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Short Communication

Interleukin 4 (IL-4) gene promoter polymorphisms in *Rhombomys opimus*, the main reservoir of zoonotic cutaneous leishmaniasis

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ABSTRACT

Great gerbils (*Rhombomys opimus*) are the most common gerbils in center to northeast of Iran as well as central Asia and serve as reservoirs for the zoonotic agents, including *Leishmania major*, the principal etiologic agent of zoonotic cutaneous leishmaniasis (ZCL). The outcome of *L. major* infection in gerbils is not uniform. Among several immune-related factors including cytokine genes, the polymorphism in interleukin 4 (IL-4) promoter gene showed a great impact on outcome and pathological symptoms of *L. major* infection at least in mouse model. In this study gerbils' IL-4 promoter gene polymorphism is assessed. Specific primers were designed to develop a PCR-based assay to amplify IL-4 promoter gene to possibly define IL-4 promoter gene polymorphism in great gerbil populations with a range of *Leishmania* infection and symptoms collected from different foci of the central, north and northeast regions of Iran. The results showed that the designed primers amplify 689 bp of the promoter gene. Sequence analysis of the promoter gene revealed five polymorphic sites assembly six haplotypes among the gerbil populations. Further studies are needed to assess whether or not the five polymorphisms cause different outcome phenotypes following infection with *L. major* in great gerbils. The data might be used to characterize the immune responses of *R. opimus* against *L. major* infection.

1. Introduction

Leishmaniases are parasitic diseases with a wide spectrum of clinical manifestations ranging from a self-healing skin lesion to a lethal visceral form of disease. According to WHO estimate the prevalence of leishmaniasis is 12 million with 0.9–1.6 million new cases each year. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis and about one-third of cases are reported from the Americas, the Mediterranean basin, and western Asia from the Middle East to Central Asia [1]. Main reservoir of zoonotic cutaneous leishmaniasis (ZCL) due to *Leishmania major* is the great gerbils (*Rhombomys opimus*) which transmitted by sand fly bites. It is shown that the distribution of ZCL in central, south Asian countries and north-western China overlaps with the presence of great gerbils [2–4]. *R. opimus* is widely distributed in central and northeast parts of Iran with high rate of *Leishmania* infection which might reach to as high as 85–93% in some foci [5,6].

Leishmaniasis clinical manifestation depends upon *Leishmania* species and host genetic background which governs generation of the type of immune response [7]. IL-4 plays an important role in various biological activities including the type of immune response development, IL-4 polymorphism is reported from different populations; correlations between IL-4 polymorphism and different form of leishmaniasis such as visceral leishmaniasis (VL) and CL are reported [8]. In a study which was completed by the same group, it was shown that exposure of *R. opimus* populations to *L. major* resulted in a wide range of reactions; out of 194 gerbils which were examined, *L. major* and *L. turranica* were isolated from 80 (41.23%) of the animals, 55 (68.75%) of the infected animals showed no skin lesion (asymptomatic), and only 25 (31.25%) of the animals showed skin lesions (ear papules and nodular reactions) and even the rate of parasitemia was not uniform [9]. In the current study it was proposed to investigate the IL-4 gene polymorphisms and determine the differentiations of IL-4 gene between the great gerbil haplotypes/populations and also check a possible correlation to the outcome of *L. major* exposure in various *R. opimus* populations originated from wide geographical ranges in Iran, the information might help to explain the outcome of the disease in the main reservoir of the ZCL.

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2. Materials and methods

2.1. Rodent collection and identification

In the present study, 111 *R. opimus* were caught from approximately all known ecological niches in Iran where the great gerbils are distributed. The specimens originated from 11 localities in central, north, and northeast regions of the country. Animals were collected using Sherman live-trap baited with a mixture of walnut, cucumber, tomato, and bread dabbed with sunflower oil. Approximately 30–40 live traps per night were used in each location in monthly collections. The traps were set close to the gerbils' burrows entrance 2–3 h before the dusk and the traps were checked in the next morning after sunrise. A total of 56 female and 55 male *R. opimus* were caught. The captured animals were transported to the laboratory, anesthetized using a mixture of 150 mg/kg ketamine 10% and 15 mg/kg xylazine 2%, blood samples (200–1000 μL) were taken from the tail vein in tubes containing 20–30 μL anticoagulants and stored at −20 °C until use. Morphological evaluations were also performed while the animals were anesthetized. The genus and species of the captured rodents were determined by external morphological characteristics including color, length of head and body, ears, tail, hind feet, and skull, and grooves on the incisor teeth [10]. The edge of each ear and muzzles of the caught rodents were physically examined for the present of skin lesions such as papules and nodular reactions. All experiments on the rodents were performed according to the guidelines of the Ethical Board of Tehran University of Medical Sciences, Iran.

2.2. PCR amplification of IL-4 gene promoter region

Frozen blood samples were thawed and 200 μL of the blood samples were treated with proteinase K, and then the genomic DNA was extracted using G-spin Tissue Spin Kit (G-spin, South Korea) according to the manufacturer's instructions.

A set of primary forward and reverse primers were designed based on sequence homology of six rodent IL-4 sequences available in genbank including *Mus musculus, Rattus norvegicus, Sigmodon hispidus, Meriones unguiculatus, Peromyscus maniculatus* and *Cricetinae* sp. To design the forward primer, the two available data of *R. norvegicus* and *M. musculus* which the flanking region was complimentary of a conserved region of −650 to −700 bp from transcription start site of the gene were used. The reverse primer was designed using all the six rodents' sequences that the flanking region was complimentary of a conserved region of +60 to +80 bp exon1 of IL-4 gene (Fig. 1).

The sequences of the designed primers are as follow: OPIL4F2: 5′-ACCTCCMCMCTGATGCTGTAGTG-3′ and OPIL4R:5′-AYCTAGCTGGGGGRTGAGACC-3′. The primers amplify a fragment with approximate size of 800 bp. A touch-down PCR thermal program was carried out with the following profile: 95 °C for 3 min, followed by eight cycles of 30 S denaturation at 95 °C, 45 S annealing at 65–62 °C (dropped by 1 °C every second cycle), and 1 min elongation at 72 °C, then 28 identical cycles with annealing temperature which was set at 61 °C, and finally 10 min at 72 °C.

The presence of two poly-T regions in the beginning and middle of amplified fragment (Fig. 1) causes problems in DNA sequencing reactions by slippage leading to multiple peaks, a new internal primer set to amplify and sequence the locus of *R. opimus* which was designed and tested.

2.3. PCR and sequencing

Finally the set of OPIL4F: 5′-AGGCAGGCATTCCTCAGG-3′ and OPIL4R 5′-AYCTAGCTGGGGGRTGAGACC-3′ primers were used to amplify a region of 689 bp. The thermal program was performed as: 95 °C for 3 min, followed by 35 cycle of 50 S denaturation at 95 °C, 1 min annealing at 65 °C, 1 min extension at 72 °C, and finally 10 min at 72 °C. A subset of PCR products as representative of different Iranian great gerbil populations were sequenced in both directions. Sequencing was performed using an ABI 3730 sequencer machine (Bioneer, South Korea). The ambiguous sequences were corrected using Chromas program and the consensus sequences obtained using DNASTAR Lasergene (SEQMAN and EDITSEQ) and were submitted to GenBank (Table 1). The sequences were aligned using ClustalW to explore possible polymorphisms.

3. Results and discussion

PCR amplification of IL-4 gene promoter region was successfully completed on *R. opimus* collected samples. The most powerful primer set to amplify a single band of the expected size of 689 bp was OPIL4F and OPIL4R. The sequences of the locus were obtained for 13 samples as representative of the great gerbil subspecies or populations (Table 1). Sequence analysis revealed six IL-4 gene promoter region haplotypes among sequenced specimens. Sequence analysis of the samples revealed five single nucleotide polymorphisms (SNPs) assembly six haplotypes among the gerbil populations (Table 1). Four out of five SNPs (80%) were of transition type (A–G or T–C). The inter population genetic variations ranges from zero to 1%. There was no significant correlation between the haplotypes and the geographical origins or subspecies of the great gerbils but some specific geographical haplotype were seen.

Physical examination of the great gerbils' ears revealed that although all of the samples were collected from *R. opimus* which were exposed to *L. major* and/or *L. turanica* but about 77% of the animals showed no skin lesion (asymptotic) and only 23% of them presented skin lesions such as papule and/or nodule (symptomatic) (Table 1). Parallel studies performed by Hajjaran et al. [9 and unpublished data] showed that only 77% (10/13) of the specimens were infected to *Leishmania* spp. whereas among the asymptomatic animals, 70% (7/10) were infected with *Leishmania* (the ones which are marked by * in Table 1).

The results of alignment showed the approximate position of 5′-UTR and ORF of the gene (Fig. 1). Approximate 5′-UTR, ORF and partial promoter are 60, 27 and 600 bp, respectively.

In the current study, five single nucleotide polymorphisms (SNPs) in the IL-4 promoter gene of the great gerbil populations were identified which might be related to the pathogenesis and clinical outcome of *Leishmania* infection in this species of reservoir animals. Of course the impact of each genotype on the outcome of the disease should be tested independently because all of the mutations in cytokine genes might not have a similar influence on the outcome of the infection. There is a report that in human IFN-γ + 874 A → T polymorphism influences the progression of the disease towards chronic CL while IL-4 −590 C → T polymorphism increases the risk of developing CL [8]. Of course, this is a single report from a specific endemic area and might not be a reflection of the whole story. In spite of having found five SNPs at 77, 103, 263, 324 and 583 nucleotide positions, no SNP was seen in −590 position of *R. opimus* IL-4 promoter gene.

In another study performed in human VL, it was shown that IL-4 polymorphism but not IL-9 influences VL incidence and clinical phenotypes due to *L. donovani* infection in human [11]. In contrast, IFNGR1 polymorphism was linked and associated to post-Kala-azar dermal leishmaniasis (PKDL) but not VL. The authors concluded that polymorphism in a type 2 cytokine gene influences underlying susceptibility to VL, whereas IFNGR1 is specifically related to susceptibility to PKDL. In a study performed in CL due to *L. braziliensis*, it was shown that a SNP in macrophage inhibitory factor (MIF-173C) favored CL infection and disease progression [12].

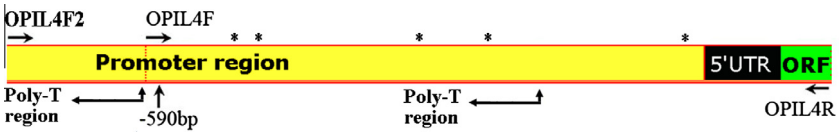

Fig. 1. Details of a partial promoter region and partial Exon1 of IL-4 gene in *R. opimus*. The primers OPIL4F and OPIL4R were designed to amplify this fragment. The length of 5′-UTR, partial promoter and partial ORF was obtained about 60, 600 and 27 bp, respectively, approximate position of −590 bp is marked. Nucleotide polymorphisms between OPIL4F and OPIL4R primers are shown with "*". Two poly-T regions are positioned nearby the −590 and middle of the locus.

Table 1
Details of *R. opimus* specimens and the five single nucleotide polymorphisms (SNPs) throughout 689 bp of IL-4 promoter gene. The star marked specimens * were infected with *L. major* and/or *L. turanica* [9] and unpublished data).

Subspecies	Longitude–latitude	Genbank accession number	District/province	Symptomatic/asymptomatic (S/A)*	Haplo-type	SNPs position				
						77	103	263	324	583
Sargadensis	32.6–52.0	JX888484	Badrood/Esfahan	A*	I	G	G	C	G	G
Sargadensis	33.3–52.5	JX888480	Habibabad/Efsfahan	A*	II	G	G	C	A	T
Sargadensis	36.0–54.3	JX888483	Damghan/Semnan	A	III	G	A	C	G	T
Sargadensis	37.0–57.1	JX888481	Esfarayen/Khorassane-Shomali	A*	III	G	A	C	G	T
Sargadensis	33.3–52.5	JX888477	Habibabad/Esfahan	A*	IV	G	G	C	G	T
Sodalis	36.2–60.5	JX888475	Sarakhs/Khorassane-Razavi	A*	IV	G	G	C	G	T
Sodalis	37.5–55.3	JX888479	Maraveh-Tappeh/Golestan	A*	IV	G	G	C	G	T
Sargadensis	37.2–57.7	JX888485	Shirvan/Khorassane-Shomali	S*	IV	G	G	C	G	T
Sodalis	36.2–60.5	JX888474	Sarakhs/Khorassane-Razavi	S*	IV	G	G	C	G	T
Sodalis	36.2–60.5	JX888478	Sarakhs/Khorassane-Razavi	A	IV	G	G	C	G	T
Sargadensis	NA	JX888482	Lab – hybrid	S*	IV	G	G	C	G	T
Sodalis	36.2–60.5	JX888476	Sarakhs/Khorassane-Razavi	A*	V	A	G	T	G	T
Sodalis	37.3–54.5	JX888473	Gonbad-e Qabus/Golestan	A	VI	A	G	C	G	T

In the current work, a different clinical symptom was shown in the gerbils' population. The number of asymptomatic infected animals was more than twice than symptomatic ones. However, due to low number of available sequences, and a considerable SNPs/haplotypes within the specimens, it was not possible to correlate between the SNPs/haplotypes and the outcome of *Leishmania* exposure in the gerbils. It is worth to further elucidate a possible correlation. Laboratory rodents proved to be invaluable for ascertaining the function of genes involved in the immune responses to the infection [13]. Approaches to develop an effective vaccine against leishmaniasis were aimed to develop a vaccine which is capable of induction of Th1 response with low amount of IL-4 production similar to individuals with history of CL [14,15].

In conclusion, further studies needed to elucidate SNPs impressing the disease in the reservoirs particularly in *R. opimus* which is the main ZCL host. The generated data might lead to develop a novel control strategy against ZCL.

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