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Determination of Leishmania Parasite Species of Cutaneous Leishmaniasis Using PCR Method in Central County, Qom Province

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Abstract

***Background*:** Leishmaniasis is one of the most important health problems in many areas of Iran. This study aimed to identify leishmania species in patients with cutaneous leishmaniasis using PCR teqnique based on sequencing ITS1 primers in villages of the central county of Qom province.

***Materials and Methods*:** This descriptive cross-sectional study was done on 169 patients who with leishmaniosis, based on clinical and laboratory confirmation treated and were followed in health center of Qomrood in 2010. This data was recorded in epidemiologic data forms. The DNA was extracted by KIAGEN kits. The extracted DNA was exploited to identify the parasite by PCR technique. The data were analyzed by SPSS-17 software. Chi square and Fisher's exact test were used for the evaluation of the hypothesis.

***Results*:** The PCR result confirmed the parasite positive slides and obtained bands from these slides were seen in the range of 350 bp which are expected band for the leishmania major parasite. The most frequent age group was above 15 years old (71.5%). Hands and feet were the most common sites of ulcer (78.8%). 19.5 of patients had 3 ulcers and more. Highest disease prevalence was observed in months October, December and November.

***Conclusion*:** The PCR results illustrated that the prevalent CL in the central county of Qom province is Zoonotic type (ZCL), and the agent of disease is leishmania major parasite. In conclusion, identification of the disease and parasite type can help the health officials to make appropriate strategies for its prevention and control.

Introduction

Leishmaniases are parasitic diseases transmitted by many kinds of *Phlebotomous sandflies*. They occur in three main clinical forms, namely cutaneous (anthroponotic and zoonotic), visceral (Kalazar) and mucosal (Aspundia). Leishmaniasis has been listed by the WHO as one of the top 10 diseases common in tropical regions, tagging it further as non-controlled and new. The disease is caused by parasites of the genus Leishmania and the phylum Kinetoplast, and transmitted by the bite of infected female sand flies [1, 2]. Cutaneous leishmaniasis has been witnessed in both anthroponotic (ACL) and zoonotic (ZCL) forms in Iran [3]. The cause of the ZCL is *Leishmania major*, rodents serving as its main reservoir. The primary vector of the disease is the sandfly of the species *Phlebotomus papatasi*. On the other hand, in the ACL, the cause is *Leishmania tropica*, humans are the main reservoir, and *Phlebotomus sergenti* is the main vector [4-6]. Leishmaniases have been reported in 88 countries and 350 million people are at risk. Approximately 12 million people were estimated to have caught the disease so far. The prevalence rate is 2 million people per year, 1.5 million of which are infected with cutaneous leishmaniasis (CL). About 90% of cases with CL are from Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria [7-10].

In Iran, controlling cutaneous leishmaniasis in rural areas is an important health issue as it is prevalent in many rural areas of 17 provinces (30 provinces in total) [4, 11, 12].

According to the Center for Disease Control, various forms of Leishmaniasis are spreading at the rate of 20,000 per year. Yet, doubtlessly, the actual rate must be up to five times higher. The causes of this may be imputed to the rapid expansion of cities, construction of residential areas on rodent colonies, environmental changes, dam construction, abortion of spraying plans against malaria vectors in many areas, uncontrolled entrance of Afghan refugees, the problems of rapid population growth, and, generally, socioeconomic changes [13-15]. The Qom province, one of the hubs of leishmaniases, reports leishmaniasis patients especially in the central part of every year [16, 17]. Also, a leishmaniasis epidemic

occurred in this province in 2003 [18]. This study was carried out to determine the species of the leishmania parasite and the epidemiological aspects of cutaneous Leishmaniasis in the Qom province.

Materials and Methods

All of 861 households with a population of over 5,168 people reside in villages covered by Qom rood Health Center. Data checklists were used to record a summary of epidemiological information of patients with Leishmaniasis. The information included age, sex, nationality, place of residence, number of lesions, sites of lesions, season, type of diagnosis, type of treatment, history of diabetes in the family, date of disease appearance, and history of having traveled over a year ago to detected endemic districts. All research steps were carried out according to the principles of medical ethics and the Research Office of Qom University of Medical Sciences. Data was analyzed using χ^2 test and an exact test of Fisher. Patients participated in the study with informed consent. All the above analyses were performed using SPSS-17.

After the completion of the demographic and epidemiological data, microscopic slides of all individuals with suspected lesions of CL were prepared. After staining the slides in the Giemsa fashion, they were examined under the microscope to search for leishmania. After microscopic examination of the slides, their DNAs were extracted to perform molecular tests. In so doing, 45 positive slides were scraped with scalpel blades and (using Kiajen Company's kits) DNAs were extracted from the tissue. Subsequently, the DNA extractions passed the PCR test for the identification of the parasite. Finally, the reaction's products were run on 1% electrophoresis gel. The primers utilized included LITSR (5'-CTG-GAT-CAT-TTT-CCG-AT-<G>-3') and L5 8S (5'-TGA-TAC-CAC-TTA-TCG-CAC-T-<T>-3') (Sinajen, Iran). These primers were designed based on the conserved area of kDNA minicircle. Reference strains of *L. major* and *L. tropica* were selected as standard. These strains were obtained from the department of Parasitology, Faculty of Health, Tehran University of Medical Sciences. The primers required were Master Mix (HSPMM): 10 µl; LITSR, L5 8S, $MgCl_2$: 1 µ1 each; Color: 2 µ1; and DNA: 5 µl. The five thermal cycles involved Preheating, Denaturation, Annealing, Extension, and Final Extension at temperatures 95, 94, 48, 72, 72ºC and for 5 min, 30 sec, 30 sec, 1 min, and 7 min, respectively. Also, 35 cycles of the first, second, third and fourth steps were repeated. Fragments expected at bands from PCR propagation were 350 bp for *L. major*. The bands created were examined using Marker 50 bp.

Results

During the 12 months of the study (concurrent in part with 2008 and 2009), 169 patients were diagnosed with Leishmaniasis in Qomrood's Healthcare Center and, with clinical and laboratory confirmation, were prosecuted for treatment. Microscopic slides of 45 patients went through PCR molecular test. The resultant bands from these positive slides were observed within the range of 350 bp, which is similar to standard *L. major* and the band expected from *L. major* (Fig. 1). This is the first report on the tracking of parasites using molecular tests in patients with cutaneous Leishmaniasis in endemic villages of salak located in central Qom. Out of the study's cohort, 9 (53.2%) were females, 79 (46.7%) were males which does not show any statistically significant difference. Distribution of the cases in four different age groups showed that there was none below 1 year of age, 12 (7.1%) between 1-6, 36 (21.3%) between 7-14, and 121 (71.5%) above 15. No need to say that those above 15 comprised the majority of patients. Housewives were infected with Leishmaniasis more than any other group. Also, regarding the distribution of 189 lesions on parts of the body, there were 90 (47.6%) on the hands, 59 (31.2%) on the feet, 29 (15.3%) on the face, 9 (4.7%) on the trunk, and 2 (1.3%) on the head and neck. The most common site of lesions was the hands (48%). 109 of patients (64.4%) had one lesion, 27 (15%) had two, and 33 (19.5%) had three or more. Concerning the seasonal distribution, it was observed that three quarters (75.7%) had been infected in the autumn, in November month. There was no case reported in the spring, 16 (9.4%) in the summer, and 25 (14.7%) in the winter. Results indicated that 94.5% of patients had no history of having trips to endemic regions of the disease. Based on the mid-year population of Qomrood, the incidence of the disease in 2008-2009 was estimated at 32.7 people out of one thousand.

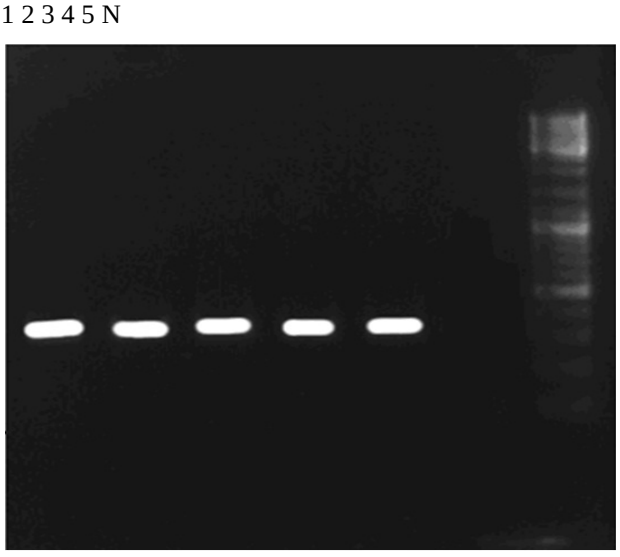

Figure 1. Bands of L. major's DNA propagation using primers ITS1 (Bands 1-4: samples from patients' lesion; Band 5: standard L. major; N: negative control, M: marker).

Discussion

Results show that patients with suspicious clinical symptoms, after microscopic examination of their slides, were diagnosed with Leishmania. PCR results corroborated this fact and also identified the parasite to be

L. major. This parasite was found to be the cause of cutaneous leishmaniasis by many studies conducted in endemic centers such as Kolaleh and Dalghan [11, 19]. In this study, 53.2% of patients were females and 46.7% were males, for which a χ^2 test showed no significant difference. Sex distribution was the same in other studies conducted in other regions such as Hormozgan's Haji Abad, Sabzevar, Bafgh, and Gonbad Kavous. However, more females than males were diagnosed with Leishmaniasis in some other regions such as Damghan (57.7% males and 42.3% females) and Kermanshah (68% males and 32% females) [7, 20-24].

As for the age groups, in this study 75% of patients were 15 and above. This is consistent with Mohammadi-Azani's study in Damghan (79%) and Yaghobi-Ershadi et al.'s study in Yazd's Khatam (85.5%) [19, 25]. Heterogeneous results come from Hormozgan's Haji-Abad with 10 to 14-year-olds having most scars and 0 to 4-year-olds having most acute lesions. Moreover, in Isfahan, which is a hyper-endemic, the disease was most prevalent among infants under one year of age [7, 26]. As noted before, in this study the fifteens and above were mostly infected and, accordingly, it can be concluded that the Qomrood region must be a hypo-endemic with regard to the endemicity classification of ZCL. As for occupations, housewives suffered from leishmaniasis more than any other occupation groups. This has been in agreement with the studies in Damghan and Kolaleh [11, 19]. By contrast, in Hamedan 85.7 of patients were plain workers, bearing in mind that Hamadan is one of the non-endemics in terms of cutaneous leishmaniasis [18]. Eighty five percent of these patients were the youth and adults who migrated in search of work to endemic provinces [27].

Turning now to lesions and scars, in this study most (48%) of damages appeared on the hands, which supports the distribution pattern of CL lesions in other centers of the disease. For example, in research by Rafati et al. in Damghan, 55.1% of lesions targeted the hands and 43.1% the feet [23]. Other studies with similar findings include Yaghoobi-Ershadi in Khatam, Yazd (45% hands, 25% feet); Rahbarian et al. in Gonbad Kavous (42.5% feet, 29.8% feet); and Zahirnia et al. in Hamadan (71.6%) [22, 25, 27]. However, one study in Haji Abad, Hormozgan Hanafi Bajd et al. found that 65.3% of lesions were on the feet and 8.7% on the hands. This corroborates the findings by other investigations in the centers of ZCL [11, 16, 26, 28].

Apart from sites of lesions, their number merits attention, as well. In the present study, 64.4% of patients bore only one, 15.9% two, and 19.5% three or more lesions. 38.9% of patients in Sabzevar, 42.7% in Damghan, 54.5% in Kermanshah, 66.6% in Bafgh, and 55% in Khatam had only one lesion, too [20-25]. Despite this large volume of research reporting the majority of cases with one lesion, in Gonbad Kavous 54.2% of patients had more than three lesions and solely 2.9% bore one. Thus, it can be concluded that sandflies have an intermittent blood feeding habit, and at every meal, bites its victim more than once, and transmits a certain number of the leishmania parasite to the host's body, hence multiple lesions.

This study showed that most cases (75.7%) were affected during November. There were none in the spring. In Sabzevar, the majority of CL patients belonged to the summer and autumn, and the lowest figure to the winter. In the same city, the season pattern of ACL was dissimilar. To be more transparent, the springtime was the peak while the wintertime was the bottom [20]. In the longitudinal research by Rafati et al. and Mohammadi-Azani, it was discovered that, during the years 1999 to 2005 in Damghan, 76.3% of patients were bitten during the autumn. Also, 82.5% of patients suffered sandfly bites in the autumn of 2008 [19, 23]. In Kermanshah, too, the largest proportion (35%) of patients was afflicted in the autumn [24]. These findings back up the results of the present study.

The highest incidence of the disease was in the months of October, November and December (autumn's month). Raf'ati et al. found that the incidence went down from October (33.1%), to November (29.5%), to September (16.2%), and to December (13.7) [23]. Mohammadi-Azani reported that October (31.6%) and November (29.8%) were the peak months of leishmaniasis incidence [19]. This study showed that the incidence rate in Qomrood was 32.7 per one thousand. Yaghoubi-Ershadi et al. reported the rate at 9.1 per one thousand in 2005 [21].

Finally, the PCR results indicated that CL common in Markazi county of Qom is the ZCL form and the agent is *L. major*. Moreover, based on the fact that the epidemiological factors differ from ZCL form to the ACL, the identification of the type of leishmaniasis by this endeavor can pave the way for the healthcare authorities to combat and eradicate the disease more effectively.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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