IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 13, Issue 1 Ver. IV (Jan. – Feb. 2018), PP 60-66
www.iosrjournals.org

Genetic Polymorphismof Iraqi*Leishmania tropica*Isolates Based on *HSP*70Gene

Duha Muhanad Bayram[1*], Hayder Z. Ali[1]
[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq
** Corresponding Author: Duha Muhanad Bayram*

Abstract: *Leishmaniasis is one of the important parasitic diseases, affecting mainly low social class people in developing countries, and is more prevalent and endemic in the tropical and subtropical regions of old world and new world. Despite ofbroad distribution in Iraq,little known about the geneticcharacteristics of the causative agents. So this study was aimed to evaluate the genetic varietyoftwo IraqiLeishmaniatropicaisolates based on heat shock protein gene sequence 70 (HSP70) in comparison with universal isolates recorded sequences data. After amplification and sequencing of HSP70 gene,the obtainedresults were alignment along with homologous* Leishmania *sequences retrieved from NCBI by using BLAST. The analysis results showed presence of particular gene mutations including substitution and insertion at 93% identity with the HSP70 gene. So,indicate the prescient of numeric variation of local isolate and this is the first step toward future phylogenic study to understand genomic diversity across Leishmania tropica parasite.*

Keywords: *Cutaneousleishmaniasis,HSP70sen/HSP70ant, BLAST, PCR, Sequencing, Baghdad.*

Date of Submission: 20-01-2018 Date of acceptance: 12-02-2018

I. Introduction

Leishmaniasis is one of the neglected infectious diseases causes serious health concern globally [1].The clinical and epidemiological characteristics, response to treatment and prognosis, can vary depending on specie [2]. At present, 53 *Leishmania* spp. are known and at least 21 of them are pathogenic to humans [3]. Although the different *Leishmania* species are morphologically very similar, they cause many clinical forms including cutaneous (CL), Mucocutaneous (MCL), and visceral leishmaniasis (VL) [4]. Cutaneous and visceral, are found in Iraq, with a population of nearly 32 million, where 23% are living below the national poverty line, has seen much strife and struggle in the past 25 years. Cutaneous leishmaniasis, widely spread specially in the central region of the country [5]. The direct parasitological methods for detection the parasite had certain limitations, so indirect ways were designed, most of them based on the detection of parasite's DNA[3].Since long years the advance of DNA amplification through PCR has facilitated the improvement of fast and sensitive methods able for molecular typing of *Leishmania* in variant biological samples.Several PCR assays for the combined detection and differentiation of parasites exist, including species-specific PCR [6, 7]. Species discrimination generally involves the following methods: direct sequencing of aPCR product , application of SSCP [8], use of species-specific restriction sites via RFLP analysis [9, 10], PCR fingerprinting [11], RAPD [12], or HRM [13]. Of these techniques, only PCR-RFLP and sequencing analysis are appropriate for the determination of all *Leishmania* sp. [4]. A more informative method is the sequencing analysis of the amplicons. The subsequent identification of single-nucleotide polymorphisms (SNPs) or comparison of the obtained sequence with available reference sequences allows for species determination followed by evolutionary relationships analysis among the sequenced isolates. In comparative sequence analysis, it is crucial to select appropriate reference sequencesand can be adopted to detect different species that belong to the same genus[14].

Sequencing of specific gene is an important task within bioinformatics, itbased on the alignments between numerousDNA sequences. One of the main goal of sequence alignment is the determination of homologous gene regions. In addition,significant important of mutation detection to access forvirulence genetic progress and useful in preventive management. So this study was aimed to determine mutation occurred on two Iraqi *Leishmania tropicaHSP*70 gene in compare with universal recorded sequences based on BLAST.

II. Materials And Methods

2.1 *Leishmania* isolates used in this study

Two *Leishmania tropica*isolates that used in this study were kindly provided by Biology Department/ College of Science/University of Baghdad.

2.2 Cultivation of isolate

Procyclic promastigotes were cultivated in M199 medium supplemented with 10% HIFBS and 1% streptomycin/penicillin (Pen/Strep) then incubated at 26°C for 3 days for obtaining massive culture [15].

2.3 DNA extraction

DNA extraction from cultures of the parasite was made using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Then DNA was stored at −20°C until use.

2.4 Oligonucleotide sequence

The primer pair HSP70sen/HSP70ant (table 1) targeting *HSP*70 gene (alpha DNA/Canada).

Table1: Primers used to detect *LeishmaniaHSP*70 gene:

Oligonucleotide	Sequence	Reference
HSP70sen/HSP70ant	5´GACGGTGCCTGCCTACTTCAA 3´	[16]
	5´ CCGCCCATGCTCTGGTACATC 3´	

2.5 PCR amplification

PCR amplification mixture which used for detection of each gene was carried out in 25 µl volume includes GoTaq® Green Master Mix, 2X (12.5 µl), 3 µl of 25 ng DNA template, 1 µl (1 Mm) of each forwarded and reversed primers and 7.5 µl of nuclease free water to complete the amplification mixture to 25µl. Amplification was performed in a thermal cycler (Eppendorf®) programmed for 35 cycles of denaturation at 94°C for 1 min, annealing at 61°Cfor 1 min, and extension at 72°C for 2 min, unprecedented by an initial denaturation of 2 min at 94°C. Final extension was for 3 min at 72°C.

2.6 Gel electrophoresis

PCR product was examined on agarose gel to confirm that there is a specific product with the desired size. The product was electrophoresed on 1% agarose gel containing Ethidium bromide (0.5 mg/ml) in Tris-Acetate-EDTA buffer (TAE buffer) and photographed under UV illumination.

2.7 Sequencing and Sequence Alignment

PCR product plus primer was labeled and sent to Macrogen Company in Korea to preform sanger sequencing by using AB13730XL, automated DNA sequencer. The result analyzed by BLAST website on NCBI.

III. Results and Discussion

The standard PCR was employed to detect *LeishmaniatropicaHSP*70 gene. The products were examined by gel electrophoresis in order to identify successful amplification of gene.The result indicate the ability of hsp70sen/hsp70ant primer pair for specifically amplified *HSP*70 gene of ~1422 bp, figure (1)

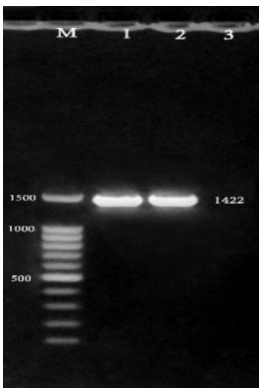

Figure 1: Agarose gel electrophoresis of PCR product of ~ 1211 bp of *HSP*70 , M-100bp Ladder, Lane (1) first isolate, Lane (2) second isolate, and Lane (3) negative control sample (No DNA template).

The *HSP70* gene has been broadly used for taxonomic and phylogenetic studies of *Leishmania*. *HSP*70 region has been found homology on chromosomes 26, 28, 30,and 35. These have been identified as suitable for PCR-RFLP diagnostics that does not need parasite culturing before amplification and so may become broadly used targets [3].

Heat-shock protein 70 gene has established to be suitable for identifying species of different geographical origins in new world and old world between numerous targets designated for *Leishmania* detection [17, 18].In addtion, previous study reported sensitivity of this primer for successful amplification in low DNA concentration of 230 pg/µl [19]

The *HSP*70 coding gene and further classes of this group like *HSP20* and *HSP23* can distinguish *Leishmania* subgenera, for example,*Leishmania*, *Viannia*, *Sauroleishmania*, and also species *L. donovani*, *L. major*, *L. tropica*, *L. guyanensis*, *L. braziliensis*, and *L. mexicana* as well as individual species [14, 20-22].

To study genetic diversity of Iraqi*Leishmaniatropica*isolates, theextracted sequences were alignment on BLAST.Initially, first and second isolate sequences were submitted into gene bank NCBI at accession number: LC328562.1 and LC328563.1 respectively.

Alignment for the above sequence was investigated against *Leishmania* complete genome by Basic Local Alignment Search Tool (BLAST) on NCBI.The alignment result for both isolates revealed high matching with the universal strain (MHOM/KE/81/NLB_030B) sequence which is recorded on NCBI as *Leishmania tropica* species (accession number:FN395026.1) at 100% query cover of 93% identify and 0% gaps.

The variations between first studied isolates of *L. tropica* sequences and (MHOM/KE/81/NLB_030B) are listed in (table 2). The sequence analysis indicated the presence of DNA substitution mutations including both type which is transition and transversion. Transition replacement of purine base with another purine at the positions244, 325, 340, 355, 396, 432,583, 724, 895, 932, 935, 908, 951, 284, 721, 745, 774, 834, 852, 945,957, 4, 136, 667, 739, 751, 893, 915, and 942. In contrast, transversion was occurred due to replacement of a purine with a pyrimidine at the positions 58, 290, 842, 877, 904, 941, 943, 965, 19, 356, 402,649, 876, 76, 238, 241, 418,573, 637,661, 823, 856, 205, 550, 211, 280, 316, 894, 572, 858, 679, and 910.In addition to previous, insertion of G, C, A, G were transpired at positions917, 936, 953, 963 respectively. The alignment between our sample and original gene in the NCBI database was shown in Figure (2):

Table 2: Genetic mutations of *Leishmania tropicaHSP*70 nitrogenous bases/ First isolate.

Type of mutation	Nucleotide Subject/ Quarry	position
Substitution /Transversions	G⟶T	58, 290, 842, 877, 904, 941, 943, 965
Substitution /Transversions	T⟶G	19, 356, 402,649, 876.
Substitution /Transversions	C ⟶G	76, 238, 241, 418,573, 637,661, 823, 856
Substitution /Transversions	A⟶C	205, 550
Substitution /Transversions	T⟶ C	211, 280, 316, 894
Substitution /Transversions	T⟶A	572, 858.
Substitution /Transversions	C⟶A	679, 910.
Substitution /Transitions	G⟶ C	244, 325, 340, 355, 396, 432,583, 724, 895, 932, 935
Substitution /Transitions	G ⟶ A	908, 951
Substitution /Transitions	A⟶G	284, 721, 745, 774, 834, 852, 945, 957.
Substitution /Transitions	C ⟶T	4, 136, 667, 739, 751, 893, 915, 942.
Insertion	G, C, A, G	917, 936, 953, 963 respectively

Score	Expect	Identities	Gaps	Strand
1442 bits(1598)	0.0	908/975(93%)	4/975(0%)	Plus/Plus

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Query  1    GGATGCCGGCACGATTGCGGGCCTGGAGGTGCTGCGCATCATCAACGAGCCGACGGCTGC  60
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Sbjct  25   GGACGCCGGCACGATTGCTGGCCTGGAGGTGCTGCGCATCATCAACGAGCCGACGGCGGC  84

Query  61   GGCCATCGCGTACGGGCTGGACAAGGGCGACGACGGCAAGGAGCGCAACGTGCTGATCTT  120
            ||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   GGCCATCGCGTACGGCCTGGACAAGGGCGACGACGGCAAGGAGCGCAACGTGCTGATCTT  144

Query  121  CGACCTTGGCGGCGGTACGTTCGATGTGACGCTGCTGACGATCGACGGCGGCATCTTCGA  180
            ||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  CGACCTTGGCGGCGGCACGTTCGATGTGACGCTGCTGACGATCGACGGCGGCATCTTCGA  204

Query  181  GGTGAAGGCGACGAACGGCGACACCCACCTCGGCGGCGAGGACTTCGACAACCGCCTGGT  240
            |||||||||||||||||||||||| ||||| |||||||||||||||||||||||||| ||
Sbjct  205  GGTGAAGGCGACGAACGGCGACACACACCTTGGCGGCGAGGACTTCGACAACCGCCTCGT  264

Query  241  GACCTTCTTCACCGAGGAGTTCAAGCGCAAGAACAAGGGCAAGGACCTGTCGTCGAGCCA  300
              || ||||||||||||||||||||||||||||||||||| ||| ||||| ||||||||||
Sbjct  265  CACGTTCTTCACCGAGGAGTTCAAGCGCAAGAACAAGGGTAAGAACCTGGCGTCGAGCCA  324
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Query  301  CCGCGCGCTGCGCCGCCTGCGCACCGCGTGCGAGCGCGCCAAGCGCACGCTGTCCGCCGC  360
            ||||||||||||||| |||||||| |||||||||||||| ||||||||||||||  ||||
Sbjct  325  CCGCGCGCTGCGCCGTCTGCGCACGGCGTGCGAGCGCGCGAAGCGCACGCTGTCGTCCGC  384

Query  361  GACGCAGGCGACGATCGAGATCGACGCGCTGTTCGACAACGTGGACTTCCAGGCCACGAT  420
            |||||||||||||||||||||||||||||||||| ||||| |||||||||||||| ||
Sbjct  385  GACGCAGGCGACGATCGAGATCGACGCGCTGTTCGAGAACGTTGACTTCCAGGCCACCAT  444

Query  421  CACGCGCGCGCGCTTCGAGGAGCTGTGCGGCGACCTGTTCCGCAGCACGATCCAGCCGGT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445  CACGCGCGCGCGCTTCGAGGAGCTGTGCGGCGACCTGTTCCGCAGCACGATCCAGCCGGT  504

Query  481  GGAGCGCGTGCTGCAGGACGCGAAGATGGACAAGCGCTCCGTGCACGACGTCGTGCTGGT  540
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  505  GGAGCGCGTGCTGCAGGACGCGAAGATGGACAAGCGCTCCGTGCACGACGTGGTGCTGGT  564

Query  541  GGGCGGGTCCACGCGCATCCCGAAGGTGCAGAGCCTGGTGTCCGACTTCTTCGGCGGCAA  600
            |||||||| ||||||||||||||||||||  ||| ||||| ||||||||||||||||||
Sbjct  565  GGGCGGGTCAACGCGCATCCCGAAGGTGCAGTCCCTCGTGTCGGACTTCTTCGGCGGCAA  624

Query  601  GGAGCTGAACAAGAGCATCAACCCCGACGAGGCTGTGGCGTACGGCGCGGCGGTGCAGGC  660
            |||||||||||||||||||||||||||||||||| |||||||||| ||||||||||
Sbjct  625  GGAGCTGAACAAGAGCATCAACCCCGACGAGGCTGTCGCGTACGGCGCTGCGGTGCAGGC  684

Query  661  GTTCATTCTGACGGGCGGAAAGAGCAAGCAGACGGAGGGCCTGCTGCTGCTGGACGTGAC  720
             ||||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  685  CTTCATCCTGACGGGCGGCAAGAGCAAGCAGACGGAGGGCCTGCTGCTGCTGGACGTGAC  744

Query  721  GCCCCTGACGCTGGGCATTGAGACGGCCGGTGGCGTGATGACGGCGCTGATCAGGCGCAA  780
             || |||||||||||||| ||||| ||||| ||||||||||||||||||||| ||||||
Sbjct  745  ACCGCTGACGCTGGGCATCGAGACAGCCGGCGGCGTGATGACGGCGCTGATCAAGCGCAA  804

Query  781  CACGACGATCCCGACCAAGAAGAGCCAGATCTTCTCGACGTAGGCGGACAACCGGCCCGG  840
            |||||||||||||||||||||||||||||||||||||||||| |||||||||| ||||||
Sbjct  805  CACGACGATCCCGACCAAGAAGAGCCAGATCTTCTCGACGTACGCGGACAACCAGCCCGG  864

Query  841  CTTGCACATCCGGGTGTACGAGGGCGAGCGCGCAAGTACGAAGGACTGCCACTCCCTGGG  900
            | ||||||||| ||| | ||||||||||||||| |  |||||||||||||   |||||
Sbjct  865  CGTGCACATCCAGGTCTTCGAGGGCGAGCGCGCGATGACGAAGGACTGCCACCTGCTGGG  924

Query  901  CACTTTCAAACTGTTCGGGCATCCCGCCGGCCCCCCCGCGTTTTGCCGCAAAATCGGGGT  960
            ||| ||| | |||| | |||||||||||||| ||  ||||   | ||||| | ||| |||
Sbjct  925  CACGTTCGACCTGTCC-GGCATCCCGCCGGCGCCG-CGCGGCGTACCGCAGA-TCGAGGT  981

Query  961  GAGCTTTCGACCTGG  975
            || | ||||||||||
Sbjct  982  GA-CGTTCGACCTGG  995
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Figure 2: Alignment of the first isolate with *Leishmania tropicaHSP*70 gene with the universal strain MHOM/KE/81/NLB_030B Sequence ID: FN395026.1. **Query:** the target (studied) nucleotide sequence.**Subject:** nucleotide sequences within a database.

The variations between second studied isolates of *L. tropica* sequences and (MHOM/KE/81/NLB_030B) are listed in (table 3). The sequence analysis indicated the presence of DNA substitution mutations including both types which is transition and transversion. Transition was determined at the positions242, 248, 329, 344,359, 401, 436,587, 728, 1004, 1029, 1062, 695, 773, 953, 1011, 1069, 1080, 1102, 3, 282, 725, 749, 856, 974, 980, 1035, 1087, 8, 140, 671,743, 755, 886, and 944. In contrast Transversions was determined at the positions 62, 294, 23, 407, 653, 1066, 80, 245, 422, 577, 641, 665, 860, 965, 1020, 1048, 209, 554, 1018, 1046, 1071, 1081, 215, 284, 320, 360, 887, 576, 683, 1054, and 1092.Also, insertion of A, G, T, G, T, A, G were occur at positions 1005, 1021, 1064, 1075, 1083, 1085, and 1093 respectively. The alignment between second isolate and original gene at NCBI database was shown in Figure (3):

Table 3: Genetic mutations of *Leishmania tropicaHSP*70 nitrogenous bases/ First isolate.

Type of mutation	Nucleotide Subject/ Quarry	position
Substitution /Transversions	G⟶T	62, 294
Substitution /Transversions	T⟶G	23, 407, 653, 1066
Substitution /Transversions	C ⟶G	80, 245, 422, 577, 641, 665, 860, 965, 1020, 1048
Substitution /Transversions	A⟶ C	209, 554, 1018, 1046, 1071, 1081
Substitution /Transversions	T⟶ C	215, 284, 320, 360, 887
Substitution /Transversions	T⟶ A	576
Substitution /Transversions	C⟶ A	683, 1054
Substitution /Transversions	A⟶T	1092
Substitution /Transitions	G⟶ C	242, 248, 329, 344,359, 401, 436,587, 728, 1004, 1029, 1062
Substitution /Transitions	G ⟶ A	695, 773, 953, 1011, 1069, 1080, 1102
Substitution /Transitions	A⟶G	3, 282, 725, 749, 856, 974, 980, 1035, 1087
Substitution /Transitions	C ⟶T	8, 140, 671,743, 755, 886, 944
Insertion	A, G, T, G, T, A, G	1005, 1021, 1064, 1075, 1083, 1085, 1093 respectively

Score	Expect	Identities	Gaps	Strand
1605 bits(869)	0.0	1029/1107(93%)	7/1107(0%)	Plus/Plus

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Query  1     CGGAGGATGCCGGCACGATTGCGGGCCTGGAGGTGCTGCGCATCATCAACGAGCCGACGG  60
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Sbjct  21    CGAAGGACGCCGGCACGATTGCTGGCCTGGAGGTGCTGCGCATCATCAACGAGCCGACGG  80

Query  61    CTGCGGCCATCGCGTACGGGCTGGACAAGGGCGACGACGGCAAGGAGCGCAACGTGCTGA  120
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Sbjct  81    CGGCGGCCATCGCGTACGGCCTGGACAAGGGCGACGACGGCAAGGAGCGCAACGTGCTGA  140

Query  121   TCTTCGACCTTGGCGGCGGTACGTTCGATGTGACGCTGCTGACGATCGACGGCGGCATCT  180
             ||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||
Sbjct  141   TCTTCGACCTTGGCGGCGGCACGTTCGATGTGACGCTGCTGACGATCGACGGCGGCATCT  200

Query  181   TCGAGGTGAAGGCGACGAACGGCGACACCCACCTCGGCGGCGAGGACTTCGACAACCGCC  240
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Sbjct  201   TCGAGGTGAAGGCGACGAACGGCGACACACACCTTGGCGGCGAGGACTTCGACAACCGCC  260

Query  241   TGGTGACCTTCTTCACCGAGGAGTTCAAGCGCAAGAACAAGGGCAAGGACCTGTCGTCGA  300
             |  ||  ||  ||||||||||||||||||||||||||||||||||  |||  |||||  ||||||
Sbjct  261   TCGTCACGTTCTTCACCGAGGAGTTCAAGCGCAAGAACAAGGGTAAGAACCTGGCGTCGA  320

Query  301   GCCACCGCGCGCTGCGCCGCCTGCGCACCGCGTGCGAGCGCGCCAAGCGCACGCTGTCCG  360
             ||||||||||||||||||  ||||||||  ||||||||||||||  ||||||||||||||
Sbjct  321   GCCACCGCGCGCTGCGCCGTCTGCGCACGGCGTGCGAGCGCGCGAAGCGCACGCTGTCGT  380

Query  361   CCGCGACGCAGGCGACGATCGAGATCGACGCGCTGTTCGACAACGTGGACTTCCAGGCCA  420
             ||||||||||||||||||||||||||||||||||||||||||  |||||  ||||||||||||||
Sbjct  381   CCGCGACGCAGGCGACGATCGAGATCGACGCGCTGTTCGAGAACGTTGACTTCCAGGCCA  440

Query  421   CGATCACGCGCGCGCGCTTCGAGGAGCTGTGCGGCGACCTGTTCCGCAGCACGATCCAGC  480
             |  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441   CCATCACGCGCGCGCGCTTCGAGGAGCTGTGCGGCGACCTGTTCCGCAGCACGATCCAGC  500

Query  481   CGGTGGAGCGCGTGCTGCAGGACGCGAAGATGGACAAGCGCTCCGTGCACGACGTCGTGC  540
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Sbjct  501   CGGTGGAGCGCGTGCTGCAGGACGCGAAGATGGACAAGCGCTCCGTGCACGACGTGGTGC  560

Query  541   TGGTGGGCGGGTCCACGCGCATCCCGAAGGTGCAGAGCCTGGTGTCCGACTTCTTCGGCG  600
             ||||||||||||  ||||||||||||||||||||  |||  |||||  ||||||||||||||
Sbjct  561   TGGTGGGCGGGTCAACGCGCATCCCGAAGGTGCAGTCCCTCGTGTCGGACTTCTTCGGCG  620

Query  601   GCAAGGAGCTGAACAAGAGCATCAACCCCGACGAGGCTGTGGCGTACGGCGCGGCGGTGC  660
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Sbjct  621   GCAAGGAGCTGAACAAGAGCATCAACCCCGACGAGGCTGTCGCGTACGGCGCTGCGGTGC  680

Query  661   AGGCGTTCATTCTGACGGGCGGAAAGAGCAAGCAAACGGAGGGCCTGCTGCTGCTGGACG  720
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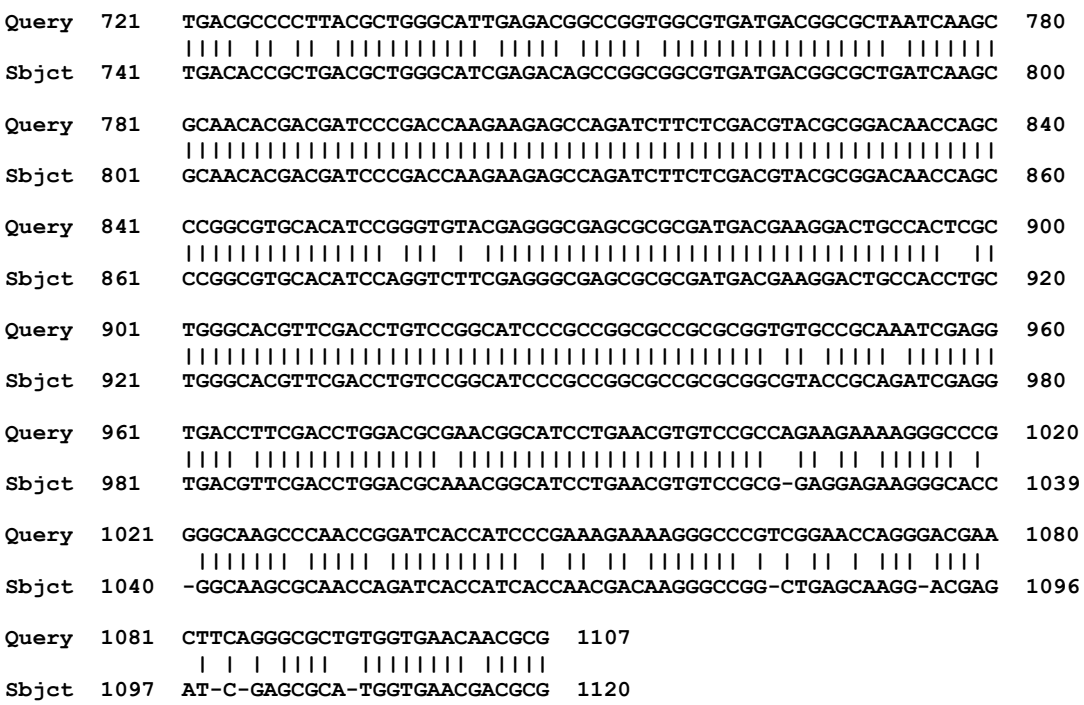

Figure 3: Alignment of the second isolate with *Leishmania tropicaHSP*70 gene with the universal strain MHOM/KE/81/NLB_030B Sequence ID: FN395026.1. **Query:** the target (studied) nucleotide sequence.**Subject:** nucleotide sequences within a database.

The alteration of nitrogen nucleotides results may be due to several reasons, comprising the geographical area differences that led to changes of genetic material of the Iraqi isolates, as a consequence of adaptation or mutations.The sequence analysis of the PCR amplicons is a more informative method. Thesubsequent identification of single nucleotide polymorphisms (SNPs) or comparison ofthe obtained sequence with available reference sequences allows species identification and tracking of evolutionary relationships among the sequenced isolates [23].

The 70 kDa heat-shock protein (*HSP*70) is conserved across prokaryotes and eukaryotes as well as its encoding gene. It has been applied in phylogenetic studies of different parasites. A previous survey [24] determined sequences and phylogenetic analysis proved that 52 *HSP*70 sequences representing 17 species commonly causing leishmaniasis both in the New and Old World. The genus *Leishmania* formed a monophyletic group with three distinct subgenera *L*. (*Leishmania*), *L*. (*Viannia*), and *L*. (*Sauroleishmania*). Another study by [14]describes*Leishmania* species diagnosis of clinical samples on the basis of partial sequencing of the heat-shock protein 70 gene (*HSP*70) dispensing classical parasite isolation.*LeishmaniaHSP*70 sequences were determined on the basis of a single PCR amplicons.The final alignment contained 207 sequences from 42 *Leishmania*-endemic countries, representing 18 species of which 15 are causing human leishmaniasis.

IV. Conclusion

This study determined sequence variants that occur in*Leishmania tropica*/ *HSP*70 gene as a result of genetic mutation and/or single nucleotides polymorphism. Sequencing analysis of genes in target provides an inclusive and controllable resource to target efforts in identifying parasite factors that influence infection. Conversely, factors that are unique to the *Leishmania* genus but common to all species may be used as prospective treatment purpose or vaccine applicants.

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Duha Muhanad Bayram1. "Genetic Polymorphism of Iraqi*Leishmania tropica* Isolates Based on *HSP70*Gene." IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS), vol. 13, no. 1, 2018, pp. 60-66.