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Molecular Diagnosis and Characterization of Leishmania Species from Clinical Specimens in Kashan, Iran 2012 - 2013

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Cutaneous Leishmaniasis is one of the health problems in Mediterranean regions including Iran. Considering the increase in the incidence of disease in Kashan, this study was designed to identify the *Leishmania* species in cutaneous leishmaniasis by PCR using primers of kinetoplastic DNA for treatment and appropriate measure for controlling of the disease. 130 patients suspected for cutaneous leishmaniosis referred to two health care centers of Kashan from 2012 to 2013 were examined. The demographic information as well as signs of the disease were recorded. The diagnosis of infection was based on observation of amastigotes within the smear of, serosity of the wound, after dying by Giemsa. Then, *Leishmania* species was identified following the extraction of DNA from serosity, and PCR with variable region of the KDNA. Expected PCR products of *L. major* and *L. tropica* were 650 bp and 760 bp respectively. The data were analyzed by SPSS using X^2 and Kappa (K). Overall of 130 specimens, 87(66.9%) cases and 98 (75.4 %) cases were positive for microscopy and PCR methods respectively. In PCR assay 70 (71.4 %) and 26 (26.6%) of the samples were identified as *L. tropica* and *L. major* respectively, and 2 (2 %) cases were detected as mix. The majority of the *L.tropica* or *L. major* had 1 to 2 wounds, but 11.5 % of *L. major* positive cases had more than 7 wounds respectively. Based on the results of this study, both species of *Leishmania* were present in Kashan, Therefore, it was suggested that careful preventive measure be taken in rural and urban parts. In addition, KDNA-PCR assay using serosity for accurate and quick diagnosis of cutaneous leishmaniasis is recommended.

Key words: Cutaneous Leishmaniasis, PCR, KDNA, Molecular Characterization.

Cutaneous Leishmaniasis is one of the health problem in worldwide particularly in countries located in Mediterranean regions including Iran. The skin lesions are different depending on the species and isolates of *Leishmania* , demonstrating such as macular, papular, wet wounds, sporotrichosis, tumor, leprosy, tuberculosis, and dermatomycosis, which need to be differential diagnosed for treatment. Even after the treatment and healing, scar will remain. In many cases, relapse of the disease has been reported that causes psychological discomfort of the patients[1-4].

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According to the world health organization report (WHO), more than 90 percent of cutaneous leishmaniasis occurs in Afghanistan, Pakistan, Algeria, Iran, Saudi Arabia, Brazil, Columbia and Syria [5]. The incident rate of the disease in Iran estimates 28 per 1000 persons and is diagnosed as Anthroponotic Cutaneous Leishmaniasis(ACL) and Zoonotic Cutane-ous Leishmaniasis (ZCL). It is reported endemic areas in various provinces of Iran, including Isfahan, Tehran, Fars, Mashhad, Khozestan and Kerman[3,6] .In ACL, the agent and reservoir of the disease are *Leishmania tropica* and human and dog respectively. In ZCL the agent and reservoir of the disease are *Leishmania major* and gerbils. In the last two decades, the cases of disease has been rising[4]. In Kashan city, the frequency rate of disease has been reported 6% in 2007, that was twofold increase compared to 1997[7].

The distinguishing between *L.tropica* and *L.major* is impossible due to the morphologic similarity. However in recent decades, different new methods , such as multilocus enzyme electrophoresis, immunologic and molecular methods have successively been evaluated for the characterization of *Leishman* species.[1,4].

In recent years, polymerase chain reaction (PCR) using kinetoplastic DNA (KDNA), Mini -exon and internal transcribed spacer (ITS) has been successful in diagnosing of cutaneous leishmaniasis[8-15]. The sensitivity and specificity of KDNA-PCR is very high and in atypical cutaneous Leishmaniasis with low rate of parasite and negative microscopic results, PCR has been positive[8,9].

Microscopic examination is the routine method for diagnosing CL, so that that the sensitivity of the test varies 42 -70 percent depending on the skill of the technicians[13, 16]. While the sensitivity and specificity of PCR with variable region of KDNA is 98.7 to 100 percent due to having 10000 copies per cell, and distributed among 10 different sequence classes. It is an ideal target that offers accurate discrimination between species[10] However, the sensitivity of PCR-RFLP using ITS1 primer is 82 to 91 percent due to 40 to 200-copies per cell whereas the specificity is 100 percent[10,11,16].

The major problem associated with CLtreatment, is incorrect diagnosis of CL in the scarce number of parasites in ZCL and the presence of false results in microscopic diagnosis, therefore, there is a need to use a sensitive method for correct diagnose of parasite species for efficient treatment and control the disease[17] . Isfahan province is one of the important endemic focus for ZCL in Iran. One of the ancient cities of this province is Kashan. Considering the contradictory reports regarding the incidence rate of *Leishmania* species in this region[18, 19], The present study was designed to diagnose cutaneous leishmaniasis and its parasite species using KDNA- PCR in Kashan city, Isfahan province, Iran. Such findings will be useful for taking proper planning to control the disease in the area.

MATERIALS AND METHODS

Study area

Kashan city is located in the north-west of Isfahan province. This is an ancient city with major tourist attractions in the central part of Iran, with an area of 8608.2 square meters. Its altitude from the sea level is 982 meter. The mean temperature is 18 degree centigrade that reaches as high as 40.8° C in summer and - 3 ° C in winter. The yearly rain fall is 116 millimeters with an estimated population of 400,000.

Patient and Methods

This cross-sectional study was performed on 130 patients suspected to cutaneous leishmaniasis from two Kashan health care centers of Zidi and Shahid Beheshti Hospital laboratory from August 2012 to September 2013. The patients were 1 to 90 years old.

The demographic information as well as the number, form and location of wounds were recorded in questioner's forms. After disinfecting the wound by 70 percent alcohol, disposable lancet was used to collect serosity from ridge wound and spreading it on two slides, after fixation and staining using Giemsa, then it was examined for amastigote presence. In addition, some amount of the wound serosity was added to 1.5 micro tubes containing 0.5 ml of sterilized normal saline and stored in -20 °C for DNA extraction.

DNA Extraction

DNA samples of patient was extracted by Kit (Bioneer; Korea) according to the manufacturer's instruction and stored in -20 °C.

PCR

The PCR was performed on all 130 DNA samples. Species-specific primers, LINR4 (forward, GGG GTT GGT GTA AAA TAGGG) and LIN17 (reverse, TTT GAA CGG GAT TTC TG), were carried out as described by Aransay *et al*, to amplify the variable region of the minicircle kDNA of *Leishmania* , the length of which varies in different *Leishmania* species. The product size is 650 bp and 720 bp for *L. major* and *L. tropica*, respectively. PCR was carried out in a 20 µl reaction mixture contained 1.5 mM MgCl2, 0.2mM dNTPs, 1 mM LINR4, 1 mM LIN17, 1 U Taq DNA polymerase (Bioneer ,Korea), 100pg DNA (2 µl), and 1X PCR buffer.(Corbett Research, Sydney, Australia). The reaction mixtures were incubated at 94°C for 5 min, followed by 30 cycles, each of 30 s at 94°C, 30 s at 52°C and 40 s at 72°C, and then a final extension at 72°C for 5 min. PCR products were electrophoresed in 1.2% agarose gel. Reference strains *L. tropica* (MHOM/IR/89/AR2) and *L. major* (MHOM/IR/54/LV39) were used as positive controls. Sample from lesion with known skin conditions other than CL served as negative control.

Statistical analysis

All data were analyzed by SPSS version 13.5(SPSS Inc.,Chicago,IL) using chi square and chi square (exact sig). The degree of agreement between PCR and Microscopy was determined by calculating Kappa (k) value. PCR products of two isolates of *L.tropica* and *L.major* were prepared and have been sequenced and submitted in DDBJ Gen bank .

This study was approved by the ethical committee of Kashan University of Medical Sciences, Iran.

RESULTS

The analysis of results showed that the mean age of the patients was 40.3 ± 21.6. Out of 130 suspected patients, using Microscopic examination, 87 (66.9%) cases were diagnosed positive, while by PCR with KDNA primer, 98 (75.4%) cases were diagnosed positive.

13 (13.3 %) cases were identified as negative by microscopic method, but positive through PCR(table 1). Kappa value was 0.759 (P<0.001).

Out of the 98 positive PCR, 70 (71.4%) cases showed 760 bp band and 26 (26.6 %) cases identified as *L.tropica* and *L.major* respectively (Figure 1). In two (2 %) cases both bands related to *L.tropica* and *L.major* were observed and it was identified as mix, since in cases that several wounds was present, from all of them serosity was collected and DNA was extracted.

CL was present in 46 (73%) women and 52 (77.6 %) men (P=0.54). The highest frequency of positive PCR was observed in age group 20-39 years (87%) and the lowest rate was detected in age group 80 and over (50%) (P=0.06). From the 98 positive PCR, the highest frequency of CL was observed in individuals with elementary education level (50%) and the lowest rate (10.2 %) was seen in individuals with college education (P =0.02). The main characteristics of *Cutaneous Leishmaniasis* according to *Leishmania* species were shown in (table 2) .

Out of 70 *L.tropica* cases, 59 (84.3%) and 11 (15.7 %) cases were dry and wet wound respectively , whereas in 26 of *L. major* ,14 cases (53.8 %), and 12 cases (46.2 %) were dry and wet wounds respectively. The difference was statistically significant (P=0.005). (Table 2). In regard to the of form of CL lesions , out of 70 CL patients identified as *L. tropica*, 46 (65.8 %) cases were papular or pustular and 15 (21.5 %) cases had wounds . But from 26 patients with *L.major* , 12 (46.1 %) cases had wound and 53.8% had papular or pustular lesions (P= 0.1) .The majority of the *L.tropica* or *L. major* had 1 to 2 wounds, but in 3 cases of *L. major* (11.5 %) there were more than 7 wounds. The highest rate (57.7 %) of *L.major* was in fall and the lowest rate (3.8%) was in spring, but *L.tropica* was detected in all seasons. (P= 0. 003)

Identifying the Leishmania species can be important for effective treatment and the design of an appropriate and efficient programme for the control of the disease[20].

Therefore, applying a highly sensitive method to diagnose the disease and then using effective treatment of the patient definitely help to prevent the spread of the disease and decrease in source of disease. Since Kashan is a tourist attracting location, it is necessary to create a safe and healthy environment for the tourists.

Based on the results of this study, out of 130 suspected patients, 87 (66.9%) cases were diagnosed positive by microscopy, whereas 98 (75.4%) %) cases were diagnosed positive by KDNA –PCR.,

The results showed that there was a relatively good concordance between KDNA –PCR and parasitological results (k= 75.9%) .

It is worth nothing that in the present study, PCR was positive in 13 cases whease they were identified as negative by direct microscopy (Table 1). However, ITS PCR-RFLP assay missed 9 of the 60 confirmed microscopy smears and showed false negatives results[14]. The Kappa value was 61.8 % which was less than the results of this study[14]. In present research the results of microscopic technique were in agreement with what was reported by Pourmohammadi (65.5%)[21] and Kumar (76%)[16]. The microscopy results reported by Fuladi and Khademvatan were 38.4% and 46.7% respectively, that is less than the results of this study. Khademvatan examined 216 suspected patients for CL in the Ahvaz using Mini -exon-PCR method and reported the frequency of 70.3%[23].The rate of infection by KDNA- PCR in the present study was similar to the results by Mini-exon-PCR in the Ahvaz[23].

According to the results of research conducted by Pourmohammadi and kumar etal, the rates of 93.6% and 87.5%have been reported in Shiraz (Iran) and India, respectively[21, 16]. These results are higher than the present research, probably due to the larger sample size, kits used and better diagnosis of physician. Farahmand examined 200 suspected patients for CL by KDNA-PCR method and found that 50 cases (25%) were positive[24] . Fuladi reported the rate of CL by PCR equal to 55.5% [22]. These results were lower than

Table 1. Relationship between PCR results with grading of *Leishmania amastigote* numbers on Giemsa stained slides

PCR	Grading of *leishmania* parasites					Total
Result	Negative	+1	+2	+3	+4	
Positive	13 (13.3)	15 (15.3)	14(14.3)	17(17.3)	39 (39.8)	98 (100)
Negative	31(96.9)	0	0	0	1 (3.1)	32 (100)
Total	44 (33.8)	15 (11.5)	14(10.8)	17(13.1)	40 (30.8)	130(100)

Table 2. The main characteristics of *Cutaneous Leishmaniasis* according to *Leishmania* species

Leishmania sp. identification.	*L.tropica* No (%)	*L.major* No (%)	Mix No (%)	Total No (%)
	70 (71.4)	26 (26.6)	2(2)	98 (100)
Type of sore				
Dry	59 (84.3)	14(53.8)	2(100)	75 (76.5)
wet	11 (15.7)	12 (46.2)	0	23(23.5)
total	70 (100)	26 (100)	2(100)	98 (100)
Lesion location				
Hand	56 (80)	9(34.6)	0	65(66.3)
Legs	6 (8.6)	13(50)	2(100)	21(21.4)
Other parts of body	8 (11.4)	4(15.4)	0	12(12.3)
total	70 (100)	26(100)	2(100)	98 (100)
Study place				
Ravand	30 (42.9)	3(11.5)	0	33 (33.7)
Fin	11(15.7)	0	1(50)	12 (12.2)
City center	27(38.6)	14(53.9)	1(50)	42(42.9)
Rural area	2(2.8)	9(34.6)	0	11(11.2)
Total	70 (100)	26 (100)	2(100)	98 (100)

the rate reported by the present study. In this study, from the total of 98 positive cases diagnosed by PCR, the highest frequency was observed in age group 20 to 39 years and the least frequency was in age group 80 and over (P=0.06). This result is in agreement with the result of study reported by Farahmand and Khirandish[24, 25]. This age group is more exposed to the risk of affliction due to the work condition. However, Marghehee[12], reported that the most rate of the infection was in age group less than 10 years.

The frequency of infection in men and women was similar, this results is in agreement with the result of Hajjaran[14]. Farahmand's study, however reported a significantly higher rate of infection for men compared to women[24]. The frequency of infection in individuals with college education was significantly less than the less educated subjects (P= 0.02) due to the more health care behavior..There was significant relation between Season and *Leishmania species* (P=0.003). In the present study, out of the 130 suspected patient for CL , 98 cases were diagnosed positive by KDNA- PCR method, that 70 (71.4%) cases were *L. tropica*, whereas 26 (26.6 %) cases were identified as *L.major* and 2 cases (2 %) were mix, whereas Shiee using ITS PCR-PFLP reported that 58 (92.1%) cases were *L.tropica* and 5 (7.9%) cases were *L.major* (19). According to the results conducted in Aran and Bidgol Kashan, out of 14 cases examined, 74 % of human isolates and the majorities of the rodent cases were *L.major* (18). In regard to the location of study, the majority of the cases of *L.tropica* were observed in Ravand and city center, whereas the *L.major* was found in city center and rural region.

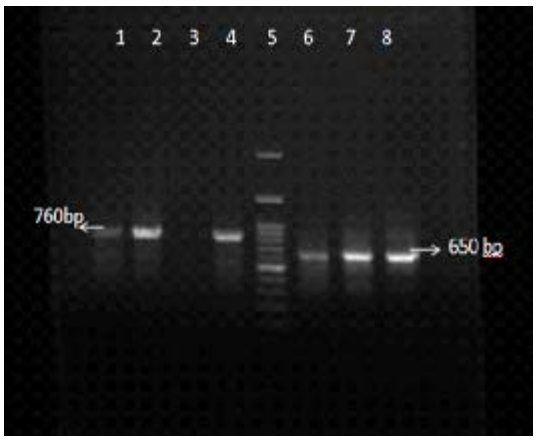

Fig. 1. Gel electrophoresis of PCR products of *Leishmania* isolates using LINR4 and LIN17 primers.. Lanes 1 and 2, *L.tropica* of patients' samples. Lane 3 Negative control. Lane 4 positive control of *L. tropica* (MHOM/IR/89/AR2). Lane 5 Marker 100bp ,Lane 6 positive control of *L.ma*jor ((MHOM/IR/54/LV39). Lane 7-8 *L.major* patients' samples. Two isolates of *L.tropica* and *L.major* sequenced and submitted in DDBJ Gene bank, with accession number AB901375 and AB901376 respectively.

Therefore, considering the fact that Isfahan province is the endemic ZCL focus and based on the present research findings both species of *L.tropica* and *L.major* are present in the Kashan, it seems necessary to take appropriate measure to control both form of the disease. It have been reported predominant species of CL In Ahwaz and Shiraz,Iran , *L.major*[9,15] ,while in Mashhad and Khorammabad of Iran were *L.tropica* [25,26]. The predominant species of CL In Turkey were *L.tropica* , *L.infantum*[27], in India *L.tropica*[16] and in Israel *L.major* ,*L.tropica* respectively[11] .

CONCLUSION

Based on the results of the present research, both species of *L.tropica* and *L.major* are prevalent in the Kashan. Since this city is an endemic focus, and CL is on the rise, appropriate measure for controlling ACL and ZCL is necessary. In addition the majority of CL was observed in illiterate individuals, thus, health educational to prevent the disease is recommended. Also, KDNA-PCR assay using serosity for accurate and quick diagnosis of cutaneous leishmaniasis is recommended.

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