatures ranged from 37.7 to 38°C with humidity at 6 to 15% (Table 2).

Table 1. Total number & species of sandflies caught in Kavirat District, Aran and Bidgol County.

Genus	No. (%)	Species	No. (%)
Phlebotomus	Total 774 (37.75)	*Ph. papatasi*	582 (28.39%)
	Female 324 (41.86)	*Ph. sergenti*	192 (9.36%)
	Male 450 (58.14)		
Sergentomyia	Total 1276 (62.25)	*S. sintoni*	554 (27.02%)
	Female 715 (56.03)	*S. tiberiadis*	410 (20%)
	Male 561 (43.96)	*S. sumbarica*	186 (9.07%)
		S. clydei	126 (6.15%)

Molecular study

From 324 samples of female *Phlebotomus* sandflies from the three study areas, sandflies were sorted by region, genus, and species. 15 *Ph. sergenti* specimens, 17 *Ph. papatasi* specimens, and 2 *Sergentomyia* specimens were chosen for PCR tests. PCR test results revealed 6 out of 34 selected samples were infected with the *Leishmania* parasite (Fig. 2).

After blasting the data, the obtained results had 99-100% homology with the isolates registered as *L.major* species in the Genbank. Also, the phylogenetic tree of the identified isolates was drawn in Figure 3. Based on the OMEGA CLUSTAL multiple alignment results of the EBI site, it was determined that the isolates numbered 348 and 324 belonged to *L.major* and *L. turanica* respectively. The phylogenetic tree of positive samples is depicted in Fig. 3. *L. major* (Code 348) was found in blood-fed *Ph. papatasi* sandflies between Abuzeidabad and Mohammadabad villages, within 100 meters of houses and rodent colonies. The rate of *Ph. papatasi* infection with *L. major* parasite in the entire area was 5.88%. Additionally, one *L.turanica* (Code 324) was found in a *Ph. papatasi* specimen collected from outdoor areas and near rodent settlements.

Discussion

Isfahan Province is a key area for ZCL in Iran (Shirzadi *et al.*, 2015), with *L. major* being the predominant species (Yaghoobi-Ershadi *et al.*, 2001; Parvizi *et al.*, 2005; Arjmand *et al.*, 2014; Azizi *et al.*, 2013). In this province, Isfahan City, Natanz, and Ardestan are the primary locations for ZCL (Yaghoobi-Ershadi *et al.*, 2001; Karami *et al.*, 2013; Parvizi *et al.*, 2012).

Table 2: Characteristics of sandflies of the genus *Phlebtomus* according to indoor and outdoor locations, temperature and humidity of the studied area, and date of capture in Kavirat District, Aran and Bidgol County, Isfahan Province.

		Months							
Parameter		April	May	June	July	Aug.	Sept.	Oct.	Nov.
No. of sandflies traped in	Ao	0	2	7	22	11	7	1	0
each	ai	0	1	6	29	17	9	2	0
points	Bo	0	1	9	27	10	8	2	0
	bi	0	3	5	31	17	11	1	0
	Co	0	0	4	18	5	4	0	0
	ci	0	1	3	26	13	10	1	0
Temp. (C°)	morning	10	18.7	20.6	33	31.6	26.1	23	20
at	evening	19.4	32.3	35	38	37.7	35.3	26	22
Humidity (%)	morning	55	21	45	13	28	20	18	50
at	evening	27	19	10	6	15	11	12	30
No. of sandflies	*Ph. papatasi*	0	7	29	139	69	45	6	0
	Ph. sergenti	0	1	5	14	4	4	1	0
	Total	0	8	34	153	73	49	7	0

Sampling points Abuzeydabad city: Ao (outdoor) and Ai (indoor), the villages of Mohammadabad: Bo (outdoor) and Bi (indoor), and Kaghazi: Co (outdoor) and Ci (indoor)

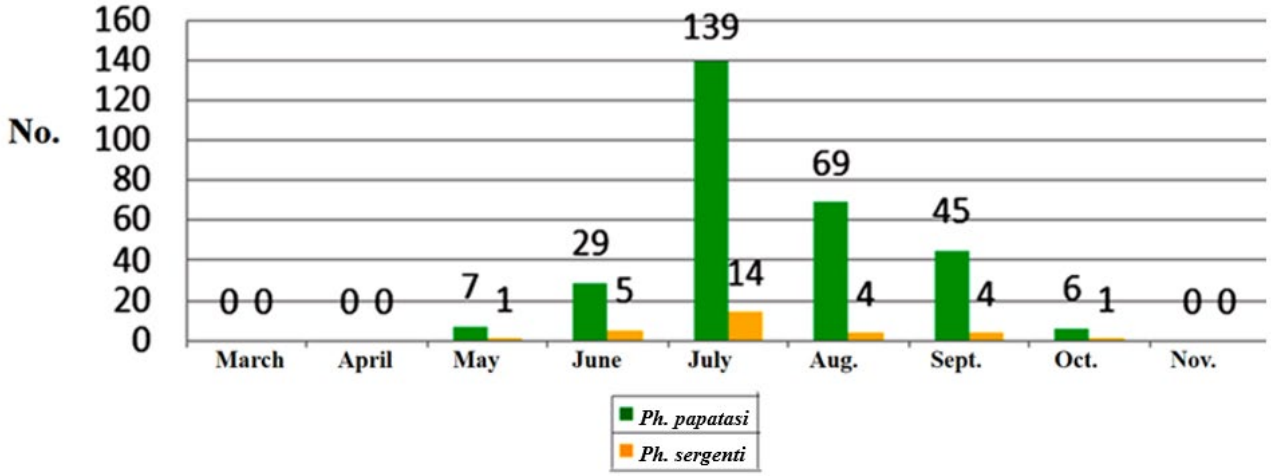

Fig. 2: Abundance and activity of sandflies caught in different months of the year in the Kavirat district of Aran and Bidgol County.

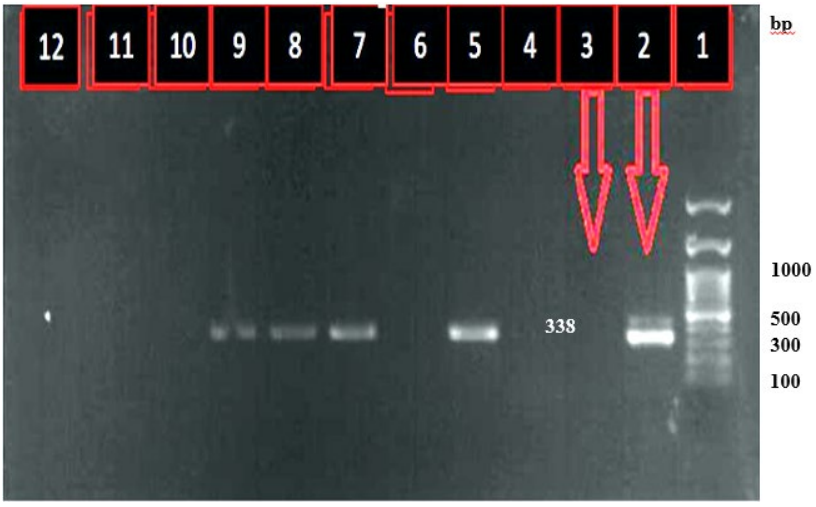

Fig. 3. PCR product electrophoresis of *Phlebotomus* samples infected by *Leishmania.* 1: Ladder 100 bp DNA ; 2: positive control (338 bp); 3: negative control; 5: positive sample code 182; 6: negative sample; 7: positive sample code 27; 8: positive sample code 324; 8: positive sample code 348.

Studies have identified *L. major* as the main cause of cutaneous leishmaniasis in Isfahan, particularly in Aran and Bidgol County (Azizi *et al.*, 2013; Mohammadi *et al.*, 2012). *Ph. papatasi* and *Rhombomys opimus* serve as the main vector and reservoir host for *L. major* in this region (Doroodgar *et al.*, 2015). The study also reveals the prevalence of various sandfly species in Aran and Bidgol, with *Ph. papatasi* and *S. sintoni* being the most common. Additionally, some research conducted in Kashan has identified several species of sandflies, with *Ph. papatasi, Ph. sergenti*, and *S. sintoni* being predominant among the samples collected (Hesam-Mohammadi *et al.*, 2014). In another study in Kashan, *S. sintoni* was found to be the most prevalent species among the sandflies collected (Doroodgar *et al.*, 2015). *Phlebotomus papatasi* infection with *L. major* and *L. turanica* (5.88%) yielded results akin to Rafizadeh *et al.*'s research in Mashhad and Rassi's in Semnan (Rafizadeh *et al.*, 2016; Rassi *et al.*, 2012). Doroodgar *et al.* (2015) indicated 1.9% of female *Ph. papatasi* were *L. major*-infected using standard RAPD-PCR (Doroodgar *et al.*, 2015). Various studies have likewise documented *L. major* infections in *Ph. papatasi*.

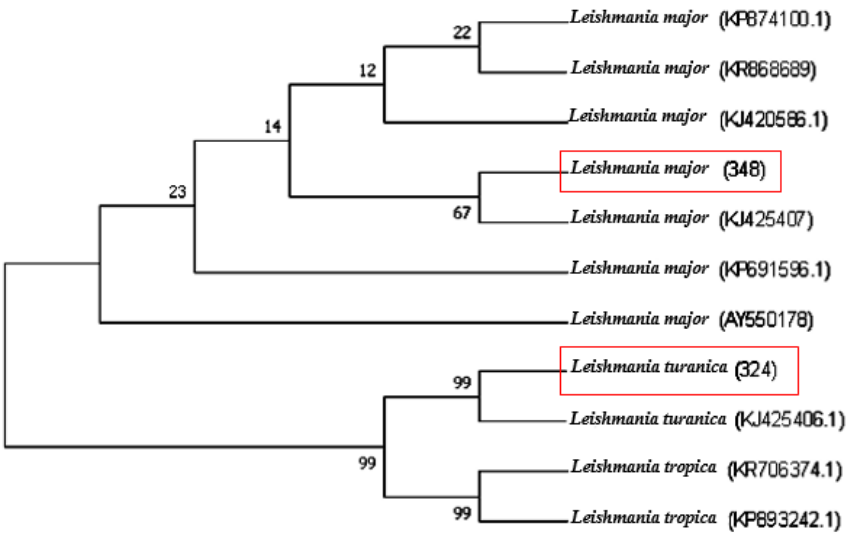

Fig. 4. Phylogenetic tree inferred of ITS1 gene sequences of *L. major* and *L. turanica* isolates from *Phelebotomus papatasi* in the present study and other *Leishmania* species obtained from GenBank using MEGA software and maximum likelihood algorithm and bootstrap 500.

In our research, *Ph. papatasi* emerged as the main sandfly species carrying *L. major*, underscoring the importance of prioritizing rural cutaneous leishmaniasis in control initiatives. Zahraei Ramezani & Sedaghat (2016) also recognized *Ph. papatasi* as the main vector for rural cutaneous leishmaniasis and *Ph. sergenti* as the key vector for urban cutaneous leishmaniasis across all regions of Isfahan Province (Zahraei-Ramazani & Sedaghat, 2016).

In the present study, the species *L. turanica* has been observed for the first time in Aran and Bidgol County. This species was first reported by Strelkova *et al.* in 1990 from large gerbils in Turkmenistan, Uzbekistan, southern Kazakhstan, and also in Mongolia of the former Soviet Union (Strelkova *et al.*, 1990). Later, this species was also reported from other parts of the world, including Iran (Bordbar *et al.*, 2014; Rassi *et al.*, 2011; Rafizadeh *et al.*, 2016). Sufficient evidence has been provided to differentiate between *L. turanica* and *L. major* using various methods such as enzyme electrophoresis (Strelkova *et al.*, 1990), genotyping (Novozhilova *et al.*, 2023), immunological assays (Guan *et al.*, 1995. *L. turanica* although restricted to gerbils, but causes mild self-healing symptoms in humans in experimental infections (Strelkova *et al.*, 2001; Akhavan *et al.*, 2010).

The increased activity of *Phlebotomus* sandflies in July and August suggests that they are most active at temperatures between 32 to 38°C and relative humidity of 15 to 32%. Hesam Mohammadi *et al.* (2014) noted that in Kashan County, the peak sandfly activity occurred before midnight (between 22:00–24:00 PM), with an average temperature of 26.92°C and relative humidity of 26.5% (Hesam-Mohammadi *et al.*, 2014). It appears that low humidity, gentle wind, and high temperatures are crucial factors influencing sandflies' night-time activity (Roberts, 1994; Guernaoui *et al.*, 2006). Research on the impact of climate elements on sandfly distribution in arid regions of India revealed that most species thrive in relatively higher temperatures and lower relative humidity, essential for survival in arid and semi-arid zones Singh (1999). Shirzadi *et al.* (2020) investigated the influence of environmental and climate factors on the spatial distribution of cutaneous leishmaniasis in northeastern Iran, encompassing North Khorasan, Razavi Khorasan, and South Khorasan provinces. They found higher disease incidence in North Khorasan and the northern parts of Razavi Khorasan, with decreased incidence in southern areas as temperature increased (Shirzadi *et al.*, 2020). A study in Naein County indicated that the peak monthly activity of *Ph. papatasi* and *Ph. sergenti* occurred in June. However, the low density and absence of *Leishmania* infection categorized this area as low-risk (Jafari *et al.*, 2020). In conclusion, the activity of *Ph. papatasi* was observed in Aran and Bidgol from May to October 2020. To prevent and manage Cutaneous Leishmaniasis, particularly in July and August, a suitable health program implementation is essential. Examining abiotic factors like humidity, temperature, cloud cover, and wind speed can enhance understanding of sandflies' nocturnal activity and help establish better, science-based control strategies.

Author's Contributions

Mohsen Akbarian: methodology, data collection, formal analysis, investigation, original draft. **Abdolhossein Dalimi:** conceptualization, methodology, resource, reviewing and editing, supervision, and project administration.

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Data Availability Statement

All data supporting the findings of this study are available within the paper.

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Ethics Approval

This study was approved by the ethics committee of Tarbiat Modares University with code number TMU-1397.218

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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بررسی خصوصیات مولکولی گونه لیشمانیا آلوده کننده پشه خاکی در مرکز ایران

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چکیده: لیشمانیوز جلدی، یک بیماری مشترک بین انسان و دام است که توسط تک یاخته لیشمانیا ایجاد می شود و در سراسر جهان از جمله ایران شایع است. این مطالعه با هدف بررسی ترکیب گونه پشه خاکی و آلودگی آن به لیشمانیا در منطقه کویرات شهرستان آران و بیدگل (استان اصفهان) انجام شد. پشه خاکی ها از مارس تا اکتبر ۲۰۲۰ هر دو هفته یکبار با استفاده از تله های چسبنده به دام افتادند. در هر منطقه، ۳ خانه مسکونی انتخاب شد که ۳۰ تله چسبنده داخلی و ۳۰ تله چسبنده در فضای باز در نزدیکی کلنی های جوندگان در شعاع ۳۰۰ متری آخرین خانه قرار گرفتند. نمونه های پشه با استفاده از کلیدهای استاندارد شناسایی شدند و اجسام پشه خاکی برای لیشمانیا با استفاده از روش PCR و توالی یابی مورد بررسی قرار گرفتند. در مجموع ۲۰۵۰ پشه خاکی صید شد که ۷۷۴ (۵۸.۱۴٪) *Phlebotomus* (43.96%) *Sergentomyia* و ۱۲۷۶ (۴۳.۹۶٪) *Sergentomyia* شناسایی شد. *Leishmania major* و *L. turanica* هر کدام در یک پشه خاکی شناسایی شدند. *L. turanica* برای اولین بار از شهرستان آران و بیدگل گزارش می شود. گونه غالب پشه خاکی *Phlebotomus papatasi* شناسایی شد. فعالیت گونه *Ph. papatasi* از ماه می تا اکتبر بوده است. برای مبارزه موثر با لیشمانیوز جلدی، به ویژه در طی ماه های ژوئیه تا آگوست، اجرای یک برنامه بهداشتی قوی ضروری است.

کلمات کلیدی: فلبوتوموس پاپاتاسی، فلبوتوموس سرجنتی، لیشمانیا ماژور، لیشمانیا تورانیکا

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