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Short communication

High infection frequency, low diversity of *Leishmania major* and first detection of *Leishmania turanica* in human in northern Iran

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ABSTRACT

Smears of suspected patients infected with zoonotic cutaneous leishmaniasis (ZCL) were stained and examined under a light microscopic observation. DNA of parasites within human ulcers was extracted directly from their smears. Nested PCR was used to amplify a fragment containing the internal transcribed spacers of the ribosomal RNA genes (ITS-rDNA) of *Lesihmania* parasites in human from Turkemen Sahara located in the northeastern part of Iran. Based on RFLP method by digesting BsuRI restriction enzyme and more precisely sequencing of DNA ITS-rDNA was shown to be species-specific. The infection rates of *Leishmania* parasites were high with 154 (93.9%) infections out of 164 suspected patients using microscopic observations. Only from 128 suspected patients out of 164, ITS-rDNA fragments were amplified and 125 samples had enough DNA to digest BsuRI restriction enzyme and do DNA sequencing. The Nested PCR assays detected not only *Leishmania major* but also *Leishmania turanica* for the first time, another parasite of the great gerbil in human. The density of *L. major* was high but the diversity was low with only 2 haplotypes. The overall ratio of *L. major* (123 infections) to *L. turanica* (2 infections) was significantly higher (Chi-squared test: $p < 0.05$). Infections of *L. turanica* are not reported only and/or not known to cause human disease. Our analytical framework conveys a clear understanding of both *L. major* and *L. turanica* which can only be approved as causative agents of ZCL by more extensive sampling and followed by standardized molecular diagnosis.

1. Introduction

The incidence and prevalence rates of ZCL have recently had a sharp increase and outspread in the world (Alvar et al., 2012) and many parts of Iran and substantially in several areas of Turkemen Sahara, in Golestan province, one of the most important endemic foci of Zoonotic Cutaneous Leishmaniasis (ZCL). ZCL originally appears as a disease of gerbils. *Leishmania* parasites are transmitted by sandflies that live and breed in gerbil burrows (Mirzaei et al., 2013; Ready, 2013).

Identifications of *Leishmania* species are important because of clinical and epidemiological reasons. As a result of morphological similarities, *Leishmania* parasites species could not be firmly identified using conventional methods.

The first objective of our research is to perceive the possibility of *L. major* detection in humans and also characterization of *Leishmania* parasites in a well known endemic ZCL of Iran. The last, is to find out the duration feasibility of mammals *Leishmania* species in human such as *L. turanica*, *L. gerbilli* and *Leishmania* species near *L. gerbilli* which were found previously in Iranian *Phlebotomus* and/or in rodents (Bordbar and Parvizi, 2014; Mirzaei et al., 2013; Parvizi and Ready, 2008). There is no record for detecting *L. turanica* and *L. gerbilli* in human.

2. Materials and methods

2.1. Sampling and DNA extraction of Leishmania from suspected patients

Samples were prepared from suspected patients as described before (Mirzaei et al., 2013) and collected during 2-year from 22 surveyed villages of Golestan province. DNA extraction of humans were performed as previously explained (Bordbar and Parvizi, 2014; Parvizi and Ready, 2008).

2.2. Identification of Leishmania parasites by PCR amplification, sequencing and phylogenetic analysis

The ITS1-5.8S rRNA fragment was 480 bp (including primers) which were subjected to nested PCR, RFLP and Sequencing. MEGA

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Table 1
Directly observed score ulcers of 138 patients, detailed features of patients' lesion based on Clinical features and Skin lesion site (M: male; F: female).

Number of lesion	Number of patients											Total
	Collection sites		Sex		Total of samples with each lesion (%)	Skin's lesion type (sign and symptom)		Toll place				
	Maraveh Tapeh	Gonbad Kavous	M	F		Dry	Wet	Body	Leg	Hand	Face & neck	
1	59	19	33	45	78(56.5)	19	59	2	19	20	37	78
2	24	8	19	13	32(23.2)	17	47	3	17	19	25	64
3	8	3	5	6	11(8)	10	23	3	7	10	13	33
4	6	1	3	4	7(5.1)	9	19	0	12	7	9	28
5	1	2	2	1	3(2.2)	4	11	1	6	3	5	15
6	5	0	3	2	5(3.6)	6	24	2	13	8	7	30
7	2	0	2	0	2(1.4)	5	9	3	5	5	1	14
Total	105	33	67	71	138	70	192	14	79	72	97	262
Undefined[a]	19	7	11	15	26	8	18	–	–	–	–	26
Total	124	40	78	86	164	78	210	–	–	–	–	288

[a] Samples were collected from regional public health center.

4.0 software was used for phylogenetic analyses (Bordbar and Parvizi, 2014; Parvizi and Ready, 2008).

3. Results

3.1. Microscopic observation of Leishmania parasites in suspected patients

Among 164 suspected people (124 Maraveh Tapeh and 40 Gonbad Kavous) having acute lesions, smears were taken and examined microscopically for *Leishmania* infections. 128 (78%) out of 138 (84.14%) freshly Giemsa-stained directly sampled were identified as *Leishmania* positive and prepared from people who were locality examined as present patients while 26 (15.85%) specimens was screened from remained smears in regional public health services of Gonbad Kavous and Maraveh Tapeh in which there was no adequate information about the locations of inhabitants (164; 93.9%).

This comprehensive inquiry has shown that the number of people who had *Leishmania* lesions in their bodies were more in Maraveh Tapeh (105) than Gonbad Kavous (33).

3.2. Leishmania parasites identification with RFLP and sequencing of ITS-rDNA fragment

DNA of 262 freshly ulcers were directly sampled from 138 suspected patients and screened to amplify ITS-rDNA of *Leishmania* parasites.

128 suspected patients of 164 individuals were *Leishmania* positive by amplifying ITS-rDNA fragment in agarose gel 1.5%. 125 *Leishmania* positive out of 128 were digested by *B*suRI (HaeIII) and sequenced to identifying species and characterization of *Leishmania* parasites. Three out of 128 samples did not have enough DNA to digest by *B*suRI (HaeIII) and/or sequence. 123 of 125 positive samples were firmly established to identify as *L. major* and two others as *L. turanica* for the first time (Table 1).

120 out of 123 *L. major* had one common haplotype which previously distinguished from sandflies, rodents and humans in this region and elsewhere (GenBank accession no. EF413075). Three remaining *L. major* had a unique haplotype which is a novel haplotype (GenBank accession no. KF152937) with two nucleotides different from a common haplotype.

The ITS-PCR products of two different sequenced species were compared with GenBank sequences in case of similarity at which 120 *Leishmania* sequences had significant homology and were found as *L. major* (approximately 462 bp including primers; 123 infections).

The majority of *Leishmania* species were identified as *L. major* excepting two *Leishmania* species which were remained ambiguous (*L. tropica* or *L. turanica*).

Two suspected isolates were again examined by Nested PCR-PFLP and sequenced several times. The sequences finally was again identified as *L. turanica*, haplotype TurkH03 (GenBank accession no. EF413078) which previously isolated and identified from sandflies and rodents in this region and elsewhere (Rouhani et al., 2014; Sharbatkhori et al., 2014; Jafari et al., 2013; Mirzaei et al., 2013)

4. Discussion

In our current investigation of *Leishmania* detection in humans of Turkemen Sahara in Golestan province, *L. major* (98.4%) and for the first time *L. turanica* (1.6%) were identified. 125 out of 128 ITS-rDNA *Leishmania* positive were used for RFLP and sequencing because three samples did not have enough DNA to do RFLP or sequencing. *L. major* was high in density with 123 out of 125 and *L. turanica* had low density with two amplified samples. These species of *Leishmania* parasites were sequenced and typed molecularly. The overall ratio of *L. major* infections was significantly higher (Chi-squared test: $p < 0.05$) than *L. turanica* infections. Previously, we showed the more diversity of ITS-rDNA gene of parasites in *L. major* than other mammals' *Leishmania* (*L. turanica* and *L. gerbilli*) in sandflies (Parvizi and Ready, 2008; Mirzaei et al., 2013).

Among 123 patients have been infected with *L. major*, two haplotypes of ITS-rDNA gene were identified by aligning sequences (Fig. 1). 120 out of 123 patients had haplotype TurkH01. This haplotype is a prevalent haplotype of *L. major* which was previously reported from humans, sandflies and gerbils originating from Iran and elsewhere (Hamarsheh, 2011; Bordbar and Parvizi, 2014). The second haplotype TurkH02 of *L. major* with 3 patients was found to be novel (GenBank accession no. KF152937). These two haplotypes with those sequences of the ITS-rDNA has already submitted in GenBank and were aligned for phylogenetic analysis in MEGA (Fig. 1).

In phylogenetic tree, most *Leishmania* species of old world including our two haplotypes were grouped in three main branches. The first prevalent main branch (including species-specific subgroups) containing agents of zoonotic cutaneous leishmaniasis and mammalian infections, and the second one consists of agents of visceral leishmaniasis and the third one is related to the agents of cutaneous leishmaniasis (Fig. 1).