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DNA Sequencing of Leishmania Sp. Isolated from Patient with Visceral Leishmaniasis in Wasit Governorate of Iraq

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Abstract: Leishmania sp. is responsible for visceral leishmaniasis, a neglected and lethal parasitic disease. Leishmania strain obtained from Al-karama Hospital, in Wasit Governorate of Iraq, isolated from patient infected with visceral leishmaniasis. PCR technique was done for detection VL based on the kinetoplast DNA (kDNA). The current study also included DNA sequence analysis where the phylogenetic tree was constructed using Maximum Likelihood method in (MEGA. X version). The *Leishmania donovani* isolate kinetoplast minicircle DNA gene were showed closed genetic related to *Leishmania donovani* strain MHOM/SD/95/SIGIN kinetoplast minicircle DNA, complete sequence (AF169136.1) at total genetic changes (0.01%).The NCBI BLAST Homology identity (99%) of *Leishmania donovani* kinetoplast minicircle DNA, partial with NCBI *Leishmania donovani* strain MHOM/SD/95/SIGIN kinetoplast minicircle DNA (AF169136.1).

Introduction

Leishmaniasis is a zoonotic of a worldwide spectrum and supposed to be a public health concern for numerous countries in the world. Natural reservoir hosts include anthroponotic cycle , human–sand fly–human (Khalil et al., 2017).

Leishmania donovani is the zoonotic protozoal disease that causes visceral leishmaniasis (VL). The pathogen spreads to visceral organs inside macrophages that survive within phagolysosomes and elude the host defense mechanism(Dirwal, 2019), potentially resulting in fetal infection linked to hepatosplenomegaly, lymphadenopathy, and progressive anemia (Costa et al., 2023).

Visceral leishmaniasis is also known as kala-azar or black fever/disease, which refers to the darkening of the skin caused by melanocyte activation during infection(Lidani et al., 2017). Other local names for VL include infantile splenomegaly, Dumdum fever, and Assam fever. It typically affects the spleen, liver, bone marrow, or other lymphoid tissues and is the most severe form of leishmaniasis (Ebrahim et al., 2017).

Molecular techniques have become increasingly relevant due to the remarkable sensitivity, specificity, the various choices of used samples, and also due to the limitations that are usually demonstrated by the conventional parasitological and serological methods (Ranjan et al.,2015). They have been successfully used to detect asymptomatic Leishmania infection and for the diagnosis and follow-up of VL patients, since they are capable of identifying relapses and reinfections in treated VL patients (Cota et al., 2013).

The conventional PCR and the variations such as nested-PCR, semi nested-PCR and quantitative real-time PCR have been demonstrated as important diagnostic tools, these can be performed on bone marrow, splenic, lymph node smears, peripheral blood and serum samples(Abass et al., 2015). PCR on blood and bone marrow samples is a highly sensitive method; while in immunocompetent individuals the sensitivities for both samples are almost equal (De Ruiter et al., 2014). In the last decade, urine samples from immunocompetent patients have shown great sensitivity (96.6%) and specificity (100%) using a recently developed PCR (Sakkas et al., 2008). More recently, an oral fluid-based real-time quantitative PCR showed sensitivity of 94.6%, with a specificity of 90% (Galai et al., 2011).

The aims of this study are:

1- Detecting DNA Sequencing of Leishmania Sp. In Iraq isolated from Patient with Visceral Leishmaniasis in Wasit

2- Detecting The phylogenetic tree for the parasite

Materials and Methods

Sample Collection:

Leishmania strain obtained from Al-karama Hospital, in Wasit Governorate of Iraq ,isolated from patient infected with visceral leishmaniasis and identified by PCR technique.

Nested PCR

The Nested PCR technique was done for detection VL based on, the kinetoplast DNA (kDNA). This method was approved according to the method defined by (Noyes and colleagues, 1998) as following steps:

1 **.Genomic DNA Extraction**

Using the gSYAN DNA extraction kit, (Frozen blood protocol) Geneaid. Taiwan, and complete according to the protocol of the manufacturer

2. Genomic DNA examination

After finishing the DNA extraction, the concentration and purity of DNA were measured by Nano drop, the result showed a concentration of (8-10 ng/µl), and purity (1.6-1.8).

3. Nested PCR master mix preparation

The Nested PCR master mix was by using Maxime PCR PreMix and complete according to company instructions, as the following table:

Table (1) External PCR Master Mix Components

PCR master mix	Volume
Genomic DNA 5-50ng	5µL
Primary primers forward (10pmol)	1µL
Primary primers reverse (10pmol)	1µL
PCR water	13 µL
Total	20µL

After that, these PCR master reaction components, which were described above, were placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all the other

components necessary for PCR reaction, such as (Taq DNA polymerase, dNTPs, Tris-Hcl, PH:9.0, KCl, MgCl2, Stabilizer, and tracking dye). Then the tube was placed for 3 minutes in an Exispin vortex centrifuge, then transferred to thermocycler for PCR.

4. External thermocycler reaction conditions

PCR Thermocycler conditions were designed, for external primer was complete according to (Noyes and colleagues, 1998) as following table:

Table (2) External PCR cycle condition

PCR cycle	Repeat	Temp.	Time
Initial denaturation	1	95C	5min
Denaturation	30	95C	30sec.
Annealing		55C	30sec
Extension		72C	1min
Final extension	1	72C	5min
Hold	-	4C	Forever

5. Internal Nested PCR reaction

Table (3) Internal PCR Master Mix Components

PCR master mix	**Volume**
Primary round PCR product	3µL
Secondary primers forward (10pmol)	1µL
Secondary primers reverse (10pmol)	1µL
PCR water	15 µL
Total	20µL

After that, these PCR master reaction components, which were described above, were placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all the other components necessary for PCR reaction, such as (Taq DNA polymerase, dNTPs, Tris-Hcl, PH:9.0, KCl, MgCl2, Stabilizer, and tracking dye). Then the tube was placed for 3 minutes in an Exispin vortex centrifuge, then transferred to thermocycler for PCR.

6. Internal thermocycler reaction conditions

PCR Thermocycler conditions were designed, for internal primer was complete according to (Noyes and colleagues, 1998) as follows table:

Table (4) Internal PCR cycle condition

PCR cycle	Repeat	Temp.	Time
Initial denaturation	1	95C	5min
Denaturation	30	95C	30sec.
Annealing		55C	30sec
Extension		72C	1min
Final extension	1	72C	5min
Hold	-	4C	Forever

7. Gel electrophoresis

PCR products were examined by loading in 1% agarose as following steps:

1- (1%) agarose gel was prepared in 1X TBE and dissolved for 15 minutes in water bath at 100 ° C, after which it was left to cool at 50 ° C.

2- Then, ethidium bromide stain (3µl) was added to the agarose gel solution.

3- Agarose gel solution was poured into the tray, after the comb was fixed in the correct position, then left to solidify at room temperature for 15 minutes, then the comb was gently removed from the tray and (10µl) of the PCR product was well added to each comb and (5µ) of (100bp Ladder) was added to one well.

4- The gel tray was fastened to the electrophoresis chamber and filled by a 1X TBE buffer, then electricity was conducted at 100 volts and at 80 am for 1 hour.

Use of an ultraviolet transilluminator to visualize the PCR components

DNA sequencing method

All sequences obtained by PCR product have been sent for Sanger sequencing using ABI3730XL, DNA Sequencer Automated, by Macrogen Corporation Korea. The results have been received by email, The data was analyzed expending genius software. The sequencing data have been related with the Gen Bank database (http.:/www.ncbi.nlm.nih.gov/BLAST/), expending the nucleotide BLAST program to recognize homology between the PCR fragments and sequences in the Gene Bank database. Sequences have been deposited in (NCBI) the National Center for Biotechnology Information, Gene Bank database under accession numbers AB13730XL

Results and Discussion.

The results of DNA sequencing analysis are shown in figure (1) In which The Maximum Likelihood approach was used to create the phylogenetic tree in the MEGA. X version. The *Leishmania donovani* IQ isolate kinetoplast minicircle DNA gene were showed closed genetic related to *Leishmania donovani* kinetoplast minicircle DNA strain MHOM/SD/95/SIGIN, complete sequence (AF169136.1) at total genetic changes (0.01%).

The NCBI BLAST Homology identity (99%) of *Leishmania donovani* kinetoplast minicircle DNA, partial cds with NCBI Kinetoplast minicircle of *Leishmania donovani* strain MHOM/SD/95/SIGIN DNA (AF169136.1).

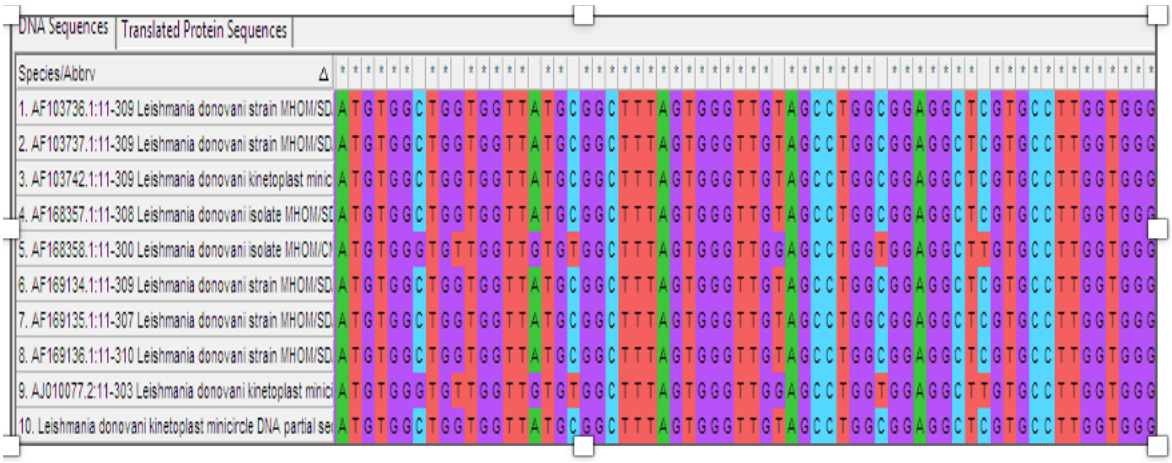

Figure (1): Multiple sequence alignment analysis of the partial kinetoplast minicircle DNA, non-protein coding region sequence in local *Leishmania donovani* IQ isolates and NCBI-Genbank *Leishmania donovani* submitted isolates based ClustalW alignment analysis by using (M`EGA 6.0, multiple alignment analysis tool). The multiple alignment analysis were showed the nucleotide sequence similarity (*) and substitution mutations in kinetoplast DNA nucleotide sequences.

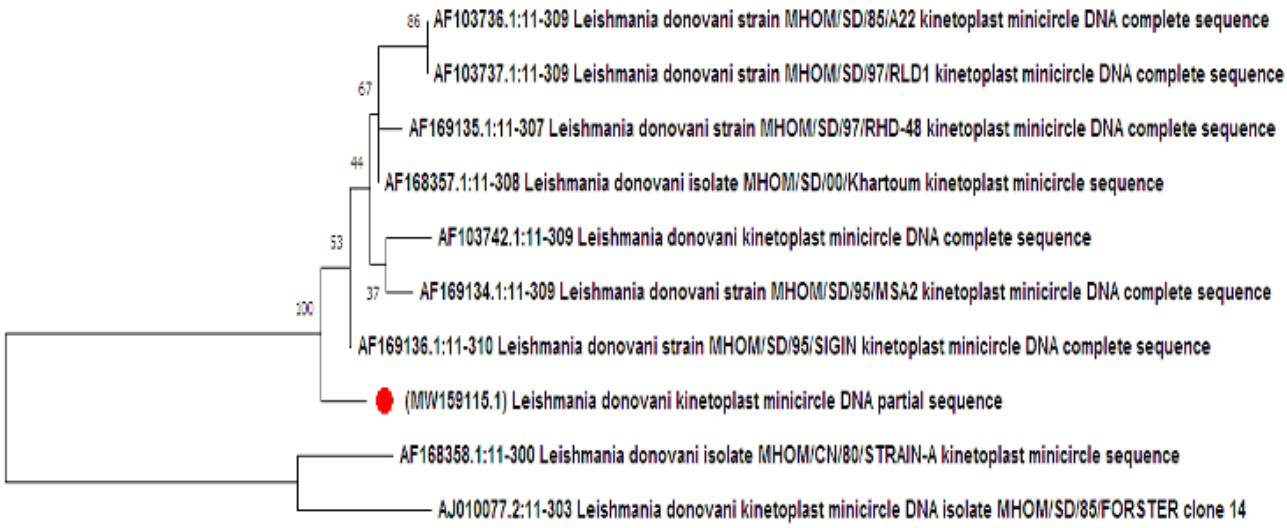

Figure (2): Phylogenetic tree analysis based on *Leishmania donovani* kinetoplast minicircle DNA, partial sequence that used for genetic relationship analysis . The phylogenetic tree was constructed using Maximum Likelihood method in (MEGA. X version). The *Leishmania donovani* IQ isolate kinetoplast minicircle DNA (MW159115.1) were showed closed genetic related to *Leishmania donovani* strain MHOM/SD/95/SIGIN kinetoplast minicircle DNA, complete sequence (AF169136.1) at total genetic changes (0.01%).

Table (5): NCBI-BLAST Homology Sequence identity between local *Leishmania donovani* isolate and NCBI-Genbank *Leishmania donovani* isolates:

CBI-Genbank *Leishmania donovani* isolates	Genbank submission accession number	NCBI-BLAST Homology Sequence identity (100%)
Leishmania donovani strain *MHOM/SD/95/SIGIN*	AF169136.1	99.00%
Leishmania donovani isolate *MHOM/SD/00/Khartoum*	AF168357.1	98.00%
Leishmania donovani strain *MHOM/SD/85/A22*	AF103736.1	97.67%
Leishmania donovani strain *MHOM/SD/97/RLD1*	AF103737.1	97.67%
Leishmania donovani strain *MHOM/SD/95/MSA2*	AF169134.1	97.67%
Leishmania donovani	AF103742.1	97.33%
Leishmania donovani strain *MHOM/SD/97/RHD-48*	AF169135.1	97.33%
Leishmania donovani isolate *MHOM/SD/85/FORSTER*	AJ010077.2:	89.90%
Leishmania donovani isolate *MHOM/CN/80/STRAIN-A*	AF168358.1	89.23%

Score	Expect	Identities	Gaps	Strand
544 bits(294)	**4e-159**	**300/303(99%)**	**0/303(0%)**	**Plus/Plus**

Query 11

TAAAATAGGGCCGGGTGGTGGCTGGAAATGGGCTCCCCTGGGCTGGATTGGGCTT CCCTGGGCTGGATTGGGCTGGAACCCTGGGGTTGGAGGCTTGGTTTTGGGTGGG CTAGCATTTTT 130

Sbjct1 ....T..... 120

Query 131

GGTGTTTTAATGGTTCGGGTGCCTTTGATGTGGCTGGTGGTTATGCGGCTTTAGTG GGTTGTAGCCTGGCGGAGGCTCGTGCCTTGGTGGGGGTGCCCTGGTGGGGGGTG CTTCGGGTTA 250

Sbjct121 240

Query 251

CTCTACGGGTGTCTTTGATGATGATGGGGTGGTTGGCTTTGGTGTTGGGGTTGATC GATGG 313

Sbjct241 .....C.....A..... 303

The study of *L. donovani* has been hindered by the lack of a high-quality reference genome and this can impact experimental outcomes including the identification of virulence genes, drug targets and vaccine development(Lypaczewski, et al.,2018). Thus, deep sequencing employing a combination of second generation (Illumina) and third generation (PacBio) sequencing technology produced a complete genome assembly(Coughlan, et al. 2017).

Compared to the current *L. donovani* assembly, the genome assembly reported within resulted in the closure over 2,000 gaps, the extension of several chromosomes up to telomeric repeats and the re-annotation of close to 15% of protein coding genes and the annotation of hundreds of non-coding RNA genes (Fernandes et al., 2013). It was possible to correctly assemble the highly repetitive A2 and Amastin virulence gene clusters. A comparative sequence analysis using the improved reference genome confirmed 70 published and identified 15 novel genomic differences between closely related visceral and atypical cutaneous disease-causing *L. donovani* strains providing a more complete map of genes associated with virulence and visceral organ tropism(Lypaczewski,2020). Based on mutation severity, gene conservation, and function, a list of putative virulence genes was ranked using bioinformatic tools such as a protein variation effect analyzer and a basic local alignment search tool (McCall et al., 2010). Finding new therapeutic targets and creating a vaccination against *L. donovani* will be aided by this full genome assembly and fresh data on virulence factors (Lypaczewski et al., 2018).

This full genome assembly and new virulence factor data will help with the identification of Combining second and third generation sequencing technologies has made it possible to create a complete genome assembly for *L. donovani*. Similarly, resequencing the genome of L. infantum recently produced a complete assembly, demonstrating the value of PacBio sequencing for Leishmania genomes, the identification of novel therapeutic targets, and the creation of a vaccine against *L. donovani*. (González-De La Fuente, 2017). As a result, the genome was more accurately annotated, identifying new mutations and polymorphisms linked to virulence and expanding the number of putative protein-coding genes(Lugo-Reyes,et al.,2021). The fact that the current assembly led to annotation modifications in nearly 15% of the genome, which corresponds to 1087 protein-coding genes, was astounding (Gannavaram et al., 2017).

Although 13 degenerate pseudogenes are identified, more do exist since our annotations derived from functional genes in L. major and therefore genes functional in other species were not identified

(Gannavaram et al., 2017). Additional single nucleotide polymorphisms (SNPs) have been found through this updated genome annotation, including in genes that may be implicated in visceral illness. Additionally, a number of non-coding genes have been annotated, enabling future research on *L. donovani* that goes beyond protein-coding genes (Madeira da Silva and Beverley, 2010).

It has also been possible to assemble known virulence factor gene families in *L. donovani* including the A2 and Amastin gene families(Samarasinghe,et al.,2018). The results of genomic, functional genomic, and proteomic studies will be much enhanced by this version of the *L. donovani* genome assembly, which will also aid in the discovery of therapeutic targets and vaccine development(Zhang et al., 2017). Additionally, this assembly offers a wider range of target DNA sequences to find markers for the diagnosis and prognosis of illness development(Pei, et al.,2023). Given the recent interest in generating genetically modified live attenuated parasites as vaccine candidates, a complete genome assembly will permit the verification that genetic modifications target intended genes with no off target mutations (Gannavaram et al., 2017).

Conclusion

The *Leishmania donovani* isolate kinetoplast minicircle DNA gene were showed closed genetic related to *Leishmania donovani* strain MHOM/SD/95/SIGIN kinetoplast minicircle DNA, complete sequence (AF169136.1) at total genetic changes (0.01%).The NCBI BLAST Homology identity (99%) of *Leishmania donovani* kinetoplast minicircle DNA, partial with NCBI *Leishmania donovani* strain MHOM/SD/95/SIGIN kinetoplast minicircle DNA (AF169136.1).

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