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Original Article

The Problem of Mixing up of *Leishmania* Isolates in the Laboratory: Suggestion of ITS1 Gene Sequencing for Verification of Species

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(Received 25 Apr 2010; accepted 22 Jan 2011)

ABSTRACT

Background: Leishmaniasis is endemic in Iran. Different species of *Leishmania (L.)* parasites are causative agents of this disease. Correct identification of *Leishmania* species is important for clinical studies, prevention, and control of the diseases. Mix up of *Leishmania* isolates is possible in the laboratory, so there is need for verification of species for isolates of uncertain identity. Different methods may be used for this purpose including isoenzyme electrophoresis and molecular methods. The isoenzyme electrophoresis, due to its drawbacks, is feasible only in specialized laboratories while molecular methods may be more feasible. The aim of this research was to study the application of the internal transcribed spacer 1 (ITS1) sequencing method, in comparison to isoenzyme electrophoresis method, for verification of *Leishmania* species.

Methods: Six *Leishmania* isolates were received from different research institutions in Iran. The species of these isolates were known by donating institution according to their isoenzyme profile. The species of these isolates were re-identified in Pasteur Institute of Iran by PCR amplification of ITS1 followed by sequencing and comparison of these sequences with *Leishmania* sequences in GenBank. Isoenzyme electrophoresis was performed for confirmation of the results of ITS1.

Results: ITS1 sequence showed that some isolates were mixed up or contaminated with *Crithidia*. Isoenzyme electrophoresis confirmed the results of ITS1 sequences.

Conclusion: ITS1 sequencing is relatively more feasible than the traditional isoenzyme electrophoresis method and is suggested for verification of *Leishmania* species.

Keywords: Leishmania, Crithidia, *Internal transcribed spacer (ITS), Isoenzyme electrophoresis*

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Introduction

The leishmaniases are a spectrum of diseases of humans and other mammals that are endemic in 88 countries with an estimated yearly incidence of l-1.5 million cases of cutaneous leishmaniasis and 500000 cases of visceral leishmaniasis (1). Cutaneous and visceral leishmaniases are endemic in Iran (2, 3). Different species of *Leishmania (L.)* parasites are causative agent of these diseases in Iran (4-6). *Leishmania* species differ from each other from many aspects including clinical presentation of the disease, their vectors and reservoirs, and their epidemiological criteria. Hence, identification of *Leishmania* species is important in *Leishmania* studies including clinical research, evaluation of vaccines and drugs, epidemiology, prevention and control of the diseases (7).

Different methods are used for species identification of *Leishmania* parasites including: use of monoclonal antibodies (6), study of isoenzyme (8), and molecular methods (9). Not all clinical and experimental laboratories have sufficient facilities necessary for identification of *Leishmania* parasites at species level. Laboratories lacking such facilities usually use *Leishmania* species that have already been identified by other laboratories. Hence, exchange of *Leishmania* parasites with identified species between different laboratories takes place usually. It is possible that *Leishmania* isolates are mixed up or they are contaminated with non-*Leishmania* trypanosomatids (e.g. *Crithidia*) in laboratory. Mixing up or contamination of *Leishmania* isolates in one laboratory and exchange of this parasite between laboratories can lead to incorrect data and conclusions. This pitfall can be prevented by verification of *Leishmania* isolates of uncertain identity. The gold standard for species identification is still isoenzyme electrophoresis (10). However, only specialized laboratories have sufficient facilities and expertise to perform this method. The internal transcribed spacer 1 (ITS1) sequencing is an alternative method for species identification that does not have many technical drawbacks of the isoenzyme electrophoresis.

The aim of this study was to evaluate the relative feasibility of ITS1 sequencing, in comparison to isoenzyme electrophoresis for verification of *Leishmania* species.

Material and Methods

Parasite

Six *Leishmania* isolates were received as gifts from different research laboratories (see Table 1). The species of these isolates were assumed to be known based on isoenzyme profile provided by the donating laboratories. The isolates were stored in liquid nitrogen and were recovered for the present study by thawing and culturing them in NNN media (11). Logarithmic parasites were harvested and washed by phosphate buffer saline (PBS) two times and were stored in aliquots of 100×10^6 parasites (for DNA extraction) and 1×10^9 parasites (for isoenzyme electrophoresis) in -70˚C.

Amplification of ITS1 and RFLP analysis

Genomic DNA was extracted by LiCl extraction (12). The ITS1 region was amplified using DNA extracted from parasite and ITS1 specific primers LITSR and L5.8S and analyzed by restriction fragment length polymorphism (RFLP) analysis using the restriction endonuclease *Hae*III (13).

Sequencing of ITS1

PCR amplification of genomic DNA from each of the isolates No. 1, 2, 4, and 5 resulted in a sharp single band in agarose gel electrophoresis. These PCR products were directly sent for sequencing (Macrogene, Seoul, Korea). Each PCR product was sequenced at least twice by ITS1 specific primers: once by the forward primer (LITSR) and once by the reverse primer (L5.8S). Nucleotide in each position was considered correct if the two sequencing results (which were sequenced in opposite directions) confirmed each other. The ITS1 sequences of isolates No. 3 and 6 were determined through a T/A cloning approach. This approach was done because PCR amplification of these isolates did not resulted in a single sharp band in electrophoresis; hence direct sequencing of PCR products were not sufficiently informative and conclusive. The T/A cloning was performed by using InsTA cloning kit (Fermentas, Burlington, Canada) according to the manufacturer instructions. Briefly, ITS1 fragment was amplified through PCR by ITS1 specific primers. The product of ITS1-PCR was run in agarose gel electrophoresis. The band that was compatible to ITS1 molecular weight (about 300 base pairs) was excised and extracted from agarose gel by Silica Bead DNA Gel Extraction kit (Fermentas Co.). Gel extracted DNA fragment was inserted into pTZ57R plasmid by ligase enzyme. The insert containing plasmid was transformed into DH5α strain of Escherichia coli bacteria. The transformed bacteria were cultured overnight and plasmids were purified by a plasmid purification kit (Bioneer Co.) according to the manufacturer instruction. This kit purifies the plasmids by the modified alkaline lysis method. The purified plasmids were sequenced by plasmid specific forward and reverse primers.

Isoenzyme electrophoresis

The isoenzyme study was performed using discontinuous polyacrylamide gel electrophoresis (PAGE) as described earlier (14). Each *Leishmania* strain was tested for the activity of at least four enzyme systems out of the following enzymes: glucose phosphate isomerase (GPI), malate dehydrogenase (MDH), nucleoside hydrolase (NH), malic enzyme (ME), super oxide dismutase (SOD) and phosphoglucomutase (PGM). Performance of this technique, due to fund limitation, was restricted to the isolates whose species were revealed to be different from the assumed species (i.e. declared by the donating institution) and for the only true *L. infantum* isolate studied in the present study.

Results

RFLP

RFLP patterns of isolate No.1 was compatible with *L. major*. RFLP pattern for isolates No 2 and 4 were suggestive of other trypanosomatids such as *Crithidia* species (e.g. *C. fasciulata* with GenBank accession number **Y00055.1**). RFLP patterns for isolates 3, 5 and 6 were compatible with the reported RFLP pattern for *L. tropica* or *L. infantum* (data not shown).

ITS1 sequencing

The ITS1 of the *Leishmania* isolates studied in the present report (six isolates) were sequenced and deposited in GenBank (Fig. 1). Comparison of the ITS1 sequences of the six isolates with other *Leishmania* species in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) revealed the following results: isolate 1 was 99% and 100% identical to *L. major* Friedlin strain and *L. major* MRHO/IR/75/ER strain respectively (GenBank accession numbers **CP000079.1** and **EF653269.1** respectively), isolate 2 and 4

(these two isolates showed complete identical ITS1 sequences) have 96% identity to *C. fasciculata*(accession number **Y00055.1**), isolate 3 had 100% identity with many *L. tropica* strains e.g. *L. tropica* with GenBank accession number **FJ948456.1**, isolate 5 was 100% identical to *L. infantum* MCAN/ES/98/LLM-877 strain (GenBank accession number: **AM502245.1**), and isolate 6 was 99% identity to *L. tropica* MHOM/SU/60/OD strain (accession number **EU326226.1**). It was concluded that isolate 1, 2, 3, 4, 5 and 6 were *L. major*, *C. fasciculata*, *L. tropica*, *C. fasciculata*, *L. infantum*, and *L. tropica* respectively (as are shown in Table 1). It is noteworthy that ITS1 sequences of the two *Crithidia* isolates were completely identical. This ITS1 sequence (accession number **HM004585.1**) has 11 different nucleotides in comparison to its closest match in GenBank (*C. fasciculata* accession number **Y00055.1**).

Species identification by isoenzyme electrophoresis

The isolate No. 3 was revealed *L. tropica* according to ITS1 sequence while it was stated to be *L. infantum* by the donating institution. Isoenzyme electrophoresis, as the gold standard for species identification, was performed for resolving this discrepancy. Characterization of isolate No. 3, using 4 enzyme systems (GPI, MDH, NH, and PGM), with reference strains indicated that its isoenzyme profile in all 4 enzyme systems is compatible with *L. tropica* MHOM/IR/72/NADIM3 strain. The isolate No. 5 was revealed *L. infantum* according to ITS1 sequence and according to data of the donating institution. The isoenzyme electrophoresis was performed for confirmation of species of this isolate. Isoenzyme profile of this isolate was compatible with *L. infantum* MHOM/TN/80/IPT1 strain using 4 enzyme systems (MDH, GPI, ME, and SOD). Isoenzyme electrophoresis was not done for *Crithidia* isolates (isolates No. 2 and 4) due to fund restriction and with respect to their lack of clinical relevancy. It is noteworthy that blastocrithidia form of *Crithidia* was seen in microscopical examination of Geimsa stained slides of the *Crithidia* isolate (isolate No. 2) whereas no promastigote form of *Leishmania* was observed in the slides. In addition, intravenous injection of this isolate into golden hamster did not resulted in parasite growth after 6 months.

Table 1: *Leishmania* isolates studied in the present study

Isolate No.	International code	Assumed species according to the donating institutions	Real species as identified in the present study
1	MHOM/IR/75/ER	*L. major*[a]	*L. major*
2	MHOM/IR/05/SHZ5	*L. infantum*	*C. fasciculata*
3	MCAN/IR/97/LON 49	*L. infantum*	*L. tropica*
4	Not available	*L. infantum*	*C. fasciculata*
5	MHOM/IR/04/IPI-UN10	*L. infantum*	*L. infantum*
6	MHOM/AF/88/KK27	*L. tropica*	*L. tropica*

[a]Abbreviations used: *L.; Leishmania, C.; Crithidia.*

***Leishmania major* strain MRHO/IR/75/ER (GenBank Accession: GQ471900.1)**

```
   1 ctggatcatt ttccgatgat tacaccccaa aaaacatata caactcgggg aggcttattc
  61 tatatatata tagtataggc ttttcccaca tacacagcaa acttttatac tcaaaatttg
 121 cagtaaaaaa ggccgatcga cgttgtagaa cgcaccgcct atacacaaaa gcaaaaatgt
 181 ccgtttatac aaaaaaatag acggcgtttc ggtttttggc gggagggaga gagaggggg
 241 tgcgtgcgcg tggataacgg ctcacataac gtgtcgcgat ggatgacttg gcttcctatt
 301 tcgttgaaga acgcagtaaa gtgcgataag tggtatca
```

***Leishmania tropica* strain MHOM/AF/88/KK27 (GenBank Accession: GQ913688.1)**

```
   1 ctggatcatt ttccgatgat tacaccccaa aaaaaaacat atacaaaact cggggaggcc
  61 tattatatac attataggcc tttcccacat acacagcaaa cttttatact cgaagtttgc
 121 agtaaacaaa aggccgatcg acgttataac gcaccgccta tacacaaaag caaaaatgtc
 181 cgtttataca aatatacggc gtttcggttt tgttggcggg gggtgcgtgt gtggataacg
 241 gctcacataa cgtgtcgcga tggatgactt ggcttcctat ttcgttgaag aacgcagtaa
 301 agtgcgataa gtggtatca
```

***Leishmania infantum* isolate MHOM/IR/04/IPI-UN10 (GenBank Accession: GQ444144.1)**

```
   1 ctggatcatt ttccgatgat tacacccaaa aaacatatac aactcgggga gacctatgta
  61 tatatatgta ggcctttccc acatacacag caaagttttg tactcaaaat ttgcagtaaa
 121 aaaaaggccg atcgacgtta taacgcaccg cctatacaaa agcaaaaatg tccgtttata
 181 caaaaaatat acggcgtttc ggtttttggc ggggtgggtg cgtgtgtgga taacggctca
 241 cataacgtgt cgcgatggat gacttggctt cctatttcgt tgaagaacgc agtaaagtgc
 301 gataagtggt atca
```

***Leishmania tropica* (GenBank Accession: HM004586.1)**

```
   1 ctggatcatt ttccgatgat tacacccaaa aaaaacatat acaaaactcg gggaggccta
  61 tatattatac attatatagg cctttcccac acatacacag caaacttta tactcgaagt
 121 ttgcagtaaa caaaaggccg atcgacctta taacgcaccg cctatacaca aaagcaaaaa
 181 tgtccgttta tacaaatata cggcgtttcg gttttgttgg cgggggtgc gtgtgtgtgg
 241 ataacggctc acataacgtg tcgcgatgga tgacttggct tcctatttcg ttgaagaacg
 301 cagtaaagtgcgataagtgg tatca
```

***Crithidia fasciculate* (GenBank Accession: HM004585.1)**

```
   1 ctggatcatt ttccgatgat accatacaca aaaacaaaaa ccggagggtt tgggtgtggc
  61 gtgtatgtgt gtatgtgtgt gcgtgtaaaa agcgcatgcg catatatgca tgcatagtag
 121 tgcccggctc tctacgttgg gaggagcgga aactaaacat ttccgtttct ctctaacaca
 181 taaacaaaca caacatagcc cagcgccgtt gcgtgctttc tctctctctc aactctctct
 241 cttgtggggg gtgttgtgtg tgggggtttg tgcgcgcgtg tgccggaaca aggccaatcg
 301 atgcacgtgt gtgtattgta ttgttctttc ttagagaacg atataaaaac cgcgtgcatg
 361 gatgacggct caaataacgt gtcgcgatgg atgacttggc ttcctatctc gttgaagaac
 421 gcagtaaagt gcgataagtg gtatca
```

Fig. 1: ITS1 containing sequences of *Leishmania* species studied in this study

Discussion

Feasibility of ITS1 sequencing method

Species verification is necessary for isolates of uncertain identity. Isoenzyme electrophoresis is still the gold standard for this purpose (10). This technique has many limitations: it is demanding, laborious, time consuming, requires bulk cultivation of parasites and only specialized laboratories has the expertise to perform this method (10, 15). Molecular methods are increasingly becoming more popular for identification of *Leishmania* species (10). Amplification of ITS1 by PCR followed by restriction analysis of the amplification product or sequencing is a molecular method that has proved to be useful for species identification of *Leishmania* isolates (10, 15-20). Advantages of ITS1 sequencing, in comparison to isoenzyme electrophoresis, include: 1) ITS1 sequencing does not need mass cultivation of parasite. The amount of parasite needed for this technique is reported to be as little as 0.2 parasites that can be retrieved through microscopic glass slides used for routine diagnosis of the disease in clinics or filter paper that can be used in remote areas like villages (15). This advantage is especially critical for laboratories lacking cell culture facilities. 2) ITS1 sequencing does not need use of reference *Leishmania* strains, because this method uses the reliable database of reference strains (e.g. GenBank) for the comparison. This advantage is especially important for laboratories with no access to reference strains or no facilities for their storage. These two advantages of this method show the relative ease of performance of ITS1 sequencing in comparison to the traditional isoenzyme electrophoresis. Our data confirms the relative feasibility of the ITS1 sequencing method in comparison to isoenzyme electrophoresis method.

Pitfall of mixing up or contamination of isolates

Our findings show the possibility of mixing up of *Leishmania* isolates or contamination with other trypanosomatids (e.g. *Crithidia*) in the laboratory. Therefore, verification of *Leishmania* species identity is vital for *Leishmania* researches especially research involving different species of *Leishmania.* We studied ITS1 for confirmation of species of 6 *Leishmania* isolates currently in use in different research laboratories in Iran. The results of ITS1 sequence revealed that species of some isolates were not the assumed species (See Table 1). Isoenzyme electrophoresis confirmed the results of ITS1 sequencing for isolates No. 3 (*L. tropica*) and No. 5 (*L. infantum*). So ITS1 sequence and isoenzyme profile show compatible results. Two isolates (No. 2 and 4) were labeled *L. infantum* while they were *C. fasciculata*. These two *Crithidia* isolates have identical ITS1 sequences. This ITS1 sequence (accession number **HM004585.1**) is novel and has not been already reported. These *Crithidia* species were stated to be isolated from visceral leishmaniasis patients. *Crithidia* microorganisms are reported to infect exclusively invertebrates and predominantly insects (21) and we found no report for pathogenicity of this trypanosomatid in humans. The real sources of the *Crithidia* isolates studied in the present study remain to be identified. The *Crithidia* has been reported as a contaminant in another study of different *Leishmania* isolates (22).

Use of ITS1 sequence for species verification

Verification of *Leishmania* species is needed for isolates of uncertain identity. However, species verification by isoenzyme electrophoresis method is not feasible in

many laboratories due to its limitation as discussed above. An important application of our findings is use of ITS1 sequence for verification of *Leishmania* species. As we know this is the first study to apply ITS1 sequencing for verification of species identity of *Leishmania* parasites. A shortcoming of our study is the limited number of isolates studied which was due to limitations in fund and manpower. We propose further studies with larger number of isolates for solid conclusion.

In conclusion, our results documented the possibility of mixing up or contamination of *Leishmania* isolates in the laboratory and confirm the relative feasibility of the ITS1 sequencing in comparison to the isoenzyme electrophoresis for species verification. It is suggested that ITS1 sequencing method, with regard to its high specificity and relative ease of performance, can be used for verification of species of *Leishmania* isolates of uncertain identity.

Acknowledgement

Helpful advice of Dr Gabriele Schönian (Institute of Microbiology and Hygiene, Charité Universitätsmedizin, Berlin, Germany) for species identification and generous advice and help of Dr Anis Jafari (Molecolar Biology Department, Pasteur Institute of Iran) for molecular methods are highly appreciated. This research received financial supports from Pasteur Institute of Iran (Research Project No. 314). The authors declare that they have no conflicts of interest.

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