Characterization of ITS1 gene of *Leishmania infantum* isolated from Iraqi patients with visceral leishmaniasis by PCR- RFLP and sequencing methods.

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ABSTRACT

For the best of our knowledge, there is no information about molecular characterization of Iraqi isolates of visceral leishmaniasis, the present work aimed to characterize three different Iraqi isolates of *Leishmania infantum* by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and sequencing methods.

Three isolates from bone marrow of Iraqi patients infected with kala azar were used in this study. The isolates were already diagnosed by isoenzyme as *Leishmania infantum*. Patients were inhabiting different parts of Baghdad. The samples after microscopic examination were cultured on modified NNN media. Then DNA was extracted for amplifying ITS1 (internal transcribed spacer 1) gene by PCR. Identification of samples was studied using RFLP (digestion with Apo1 restriction enzyme) and sequencing of PCR products.

The PCR of all samples showed a band under about 500 bp. The results by using PCR-RFLP method showed no restriction with digestion with Apo1 restriction enzyme. The results of sequencing showed differences with all separated gene from *Leishmania infantum* in the gene bank.

In this study we found that the sequences of ITS1 gene of *Leishmania infantum* separated from Iraqi patients are different from other samples, as there is no similarity with *Leishmania infantum* **(**MHOM/TN/80/IPI1). The more similarity is with the Iranian isolate of *Leishmania infantum* (MCAN/IR/97/LON) with 41%, whereas the similarity with *Crithidia luciliae* internal transcribed spacer 1, ITS1 is 96%.

Key words: Visceral leishmaniasis, PCR-RFLP, sequencing, Iraq

INTRODUCTION

Leishmaniasis is endemic in 88 countries and 12 million people per year have been estimated to be at risk of infection. Leishmaniasis forms a spectrum of diseases ranging from benign, self-healing skin ulcer to overwhelming lethal systemic infection (1).

The leishmaniasis comprise a group of diseases that display widely different clinical manifestations in humans, which depend not only on the species initiating infection but also on the general health and genetic make-up of the infected individual (2).

Visceral leishmaniasis is a serious health problem and an important endemic disease in Iraq. During the last years there was an increase in visceral leishmaniasis cases in Iraq (3).

Majeed *et al*. (2012) reported that in Iraq, visceral leishmaniasis incidence per 100 000 population was 2·6 in 2007, 3·1 in 2008, and 4·8 in 2009, mostly in children aged <5 years (4).

In Iraq visceral leishmaniasis is usually detected in infants and children (5, 6). According to Zukerman & Lainson at 1977, visceral leishmaniasis in Iraq is considered to be of infantile type (7).

Correct identification of the etiologic agent of leishmaniasis is important for clinical, epidemiologic reasons, prevention, and treatment. Therefore, molecular characterization based on partial sequences of Leishmania species is important.

ITS1 is a suitable target for detection, identification and characterization of Leishmania spp for this reason, this gene was used in this study. In the present study, PCR and sequencing of ITS1gene were employed to study visceral leishmaniasis of the Iraqi isolates.

Materials and Methods

1) Isolates:

Three Iraqi isolates of L. infantum was used in this study, they were collected by bone marrow aspirate from three patients living in different parts of rural areas of Baghdad.

Those patients were all less than five years old, one girl and two boys.

The isolates were cultivated on liquid NNN medium containing RPMI

so that large number of promastigote can be harvested for DNA extraction,

also Leishmania infantum strain (MHOM/TN/80/IPI1) was cultivated and used as positive control.

2) DNA extraction:

About 2×106 of promastigotes of each isolates and MHOM/TN/80/IPI1 strain were initially washed with phosphate buffered saline (PBS).

The procedure of the extraction of DNA was followed according to the manufacturer instruction of the kit (Bioneer Corporation).

The DNA extraction products were detected in 0.8% agarose gel and photographed. The results showed that concentration of the extracted DNA was high.

3) PCR amplification and gel electrophoresis

Genomic DNA from promastigotes of all isolates and Leishmania infantum strain (MHOM/TN/80/IPI1) were used as templates to amplify the ITS1 gene by PCR. The reaction was performed in 25µl of the solution containing, 3µl of template DNA, 0.5µl of dNTP (with concentration 10mM and final concentration of 200 µM), 0.5µl of Taq DNA polymerase (with concentration 5 Unit/ml), 2.5µl of 10X PCR buffer, 0.75µl of MgCl2 (50 mM), 15.75µl of distilled water and 1µl of each of primers (10Pmol/µl).

The primer sequences that we used in this study were as follows (Spanakos et al., 2008) (8):

LeF, 5_-TCCGCCCGAAAGTTCACCGATA-3and

LeR, 5_-CCAAGTCATCCATCGCGACACG-3_;

These primers could detect 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence of Leishmania spp.

The PCR program was as follow: 5 min at 94oC for initial denaturation step, then 30 cycles of denaturation at 94oC for 1 min, annealing at 54oC for 30 s, an extension at 72oC for 45 s, and a final extension at 72oC for 5 min.

The PCR products were analyzed by electrophoresis on 1.5% agarose gels, visualized by UV illumination and finally photographed.

4) Digestion with restriction endonuclease enzyme (RFLP)

Based on the sequences of L. infantum retrieved from Gen Bank, we expected that ApoI could digest the PCR product, then ApoI (Fermentase co.) was selected as a suitable enzyme used for all L. donovani complex (Spanakos et al., 2008)(8).

RFLP was performed according to the methods described by (Marfurt et al., 2003) (9). We provided total 31 µl volume, include 10 µl of PCR products (0.1-0.5 µg of DNA), 18 µl of nuclease-free water, 2 µl of 10X buffer Tango, and 1 µl of ApoI enzyme (Fermentase) at 37ºC water bath overnight (16 h). The digest was used for electrophoresis on 3.5% agarose gel. Fragment sizes were estimated by their comparison with bands of 100bp DNA length standard ladder.

5) DNA sequencing

The PCR products were excised from a 3.5% agarose gel and DNA was isolated using the QIA quick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions.

All PCR products were sequenced with dideoxy method (Fazapajouh Company) using the PCR primers. Using the Clustal W 1.8 software package.

RESULTS

1. Evaluation of the PCR Method

Based on the results of PCR product in the gel electrophoresis, all isolates showed DNA size 477 bp, whereas for MHOM/TN/80/IPI1 was about 380 bp (Fig.1).

2. RFLP

The resultant restriction fragments of samples were separated on a 3.5% agarose gel and difference was observed in amplicon size between isolates and MHOM/TN/80/IPI1 strain.

The PCR product of all isolates didn't digested by ApoI (Fig.2) but MHOM/TN/80/IPI1 was digested to two fragments (Fig.3).

3. Sequencing

PCR products containing clinical samples and strain MHOM/TN/80/IPI1 were sequenced according to F and R primers. All isolates in this study showed similarity sequences (Fig.4).

The results of sequencing indicate that the length of fragments of all clinical isolates were 477 bp. It was additionally found out that there is no restriction site of ApoI (AATT) in ITS1 sequences for all clinical isolates, whereas restriction enzyme site was shown in MHOM/TN/80/IPI1 ITS1 sequence.

Discussion

One of our findings in this study is that the sequences of ITS1 gene of the isolates separated from Iraqi patients are different from other *Leishmania infantum* strains that registered in gene bank.

ITS1 gene is a target to study the characterization of visceral leishmaniasis because ITS1 sequences are adequately polymorphic to detect different strains (Al-Jawabreh *et al.*, 2006; Fryauff *et al.*, 2006) (10,11) and so we can compare our data with many other study on Leishmania ITS1 sequences that are saved in sequences data banks (Schonian *et al.*, 2003)(12).

In this study it was found that the sequences of ITS1 gene of *Leishmania infantum* separated from Iraqi patients shows no similarity with *Leishmania infantum* strain (MHOM/TN/80/IPI1), and moderate similarity with Iranian isolate of *Leishmania infantum* (MCAN/IR/97/LON) with 41% (Fig. 5), whereas the similarity with *Crithidia luciliae* internal transcribed spacer 1, ITS1 is 96% (Fig.6). We need more molecular studies on other genes of the Iraqi *L. infantum* to explain the similarity with the genus *Crithidia luciliae* and we may explain this similarity as these isolates are new strain of *L. infantum*. The sequence variants of ITS1 might be due to the presence of multiple strains/clones or the existence of intragenomic variations in the multicopy ITS1, or a

combination of both. Finally, we may need to revise the taxonomy of the Iraqi strain of *Leishmania infantum*.

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The authors declare there is no conflict of interest.

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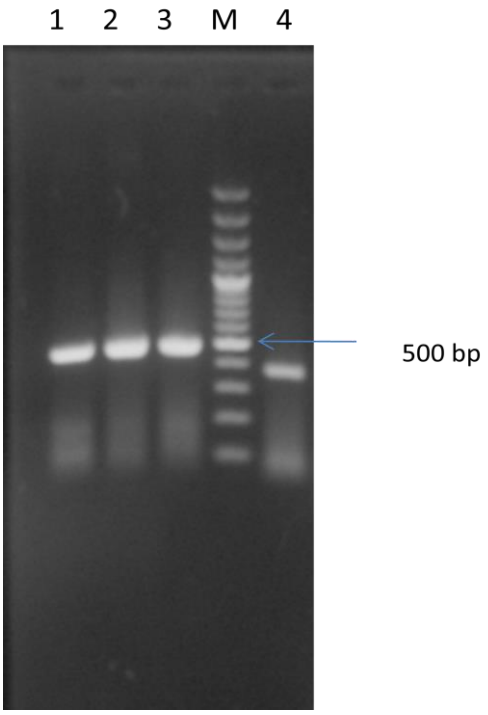

Fig. 1. Agarose gel electrophoresis of PCR products. Lanes 1 to 3: *Leishmania infantum* isolates from Iraqi patients, (Lane M) is a 100 bp ladder.Lanes 4 : *Leishmania infantum* as standard strain **(**MHOM/TN/80/IPI1).

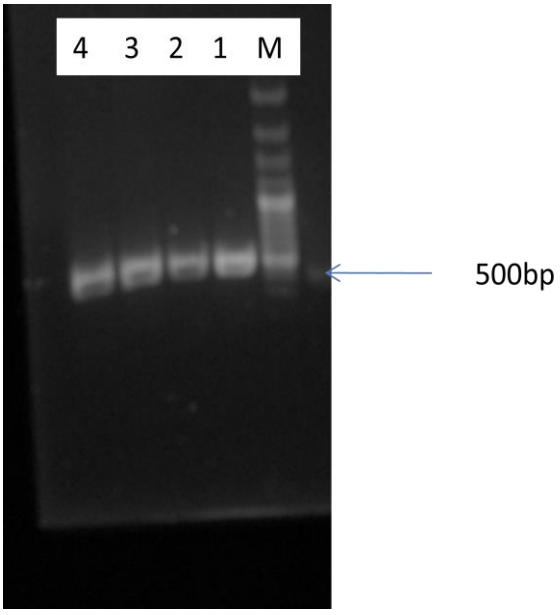

Fig.2: Agarose gel electrophoresis of PCR and RFLP products (with *Apo*I enzyme). The marker (lane M) is a 100 bp ladder.Lanes 1&3 ITS1 from patients isolates before digestion , Lanes 2 and 4 of after digestion with *APO*I enzyme.

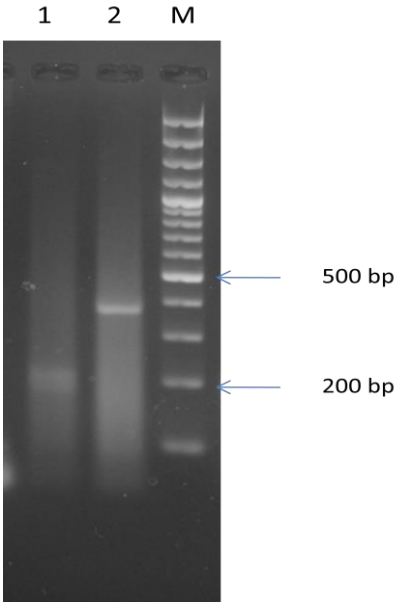

Fig.3: Agarose gel electrophoresis of PCR products and after digestion with *Apo*I. The marker (lane M) is a 100 bp ladder. Lane 1 PCR product of *Leishmania infantum* strain **(**MHOM/TN/80/IPI1) after digestion with *APO*I enzyme , Lanes 2 ITS1 gene before digestion with *APO*I enzyme.

TCCGCCCGAAAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGT
AGGTGAACCTGCAGCTGGATCATTTTCCGATGATACCATACACAAAAACAAAAACCGGAGGGT
TTGGGTGTGGCGTGTATGTGTGTATGTGTGTGCGTGTAAAAAGCGCATGCGCATATATGCATGC
ATAGTAGTGCCCGGCTCTCTACGTTGGGAGGAGCGGAAACTAAACATTTCCGTTTCTCTCTAAC
ACATAAACAAACACAACATAGCCCAGCGCCGTTGCGTGCTTTCTCTCTCTCTCAACTCTCTCTCT
TGTGGGGGGGTGTTGTGTGTGGGGGGTTTGTGCGCGCGTGTGCCGGAACAAGGCCAATCGATGCAC
GTGTGTGTATTGTATTGTTCTTTCTTAGAGAACGATATAAAAACCGCGTGCATGGATGACGGCTC
AAATAACGTGTCGCGATGGATGACTTGG

Fig. 4 : Result of PCR product contain18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence of visceral leishmaniasis isolate.

CCGATGATACCATACACAAAAACAAAAACCGGAGGGTTTGGGTGTGGCGTGTATGTGTGTA

TGTGTGTGCGTGTAAAAAGCGCATGCGCATATATGCATGCATAGTAGTGCCCGGCTCTCTA

CGTTGGGAGGAGCGGAAACTAAACATTTCCGTTTCTCTCTAACACATAAACAAACACAACA

TAGCCCAGCGCCGTTGCGTGCTTTCTCTCTCTCTCAACTCTCTCTCTTGTGGGGGGTGTTG

TGTGTGGGGGTTTGTGCGCGCGTGTGCCGGAACAAGGCCAATCGATGCACGTGTGTGTATT

GTATTGTTCTTTCTTAGAGAACGATATAAAAACCGCGTGCATGGATGACGGCTCAAA

Fig.5: The results of sequencing of ITS1 for Iraqi isolates, showed 41% similarity with Iranian isolates that this similarity is shown in the beginning and the end of ITS1 gene (underline regions).

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Query        13   TACACAAAAACAAAAACCGGAGGGTTTGGgtgtggcgtgtatgtgtgtatgtgtgtgcgt  72
                  |||||||||||| |||||||||||||||||||||||||||||||| | ||||||| |||
Crithidia    1    TACACAAAAAC-AAAACCGGAGGGTTTGGGTGTGGCGTGTATGTGTATGTGTGTGTACGT  59

Query  73         gtAAAAAGCGCATGCGCATATATGCATGCATAGTAGTGCCCGGCTCTCTACGTTGGGAGG  132
                  ||||||||||||||||||||||| |||  |||||||||||| ||||||||||||||||||
Crithidia    60   GTAAAAAGCGCATGCGCATATATACATATATAGTAGTGCCCAGCTCTCTACGTTGGGAGG  119

Query  133        AGCGGAAACTAAACATTTCC-GTTTCTCTCTAACACATAAACAAACACAACATAGCCCAG  191
                  |||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Crithidia    120  AGCGGAAACTAAACATTTCCTGTTTCTCTCTAACACATAAACAAACACAACATAGCCCAG  179

Query  192        CGCCGTTGCGTGCTTtctctctctctcaactctctctcttgtgggggggtgttgtgtgtgg  251
                  |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Crithidia    180  CGCCGTTGCGTGCTTTCTCTCTCTCTCAACTCTCTCTTTTGTGGGGGGTGTTGTGTGTGG  239

Query  252        gggtttgtgcgcgcgtgtgCCGGAACAAGGCCAATCGATGCACGTGTGTGTATTGTATTG  311
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Crithidia    240  GGGTTTGTGCGCGCGTGTGCCGGAACAAGGCCAATCGATGCACGTGTGTGTATTGTATTG  299

Query  312        TTCTTTCTTAGAGAACGATATAAAAACCGCGTGCATGGATGACGGCTCAAA  362
                  |||||||||||||||||||||||||||||||||||||||||||||||||||
Crithidia    300  TTCTTTCTTAGAGAACGATATAAAAACCGCGTGCATGGATGACGGCTCAAA  350;
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Fig.6 : Alignment of the Iraqi isolate and *Crithidia luciliae* with 96% similarity.

الملخص العربى

توصيف جين مفساح الاستنساخ الداخلي ١ (ITS1) للشمانيا الطفلية والمعزولة من مرضى عراقيين مصابين باللشمانيا الحشوية وذلك باستخدام تقنيات تفاعل انظيم البلمرة التسلسلي (PCR) ، تباين اطوال قطع التقييد (RFLP) والتتابع (Sequencing)

الهام عبدالواحد مجيد فاطمة غفاري فار ندى البشير

على حد علمنا لا تتوفر معلومات حول التوصيف الجزيئي للعزلة العراقية للشمانيا الحشوية لذا هدفت هذه الدراسة توصيف العزلة العراقية للشمانية الطفلية (*Leishmania infantum*) باستخدام تقنيات تفاعل انظيم البلمرة التسلسلي (PCR) ، تباين اطوال قطع التقييد (RFLP) والتتابع.

تم جمع ثلاثة عزلات من نخاع العظم لمرضى مصابين بمرض الكلا ازار ويقطنون مناطق مختلفة من بغداد. بعد التعرف على الطفيلي تحت المجهر تم انماءها على الوسط الزرعي المحور. استخلص الدنا (DNA) لتضخيم جين مفساح الاستنساخ الداخلي -١ (ITS 1) بواسطة تفاعل انظيم البلمرة التسلسلي (PCR). التعرف على العينات تمت دراسته بتقنية تباين اطوال قطع التقييد (RFLP) (هضم باستخدام انظيم الاقتطاع Apo 1) ثم اجراء التتابع لناتج البلمرة.

اظهرت نتائج تفاعل انظيم البلمرة التسلسلي للعينات ان هناك حزمة تحت حوالي ٥٠٠ زوج القواعد. اما نتائج تفاعل انظيم البلمرة التسلسلي مع تباين اطوال قطع التقييد فلم تظهر اي اقتطاع باستخدام الانظيم الهاضم كما كانت نتائج التتابع مختلفة عن الجين المفصول من اللشمانيا الطفلية في بنك الجينات.

يستنتج من ذلك ان تتابع هذا الجين في اللشمانيا الطفلية المعزولة من المرضى العراقيين تختلف تماما عن العزلة القياسية (IPII /80 / MHOM/TN) ولكن ظهر بعض التشابه مع العزلة الايرانية للشمانية الطفلية (MCAN/IR/97/LON) بنسبة ٤٠% بينما كانت نسبة التشابه مع احد انواع الكرايثدية (*Crithidia luciliae*) هي ٩٦%.