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Genetic Detection of *Leishmania Tropica* in Clinical Samples from Patients with Cutaneus Leishmaniasis by Using Convenntial PCR and RT-Time PCR

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Abstract

Background: cutaneous leishmaniais is an endemic parasitic disease in Iraq.

Objectives:

We applied two kinds of PCR technique: convinential PCR, and RT-PCR on paraffin-embedded skin biopsies to estimate which *Leishmania* spp. is most prevelence in iraqi population. The detection of *Leishmania* by Traditional serological methods was not accurtate, a pair of primers were used to amplify a region within 5.8s ribosomal RNA gene, then the amplified products were sequenced by macrogen company. The Real-time PCR were done to detect the presence of *Leishmania troppica* sp. by targeting spesific gene ITS. This technique were done to compare its accuricy to the conventional pcr technique. The phylogenetic tree was studided to show the distence among the spesies in Iraq and other countries.

Methods: A total of 26 specimens collected from patients with cutaneous ulcers suggestive of leishmaniasis with age ranging from 5 to 14 years. All those patients were attending hospital and health centers in north Baghdad from July to September of 2019. After the DNA extraction it has been ita has been visuilized by DNA electrophoresis, Real-time PCR has been used to explore the presence of cutaneous *Leishmania* DNA in samples. Conventional PCR were also done and the amplified products were sent to sequencing.

Results: the results proved that using ITS gene as detection gene is accurate enough. The phylogenetic tree was shown 48% share identical with isolate from Iran (MH488993) and the least identical percentage showed 20% with isolates from Spain and USA (MN604128 and FJ948452).

Conclusions: This study was proved that RT-PCR has less invasive sampling, more sensitivity, and specificity than traditional diagnostic methods and recommend it to be used for detection of

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leishmaniasis in hospitals and research centers in Iraq.

Introduction

The unicellular flagellated protozoan *Leishmania* causes Leishmaniasis, which has remains neglected and affects mostly underdeveloped and poor countries. It is estimated that annually 1.3 million new cases and up to 30 000 deaths occur according to World Health Organization (WHO) reports (Alvar, Vélez et al. 2012). *Leishmania* caused cause varied medical patterns, including cutaneous, mucosal, and potentially life-threatening visceral forms (Herwaldt, 1999)**.** The identification of species is important for the clinical treatment. Cutaneous leishmaniasis considered as a group of diseases as it has varied spectrum of clinical appearances, which include small cutaneous nodules to gross mucosal tissue destruction. Several Leishmania spp, cause cutaneous leishmaniasis such as *Leishmania major*, *L. tropica*, and *L. mexicana*. Leishmaniosis considered as neglected diseases because rarely fatal, with little interest by financial donors, public-health authorities, and professionals to implement activities to research, prevent, or control the disease (Reithinger, Dujardin et al. 2007). Leishmaniasis represent a diagnostic challenge as there are wide spectrum of clinical manifestations that they may present.

Molecular approaches are typically more sensitive, less labor-intensive, and more rapid when compared to the traditional parasitological approach for *Leishmania* species identification (Sakkas, Gartzonika et al. 2016). The main molecular diagnostic test of researcher is PCR (Reithinger and Dujardin 2007). Numerous different PCR types are available that can be used to detect and genotyping the *Leishmania sp.*, and the most used technique is the conventional PCR in which PCR product have to be detected by and visualized after ethidium bromide staining (Benassi, Benvenga et al. 2017)**.** Another approach can also be used in which PCR products are evaluated the amplification and this technique named real-time PCR. In this technique the reaction and detection are performed in a single tube setup which considered an advantage as it increase the accuracy and decrease the time consuming (Corradini 2018).

For those reasons that mentioned prevouisly this study aims to estimate which *Leishmania* spp. is most prevelence in iraqis that have been serologicaly tested for positive leishmaniasis. Additionally to determinbe the most approperait molecular test for *Leishmania* and finally to detect the genetic distance between different isolates that obtained in this study and between the isolates from the different parts of the globe obtained from the NCBI database.

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Materials and methods

A total of 26 specimens collected from patients with cutaneous ulcers suggestive of leishmaniasis with age ranging from 5 to 14 years, they were attending hospital and health centers in north Baghdad and more accurate tests were done to ensure the infection of *Leishmania*, within period of July to September of 2019. The patients complained from skin lesion in exposed part of the body mostly in the face, leg, and arm and diagnosed clinically by special dermatologist as cutaneous leishmaniasis. Biopsies from the lesions were preserved in deepfreeze (-20 °C) for molecular analysis.

Genomic DNA Extraction

In this study, samples of 26 patients dignosied by infection with cutaneous leishmaniasis were diagnosed by PCR. DNA was extracted of the 26 samples by using ZYMO research kit according to manufacturer's manual.

ribosomal RNA gene amplification

In order to target 5.8s ribosomal RNA gene within the DNA, the following primer pairs was used: The sequence of forward primer AAAAACAACACGCCGCCTC. And reverse; AAAAATGGCCAACGCGAAAT. The PCR reaction was performed in a total volume of 25 ml l containing 5 µl DNA, 1 µl of each primer, 12.5 µl Thermo-start PCR Master Mix (KAPPA, USA) and continue the volume up to 25 µl with DNase/RNase-free water. Cycling conditions was performed as follows: 95°C for 7 min, followed by 35 cycles of, denaturation at 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. the product then visualized on gel electrophorisis the product size shown 400 bp band. The target gene were sequenced by macrogen company/ Korea online at (https://dna.macrogen.com/eng/member/login.jsp.

Real- time PCR

Real-time PCR has been used to explore the presence of *Leishmania tropica* DNA in samples. Real-time PCR were done to detect the presence of *Leishmania* sp. By targeting spesific gene ITS by using forward primer; TACGAGAGGAACTCCCATGC and reverse primer; TGGGATTGGCTTCTGGCTTAG. Which specify a zone with 118bp. The RT-PCR program were as follow. First denaturation 95C for 7 min followed by 40 cycles of denaturation 95C for 30sec, annealing 60C for 30sec and final cycle extension 72C for 30 sec.

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Results and discussion

After extraction the DNA electrophoresis were done to check the extraction procedure the results of DNA extraction are shown in figure-1

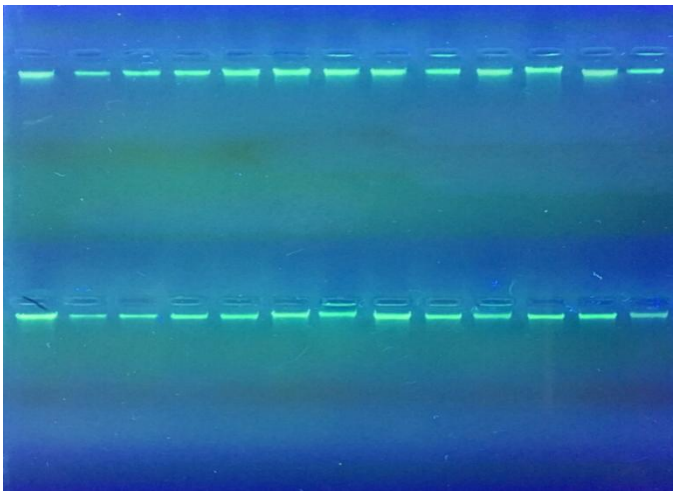

Figure 1: gel electrophoresis after DNA extraction

The results of the RT-PCR were shown in the figure-2 as curves showing the ingreasing flourecense as the amplification were going the results proved that using ITS gene as detection gene for *Leishmania tropica*

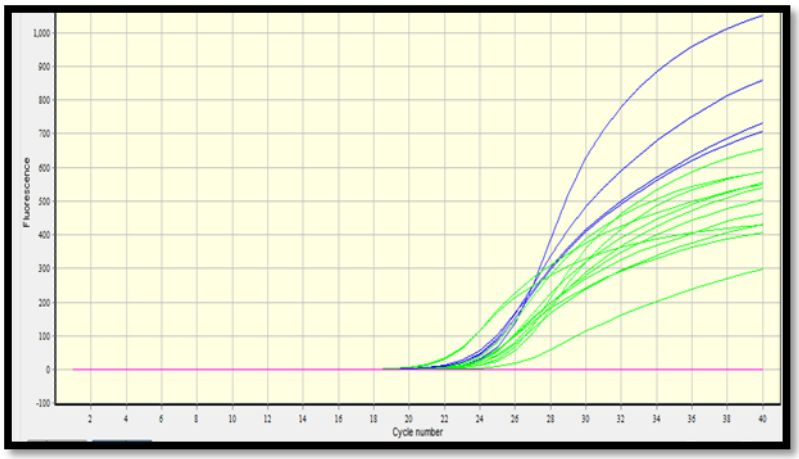

Figure 2: real time PCR curves showing the amplification of 26 samples.

Phylogenetic tree constructed from the 5.8S ribosomal RNA gene sequence of cutaneous *Leishmania* spp. using MEGA6 analysis and neighbor-joining (NJ) algorithm from the alignment (Clustal W sequence alignment) shown in Figure (3). Hierarchical cluster analysis determine the

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following Iraq isolate (2,8) the identical 100%, and both isolates shown 48% with isolate 1 all those isolates share 48% identical with isolate from Iran (MH488993) and 26% isolate from India (EU326226) and those previous isolates share 46% with isolate 3 and isolate from Iran (KY612602). The least identical percentage showed 20% with isolates from Spain and USA (MN604128 and FJ948452).

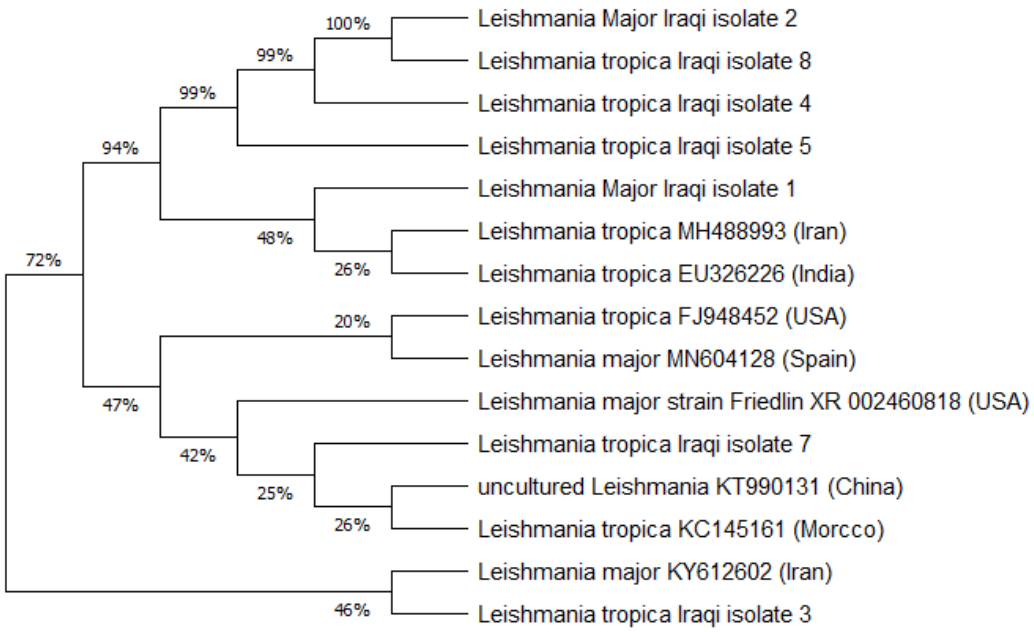

Figure 3 :Neighbor-joining tree *Leishmania* spp. of 5.8S ribosomal RNA gene.

Discussion

The diagnosis of cutaneous leishmaniasis ususally established on clinical features and numerous diagnostic methods have been using with a varied precision, including direct parasitologic examination (microscopy, histopathology, and parasite culture) or indirect testing with serology and molecular diagnostics. According to the available instruments ussually determine the diagnostic test employed not on diagnostic accuracy (Zhao, Duan et al. 2015) . In this study we employed two molecualr tests after the clinical features diagnostics.

To diagnose CL studies have been developed many molecular diagnostic tests CL, and the majority of those studies used RT_PCR and assumed to have better sensitivity and specificity than traditional diagnostic methods and allow the use of less

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invasive sampling for diagnosis (Cruz, Millet et al. 2013). In particular PCR, numerous different gene sequences have been targeted over the last decades. In this study we targeted two genes (5.8S ribosomal RNA gene have been targeted in conventional PCR while the ITS gene targeted in RT_PCR as it has conserved sequence).

In Iraq the last war against ISIS increased the migration from north of the country to middle and south regoins and because of the lack of facilities and poor of living condition led to spread the cutaneous leishmaniasis (Al-Warid, Al-Saqur et al. 2017)

Previous study performed by Hijawi (Hijawi, Hijjawi et al. 2019) showed the ITS1 region amplification technique the best for the discrimination of *Leishmania tropica* parasite. And another study in Iraq done by (Hamza, Obayes et al. 2019) also agreed with our results and the phylogenetic distence among the spesies and other part of the world. In this study the phylogenetic analysis showed high similarity among isolates from the Iraqi species which are more maching genetically with Iranian species compared with other species enrolled in this study which are being primarily calculated with the use of molecular sequencing data or morphological data matricesthe, and this variation might be related to the differences in the ecology and pathology of the isolates within the same species. The genetic structure of the population, that can be stimulated by geographical barriers, parasite distribution. Due to the progressive advancement in sequencing technology this led to significant increases in phylogenetic and population structure analysis(Assimakopoulos and Marangos 2020).

Conclusion

This study was performed to diagnoses cutaneous *Leishmania* species using molecular methods. The results were shown that using molecular methods were most accurate and specific than other convenantial serological methods. Also, the real-time technology reduce the time of analysis and the risk of contamination in order to detect the presence of *Leishmania* sp. DNA . This study recommended to use the molcular techniques in the diagnosis of cutaneous *Leishmania* and other parasitic infectious species in Iraq.

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