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Original Article

Intraspecific Variation in *Leishmania major* Isolated from Different Forms of Zoonotic Cutaneous Leishmaniasis

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Abstract

Background: Zoonotic cutaneous leishmaniasis (ZCL) is a polymorphic disease which may show various clinical manifestations. Although genetic variability of the parasite is suggested to be one of the factors influencing clinical manifestations in leishmaniasis, no data exist regarding genetic polymorphism of *Leishmania major*. Therefore, determination of genetic variation within the species of *L. major* isolated from different cases of ZCL in Isfahan, Iran and its relation to clinical manifestation of the disease is investigated.

Methods: The internal transcribed spacers (ITS) in the ribosomal operon of 9 isolates of *L. major* from three clinically different forms of ZCL were amplified by polymerase chain reaction (PCR), followed by the digestion of the PCR product with two restriction enzymes. The profiles were visualized in agarose gel under UV light.

Results: The PCR product obtained for all isolates was about 1060 bp in size. Restriction analysis of ITS with both enzymes showed identical fragment patterns in samples isolated from clinically similar forms of ZCL, but highly diverse fragment patterns in those isolated from clinically different forms.

Conclusion: *L. major* causing ZCL in Isfahan is genetically a highly polymorphic species and a correlation may exist between genetic heterogeneity of the parasite and the clinical manifestation of the disease in human.
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Keywords ● *Leishmania major* ● Polymorphism

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Introduction

The factors influencing the clinical manifestations of leishmaniasis including Zoonotic cutaneous leishmaniasis (ZCL) which is a polymorphic disease are not completely understood. However, genetic variability of parasites is one of the most likely factors and is currently the subject of much interest and controversy.[1,2] This genetic heterogeneity may produce different phenotypes which can be associated with the diversity of clinically important manifestations. In the present study restriction fragment length polymorphism (RFLP) analysis of the amplified internal transcribed spacer (ITS) in the ribosomal operon was used to investigate the genetic variations among *Leishmania major* isolates and correlated

the findings with the clinical manifestations of ZCL in Isfahan, Iran, where ZCL is a major public health problem.

Materials and Methods

Leishmania promastigotes were isolated from skin lesions of three patients with scaly flat ulcers, three patients with volcano-shape lesions and three patients with papular forms of ZCL living in rural district north of Isfahan, Iran and subcultured in NNN and RPMI-1640 culture media.[3] They were identified as *L. major* by recombinant DNA probe as compared with the reference DNAs.[4] DNA was extracted from stationary phase promastigotes with phenol/chloroform according to standard procedures.[5] The whole internal transcribed spacer (ITS) in the ribosomal operon was amplified with the primers LITSV (5′-ACACTCAGGTCTGTAAAC) and LITSR (5′–CTGGATCATTTTCCCATG) according to El Tai et al.[5] The PCR products containing the amplified ITS region were digested with the restriction enzymes, BstU1 and Cfo1, (New England Biolabs Inc, UK) under the conditions recommended by the supplier. Amplification and restriction products were separated in a 1% agarose gel and visualized under ultraviolet light after staining with ethidium bromide.[5] Fragment sizes were estimated by comparison with bands of a DNA molecular weight markers (Hyper ladder I, 1Kb DNA ladder, bioline, biology laboratory supplies and reagents, UK). Throughout the study, genomic DNA from reference strain *L. major* (MHOM/IL/85/LEM769) was used as positive control and distilled water as negative controls.

Results

The PCR products obtained for all nine isolates (Fig 1 A, lanes 3-11) and the reference strain (Fig 1 A, lanes 2 and 12) were approximately 1060 bp in size for the entire ITS amplicon which was indicative of leishmania. Amplification product were not observed when distilled water was used instead of template DNA in the negative controls (Fig1 A, lane 1). Figure 1 B and C illustrate restriction patterns of amplified ITS region of different isolates with restriction enzymes BstU1 and Cfo1. The resulting pattern of bands depends on both chosen restriction enzyme and type of isolates. However, using either of enzymes, the patterns of the bands could be classified into three groups according to the number and localization of bands. Interestingly, this classification also corresponded to the shape of skin lesions from which promastigotes were isolated. The digestion of isolates of scaly flat ulcers of group I with BstU1 showed two bands (Fig 1 B, lanes 1, 2, 3) and the isolates of volcano–shape lesions of group II showed three distinct bands (Fig 1 B, lanes 4, 5, 6). However, in isolates from papular forms of skin lesions of group III, the restriction enzyme BstU1 did not cut amplified ITS region in one isolate (Fig 1 B, lane 8), but showed two bands in two other isolates (Fig 1 B, lanes 9, 10). In the reference strain, 3 bands were observed (Fig 1 B, lane 7) which were almost similar to group II isolates from volcano-shape lesions, the typical form of ZCL, but different from group I and III (the atypical forms of ZCL). The digestion of the same nine isolates and reference strain with Cof1 demonstrated three easily distinguishable digestion

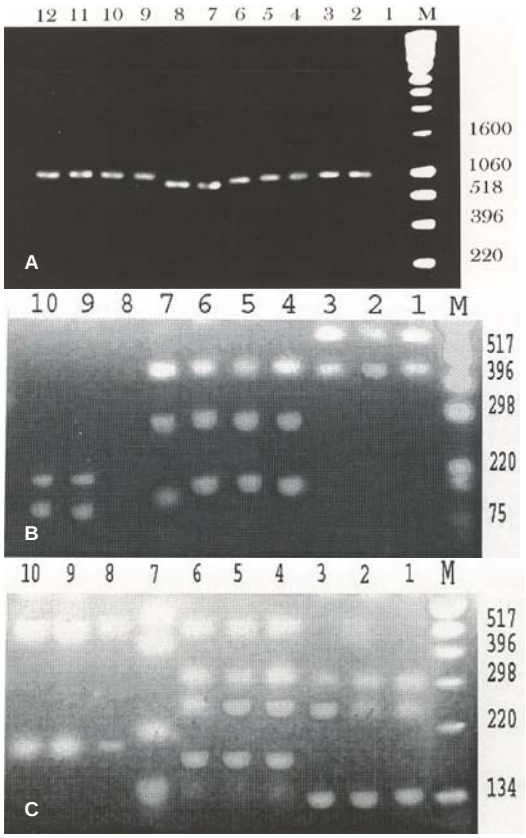

Figure 1: A ITS-PCR amplification products of *L major* isolated from different forms of ZCL. Lane M: 1 kb ladder DNA size marker. Lane 1: negative control. Lanes 2 & 12: reference strain (positive control). Lanes 3 to 5 scaly flat ulcers. Lanes 6 to 8 volcano shape lesions. Lanes 9 to 11 papular forms. **B**: Restriction of amplified ITS region of *L major* isolated from different forms of ZCL with the enzyme BstU1. Lanes 1 to 3 scaly flat ulcers (group I). Lanes 4, 5, 6 volcano shape lesions (group II). Lanes 8 to 10 papular forms (group III). Lane 7 reference strain. C: Restriction of amplified ITS region of *L major* isolated from different forms of ZCL with the enzyme Cof1. Lanes1 to 3 scaly flat ulcers(group I). Lanes4, 5, 6 volcano shape lesions (group II). Lanes 8 to 10 papular forms (group III). Lane 7 reference strain.

patterns from scaly flat ulcers of group I which had three bands (Fig 1 C, lanes 1, 2, 3) and group II from volcano–shape showed 4 intense and a faint band measuring about 134 bp which was difficult to photograph (Fig 1 C, lanes 4, 5, 6). Isolates of group III isolated from popular forms consisted of 2 bands (Fig 1 C, lanes 8, 9, 10). The restriction pattern of reference strain comprised of 4 bands which was closer to isolate of group II with respect to the length size of bands (Fig 1 C, lane 7).

Discussion

The results of this study revealed remarkable variations in the RFLP banding patterns of the ITS region of *L. major* isolated from patients with clinically different forms of ZCL in a single geographical area. In accordance with the present finding, considerable heterogeneity has been reported within the ITS–RFLP of strains of *L. tropica,*[6] *L. aethiopica*,[2] *L. donovani*,[7] and kDNA strains of *L. mexicana*,[1] *L. infantum*,[8] and *L. donovani*.[5] The results of this study indicate that the genetic variability observed among different isolates of *L. major*, may represent a heterogeneous group of organisms as observed in some other leishmania species. Although leishmania species have a clonal population structure, a possible explanation for this diversity is that, their sexual recombination is not excluded. Delgado as well as Banules and their colleagues have evidenced a hybrid formation in leishmania and occasional bouts of genetic exchange or hybridization.[9-11] Obviously occasional or rare bouts of sexual recombination in a normally asexual organisms can have a profound effect on the extent of genetic diversity.[12]

ZCL is endemic in large proportion of Afghani refugees living in the rural area of Isfahan for the past 25 years. Since transmission from person to person by sandflies in ZCL could not be ruled out, those who were infected could possibly be a new source of infection for both reservoir host and man.[11] This could lead to the possibility of mixed infection, which by itself may increase the chance of hybrid formation between two geographically different strains and consequently formation of a new type of skin lesion. It has been reported that a new form of cutaneous leishmaniasis in Venezuela is the result of hybrid formation between *L. braziliensis* and *L guyanensis.*[9] In the present study, a clear correlation was observed between the ITS-RFLP patterns of isolates and clinical appearance of skin lesions. These observations are in agreement with the reports of Brzuna-cruz et al on *L. mexicana*,[1] but inconsistent with the reports of Schonian, Schonian and his colleagues who showed that genetic variability is correlated with geographic origin of the isolates rather than clinical manifestation of the disease in *L. aethiopica*,[2] and *L. tropica*.[6] Although, it is not easy to explain this controversy, it is certain that the clinical manifestation of leishmaniasis is the result of interaction between molecules of both parasite and host cells about which very little is known.[13]

It is likely that more than one molecule is implicated in this process and it remains to be understood which genetic markers are most appropriate to ascertain the major genes determining parasite virulence and consequently the clinical manifestation of disease and its relation to geographic origin.[13,14] All nine isolates of *L. major* of this study, except of group II, when digested with BstU1 differed in their ITS–RFLP pattern from that of reference strain of *L. major*. This is in accord with the finding of Brzuna–Cruz et al. who showed that Mexican isolates of *L. mexicana* differed in endonuclease digestion pattern from that of the WHO reference strain.[1] These differences could be due to the variation of geographical origin or their evolutionary antiquity.[1,2,6] Furthermore, the reference strains of leishmania are isolated and grown in repeated *in vitro* cultures or in laboratory animals. These conditions provide an opportunity for clonal selection and consequently, the reference strains maintained *in vivo* or *in vitro* in the laboratory can differ from those isolated from naturally infected humans' host. Moreover, some of the genetic variability could be due to multiple infection and population heterogeneity when leishmania isolated from naturally infected host, since the isolates are not cloned prior to analysis. However, in the present study the use of uncloned leishmania populations was essential in order to stick with the actual situation prevailing in the filed.

Conclusion

The results of this study are suggesting that *L. major* causing ZCL in Isfahan is genetically a highly polymorphic species. Although clinical manifestations of leishmaniasis has been attributed both to differences of host response and genomic heterogeneity of the parasites,[13] the results of this study, however, are in favor of an important role for genomic heterogeneity of *L. major* in determining the clinical characteristics of ZCL. Additional *L. major* isolates from patients, reservoir hosts and vectors from different locations are needed to consolidate these findings with the degree of genetic heterogeneity and its relation to clinical manifestation and geographical distribution of the disease.

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