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# Molecular investigation of cutaneous leishmaniasis in humans in Al-Najaf Al-Ashraf governorate

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***Abstract***---The method of molecular diagnosis using PCR technology, phylogenetic tree and DNA sequencing was used for the purpose of microscopic examination and accurate characterization of L. Major parasite. Depending on the diagnostic gene (ITS1) with a size of 350 pb and 500 pb base pairs, the genetic tree was also analyzed and the similarity ratios were compared among the globally registered isolates using the program MEGA In order to compare the percentages of similarity between strains in this study with local and global isolates registered in the NCBI Global Gene Bank within the Data base , Mega 10 program was used . Global Isolates.The results of the molecular diagnosis PCR for the highest samples of the neighborhoods of the southern governorate showed (26) injuries with a percentage of 40 %, and the lowest in the governorate center (5) injuries with a percentage of 7.7 %, while the district of Kufa and the northern neighborhoods recorded (21) and (13) injuries with rates of 32.3 %, 20 And straight.

***Keywords***---molecular investigation, cutaneous leishmaniasis, humans.

## Introduction

Polymerase chain reaction (PCR) method has been used successfully recently and keeps pace with the development in the field of Molecular biology in diagnosing the type of parasite and the main cause of leishmaniasis in clinical samples, where the Deoxy DNA is amplified and doubled ribo nucleic acid) (outside the vitro system in vitro and that Using special primers designed for the gene encoding

Kineto plastic DNA (KDNA) with high sensitivity and specificity. The products of replication are electrically migrated so that the genes can be known (Akhoundi M, 2013). Prevention and control consists in preventing and controlling the house fly to humans, eliminating all stored hosts ، treating infected people, and providing health education. (Al Hilfi TK, 2013) .

**Modern techniques used in the diagnosis of cutaneous leishmaniasis**
**Polymeric chain reaction polymwrase chain reaction (PCR)**

It is the latest techniques used in diagnosis and has a very high sensitivity and is used in diagnosing leishmaniasis in many locations such as blood, lymph node biopsy, spleen, bone marrow, skin and excised parts (Alexander, 2012)، as well as identifying the parasite-bearing sand bug from uninfected others (Al-Fartusie, 2017). The development in molecular biology has provided modern methods for diagnosing and profiling Leishmaniasis to avoid traditional errors. The PCR technique is of 100 %quality (Al-Helaly, 2014) and it is suitable for all types of Leishmaniasis and various samples such as skin ulcers and others .This technique can be used to find out the reservoir or animal reservoir of the disease Leishmaniasis, and this method is very effective in epidemiological studies and detect infection in individuals and animals (Al-Hoot, 2017) .

This technique was invented by the American scientist Mellis in 1990 ,where it enabled researchers in the field of molecular biology to duplicate the DNA of millions of copies of a small piece of it .This method is a great scientific revolution in all scientific research and accurate diagnosis of living organisms according to their DNA. (Ali,2018). This technique was demonstrated based on ssur RNA ،which includes DNA in all types of Leishmania ،and sets a point of inflection with the RRNA genes and differentiates them by using a primer constructed with three complex ends of the specific genetic change site in the ssur RNA genes in Leishmania species ،and the detection of DNA and RNA . In those samples, as well as the detection of early infections, and this technique has become very important in working in most international laboratories (ALI, 2013).

DNA results were shown Leishmaniasis identified 10 months prior to diagnosis in asymptomatic cases to investigate for leishmaniasis. visceral (Allahverdiyev AM,2011) It was found that this method has a high specificity and sensitivity of up to 100 %compared to other laboratory tests and is necessary to identify the types of leishmaniasis isolated from the different clinical types of the disease (Al-Obaidi, 2016). (PCR) helps to early diagnosis of the disease and reduce the patient's stay in hospitals and the financial cost as well. Modern molecular methods such as hybridization with specific probes RFLP/multiplex/PCR-dot blot/DNA have been proven. PCR- and fingerprints for accurate identification of types of Leishmania, and this aspect is important in the epidemiological and scientific investigation in the scientific and health fields (Ali, 2016). The principle of PCR technology is to amplify a few molecules of) DNA) DNA after extracting it from bodily fluids or cells and to obtain large quantities of it and to be able to perform the analysis. as follows:

- primers: which are made up of a small number of nucleotides 18-20 Oligonucleotides

- Nitrogenous base that can bind to the DNA to be amplified at a specific and distinct location called the Highly Conserved Region.
- Excessive amounts of deoxyribonucleotide triphosphate
- (d ATP , d CTP , d GTP , d TTP) Deoxy nucleoside triphosphates (d NTPS).
- The enzyme oligomerase Polymerase resistant to high temperature Taq polymerase extracted from hot springs bacteria.
- Protective solutions or buffers.
- Suitable electrolytes, such as the magnesium ion ,Mg + 2, which represents the cofactor of the polymerase enzyme. (Allahverdiyev AM,2011).

**Copying process**

Nucleic acid to be transcribed with the primer , polymerase enzyme , and a group of nucleic acids is placed inside a tube in a thermo-controlled device and is in three independent stages.

- Denature stage: in which the temperature is raised to 94 ° C to decode the original DNA.
- Anneal adhesion stage: the temperature is lowered to a rate of 55-60 (C (for the primer to stick physically through hydrogen bonds with the original DNA.
- Extend stage: the temperature is raised to 75 ° C and polymerase is done to start the process of building new DNA. This technique relies on the ability of the DNA polymerase enzyme to make a copy of a specific region of one of the DNA strands in the presence of DNA primers and auxiliaries for the replication process to take place through (40-30) cycles in the programmed thermal circulatory system to reach and detect a quantity of the target and detect it (about a billion). copy) and doubling the sequence and specifying it. The amount of DNA output depends on the number of cycles, and this technique appears to be the most accurate of serological techniques for diagnosing disease within a few hours ( Alvar J, 2012) .

**Materials and Working Methods**

**Devices used**

Table 1-3
of the devices used in the current study

| Origin | company | the device name | T |
|---|---|---|---|
| Japan | Olympus | light microscope | 1. |
| Germany | Hettish | Cetrifuge Centrifuge | 2. |
| USA | Clearer Scientific | PCR test device | 3. |
| USA | Matini | PHmeter _ | 4. |
| Holland | Dame shipyard | Incubator _ | 5. |
| Lebanon | Concord | Deepfreeze _ | 6. |
| Korea | Bioneer | Vortex mixer | 7. |
| USA | Sony | Microscopic camera | 8. |

| | | | |
|---|---|---|---|
| USA | Biotek washing | Bioelisa washing _ | 9. |
| England | Clever | Electric relaydevice _ | 10. |
| Germany | group Gemmy indyslreal | Water bath | 14 |
| Spain | Myrl | wax casting device Embidding | 15th |
| French | Froilabo | Oven antenna | 16 |

**Chemical Materials**

Table 3-2
of the chemicals used in the current study

| Origin | company | Subject Name | T |
|---|---|---|---|
| | | PCR diagnostic kit | **1.** |
| | | | |
| India | Labort | absolute ethanol alcohol | **2.** |
| India | Labort | methanol alcohol | **3.** |
| India | Labort | Formalin 15 % | **4.** |

**Laboratory tools and supplies**

Table 3-3
of the laboratory tools and supplies used in the current study

| Origin | company | tool name | T |
|---|---|---|---|
| Jordan | AFco | blood collection tubes test tube | **1.** |
| | | | |
| China | China | 5ml and 1ml medical syringes Disposable Syringe | **2.** |
| Canada | Bio Basic | automatic tubes | **3.** |
| UK | Glasswerk | dye pot | **4.** |
| China | Citoglas | glass slides Slide | **5.** |
| China | Citoglas | Cover Slide | **6.** |
| Thailand | Sampermed latex | Gloves paws | **7.** |
| China | China | Eppendorf tube rack | **8.** |

**Blood samples**

Venous blood samples were drawn for (65) people with cutaneous leishmaniasis and from (18) healthy control group people of all ages studied, by a medical syringe size (5 ml), which was distributed to (2 ml) of blood placed in EDTA anticoagulant tubes for the procedure. Blood picture tests : Basophil, Neutrophl , monocytel , pcv , Hb , WBC, Eosinoph .(4 ml) of blood was also placed in special tubes with a red cap to extract serum and left tilted for 15 minutes at room temperature, then placed in a centrifuge Then, the sera were placed in Eppendorf tubes made of plastic, marked and stored at -20°C until use in subsequent examinations.

**Molecular study**
**Partial study**
**Tissue DNA extraction**

DNA extraction was carried out from suspicious tissue samples using the tissueDNA extraction kit supplied by the Canadian company Abm .The extraction was carried out according to the company's instructions as follows:

- 100mg of tissue abrasive sample was transferred to tubes and then 400 µl of lysis buffer was added .
- 20µl Proteinase K was added to sterile 1.5 mL tubes and mixed well with a Vortex apparatus for 30 seconds.
- Samples were incubated at°60 C for 10 minutes.
- 200µL of Binding buffer was added to each sample and mixed well with the Vortex for 10 minutes.
- Samples were incubated at°60 C for 30 minutes.
- 200µl of absolute ethyl alcohol were added and mixed with a Vortex for 15 seconds.
- The mixture was transferred to special tubes equipped with the kit called Binding column placed inside 2ml collection tubes and then these tubes were placed in a centrifuge at 8000 rpm for one minute and then the precipitate was discarded.
- 500µL of Washing buffer1 was added and these tubes were centrifuged at 8000 rpm for 1 min and then the sludge was discarded.
- 500µL of Washing buffer2 solution was added. These tubes were centrifuged at 12,000 rpm for 3 min and then the sludge was discarded.
- DNA-containing Binding column tubes were transferred to sterile 1.5 ml tubes, 50 µl of Elution buffer were added and the tubes were centrifuged at 8000 rpm for 1 minute to dissolve the DNA.
- The DNA was kept in the refrigerator until the PCR test was performed .

**Examination of the extracted DNA profile**

DNA extracted from the samples was detected using the Nanodrop spectrophotometer device for detection and measurement of the concentration of nucleic acids ) DNA $\mu$and RNA (. The absorbance at a wavelength ranging from 260-280 nm was used as follows:

- After operating the Nanodrop device ,the DNA measurement program was selected
- Zeroing the scale substrate twice using blotting paper for the device, by placing one microliter of ) ddH2O (using a sterile micropipette on the surface of the scale substrate and due to zeroing, then we clean the substrate for measuring samples.
- Pressing the OK button to start the process of measuring the concentration of DNA ,using one microliter of each sample of the extracted DNA ,and then we clean the substrate of the scale of the device again to measure the other sample.
- The purity of the extracted DNA samples was also determined by reading the absorbance of the Nanodrop Spectrophotometer at two wavelengths of

260/280 nm, as the extracted DNA is considered pure when the absorbance ratio is.(1.8)

**Examination method PCR**

PCR assay was performed using the primers specific to the genes of the samples and the following steps:

**PCR examination**

Using the 18SrRNA primer for parasite genus diagnosis yields bP size products.

**Preparation of a PCR master mix Primary**

The polymerase chain reaction mixture was prepared using the xPCR Mix2 kit supplied by the Canadian Abm Company, according to the company's instructions as follows:

Preparing the polymerase chain reaction mixture in the PCR tubes equipped with the kit and containing the components of the polymerase chain reaction. The other components were added to the reaction mixture according to the company's instructions as in Table:(6-3)

Table 1
PCR Polymerase Chain Reaction Mix

| PCR master mix | Volume |
|---|---|
| DNA template | 5$\mu$ |
| Forward primer (10 pmol ) | 3 $\mu$ |
| Reverse primer (10 pmol ) | 3 $\mu$ |
| 2x Taq polymerase | 25 $\mu$ |
| Nuclease free water | 14 $\mu$ |
| Total | 50 $\mu$ |

After completion of the polymerase chain reaction mixture preparation, the tubes were closed and carefully mixed with a Vortex mixer for 5 seconds. The tubes were transferred to the PCR Thermocycler for PCR Thermocycler conditions.

**PCR Thermocycler conditions**

PCR examination was carried out using a PCR Thermocycler ,and the device was programmed as in Table:(5-3)

Table 2
Nested PCR Thermal Cycles

| PCR Step | Repeat cycle | PCR master mix | Volume |
|---|---|---|---|
| Initial | 1 | 94 | 30 min |

| | | | |
|---|---|---|---|
| denaturation | | | |
| Denaturation | 35 | 94 | 30 sec |
| Annealing | | 51 | 30 sec |
| Extension | | 72 | 60 sec |
| Final extension | 1 | 72 | 10 min |
| Hold | - | 4 | Forever |

**Acarose gel electrophoresis Gel electrophoresis**

Electrophoresis was carried out using 1.5% agarose gel to read the result of the PCR product analysis as follows:

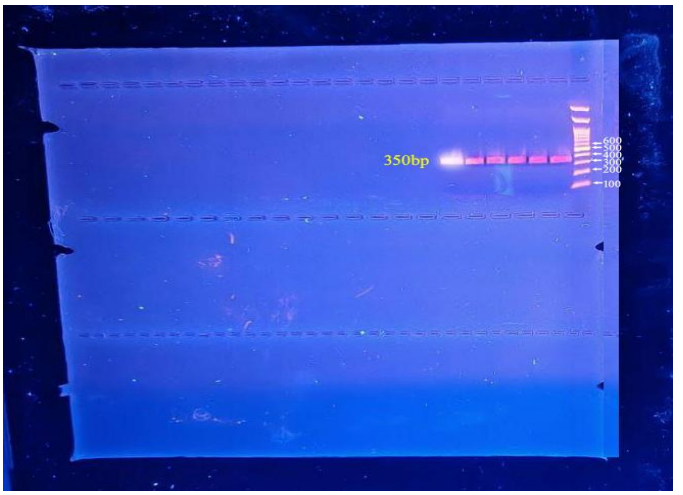
- 1.5g of acarose in 100 cm of TBE buffer The buffer at X1 concentration and using the magnetic vibrating plate until complete dissolution.
- dye Ethidium 5 . was added to the acarose microliters per 100 ml.
- Leave the agarose solution to cool until it reached a temperature of 50-55 ◦.
- Prepare the glass plate by installing the plate on the surface and fixing the comb on it.
- Pour the agarose softly and continuously to prevent air bubbles from forming.
- Leave the agarose gel at room temperature for half an hour to solidify and gently lift the comb and then transfer the agarose to the electrophoresis basin.
- agarose is immersed in a solution) TAE 1x ,(as the level of the solution is 5 mm higher than the level of the gel above the surface of the agarose.
- 10µl of DNA was added.
- 5µl )DNa Ladder 3000bp (was added to the first hole.
- Connect the power supply to the relay trough by wires and the positive and negative poles and pass the current at 100 volts for 60 minutes and relay towards the positive pole.
- After the migration process was completed, the plate was quietly removed from the basin and placed under the UV-transilluminator by exposing it to a wavelength of 260 nm to observe the amplifier bands and then photographed.

**Results**

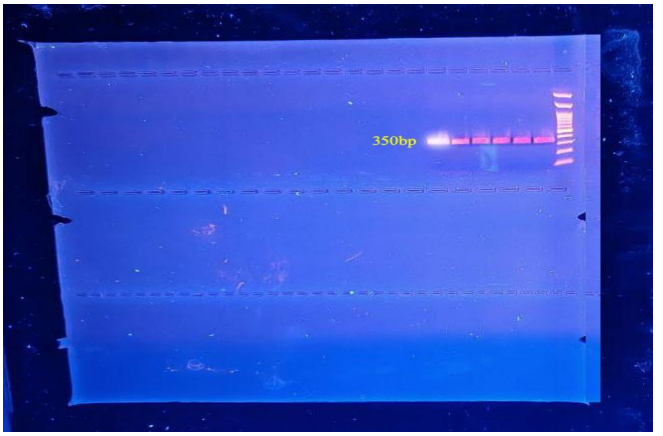
**Molecular study to diagnose cutaneous leishmaniasis by (PCR) technique**

PCR technique was used to diagnose cutaneous leishmaniasis in Najaf governorate, where the DNA of the leishmaniasis parasite was extracted .KDNA image (1-4) of all samples. The process of electrophoresis was carried out in a PCR machine ,and a specific part of the DNA was copied and numerically amplified according to the steps shown in the method of work. A positive result was recorded in the presence of DNA and a bundle of length (500bp, 350bp). base pair compared to the standard ladder and according to the result a person is as follows:

*Leishmania Major* 500bp




Picture 1-4. Electrophoresis of the results of profiling isolates from ( L.Major ) bp350 by PCR technique




Picture 2-4. Demonstrate the presence of DNA extracted from the cutaneous Leishmania parasite through electrophoresis in agarose gel

The molecular study using polymerase chain reaction technology showed that cutaneous leishmaniasis in Najaf governorate was caused by L. Major , and the highest infection rate was (40%) in the southern neighborhoods, while the governorate center recorded (7.7%) and was the lowest . ($p<0.05$) as shown in Table (1-4) .

Table 1-4
Percentages of infection Leishmaniasis according to residential neighborhood divisions

| % | Number of injured | Residential dividers |
|---|---|---|
| 20 | 13 | Northern neighborhoods |

| | | |
|---|---|---|
| 7.7 | 5 | Governorate Center |
| *32.3 | 21 | Kufa District |
| 40 | 26 | Southern neighborhoods |
| 100 | 65 | total |
| 4.2 | | Arithmetic chi- square value |
| 0.71 | | tabular chi - square P < 0.05 |

**Discussion**

**Molecular study for the diagnosis of cutaneous leishmaniasis by PCR**

Human infection with leishmaniasis is overlapping and different in terms of clinical signs and symptoms that appear on infected persons. Therefore, it is necessary to know the type of parasite that causes the disease, if it is leishmaniasis. Late visceral or cutaneous leishmaniasis , therefore, it has become important to use very accurate and highly sensitive diagnostic methods. Hence, the polymerase chain reaction ) PCR (technique was used to investigate and differentiate between leishmaniasis parasites , where DNA amplified For leishmaniasis after extraction from the samples, which has a sensitivity of 98.7% for diagnosing cutaneous leishmaniasis (Amin VI, 2012) .PCR technique in the current study demonstrated that the isolates used in the study belong to the type of Leishmania major *L. Major* with molecular weight 500bp ,350pb and it is responsible for the incidence of skin disease within the study area.

In agreement with these results, the findings of a study that showed a wide spread of Leishmaniasis *L. Major* among its other species .(Amzar, 2017) .The results of the current study also agreed with the findings of ( Andreani G, 2012) in Baghdad , which is that the parasite *L. Major is the only one diagnosed with* PCR technique . ( 98% of its samples were *L. Major* ,while the results of the current study did not agree with what was found by Al Shukr (2012) , where two types of leishmaniasis were diagnosed , namely L. Major. 620bp and L. tropica 800bp Also, the results of the current study did not agree with what was reached by Rahi etal . (2013) when using the PCR technique ,where he indicated that there are two types of Leishmaniasis in Iraq, they are (36.4) *L L . tropica And by* 63.6% )*L. Major* . (In northwestern Iran, specifically in the city of Mashhad ,Antinarelli (2018) indicated that there is a greater percentage of L. tr0pica and less *L. Major* . This is inconsistent with our current study.

Most of the studies indicated that L. Major *leishmaniasis* is the dominant and dominant disease in Najaf Governorate, compared to *L. tropica , which is* present in a small percentage, and this indicates the dominance of leishmaniasis of animal origin (ZCL) )Al Shukr 2012 .(Areas (2017) explained in Iran the use of the diagnostic technique (PCR) from the reservoir hosts as a high-accuracy method .In a scientific study carried out by Ashkani-Esfahani , (2014) results were shown for 14 cases of leishmaniasis , 12 of whom were diagnosed with L .*Major and only 2 with L. tropica* .Table ( 9-4) indicated a significant difference according to the statistical study at the level (p<o.o5) , and that the infection with the cutaneous

leishmaniasis parasite in the southern neighborhoods was at a rate of (40%) , which is the highest percentage in the governorate's regions, and the governorate center recorded the lowest infections with a percentage of (%). 7.7) These results are consistent with the findings of Al-Moussawi (2015) that the proportion of the governorate center in Karbala had the lowest number of infections (2.7%) compared to the areas of sub-districts and districts ,such as Ain Al-Tamr district with a percentage of (39.7%) and Al -Hussainiya district (28.7%) .The areas of the governorate center are scarce of animals, in contrast to the large presence of storing animals such as dogs and rodents, and this is what caused the increase in the infection rate in them as a result of the relationship between humans and the storage host (rodents and dogs) and the carrier host (sand flies) in urban areas (Azami, 2018).

**DNA Sequencer Analysis**

Through the analysis of the genetic tree, it was found that there is a high convergence of the nitrogenous bases of the type *L. Major* with a group of globally registered samples. It was noted that the cutaneous leishmaniasis samples *L. Major* in the current study were 99% close to the sample with serial number OK 560729 in the Kingdom of Saudi Arabia, and 97% approached With the sample serial number KP 773410 in Iran .study with its comparison with international samples ,as well as the identification of the sequences of nitrogenous bases of the COT gene for cutaneous leishmaniasis samples. Globalism. This is consistent with the one who showed the molecular biology approach as a tool for limited and sensitive diagnosis and as a target for accessing the genetic information of the parasite.

**Conclusions**

- Molecular diagnostics (PCR) technology, which is one of the modern and accurate techniques in diagnosis, confirmed that *L. Major* is the dominant type in Najaf governorate and is the cause of cutaneous leishmaniasis in it, and that the highest infection rate was recorded in the southern regions of the governorate (40%) and the lowest was in the governorate center (7.7%).
- The genetic tree of the cutaneous leishmaniasis parasite identified the species present in the governorate and those genetically close to it and found in neighboring countries.
- Cutaneous leishmaniasis is a common transmissible disease and is considered one of the endemic diseases in Najaf and Iraq alike.

**Recommendations**

- Using micro-molecular techniques such as real time polymerase chain reaction - PCR to find out the DNA sequences and study the molecular characterization of the multiple species responsible for cutaneous leishmaniasis in Iraq.
- Emphasizing the dissemination of health education and awareness among citizens through introductory seminars on parasite and disease and how to prevent and treat, in addition to combating the host vector and storage by spraying chemical pesticides, controlling rodents and loose dogs, urging

cleanliness and disposal of waste and organic materials that are an environment conducive to the growth of the vector (Sand fly).

- Emphasis on the development of effective treatments to reduce the severity of skin infection, and the search for plant extracts with positive efficacy to cure the disease.

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