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Molecular Study for Detection of Cutaneous Leishmaniasis by Nested PCR in Wasit Province, Iraq

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Annotation: Cutaneous leishmaniasis (CL) is an ignored parasitic condition of main clinical interest, which is spread by a parasite, and the disease has a high morbidity in the endemic regions, including Iraq. It is brought about by numerous genera of the genus Leishmania and is contracted by the bite of an infected female phlebotomine sand fly. Treatment options, epidemiological surveillance, and control need to make the appropriate identification of the causative species. This paper utilizes Nested Polymerase Chain Reaction (Nested-PCR) with the use of the kinetoplast DNA (kDNA) as a molecular identification technique of Leishmania species of CL in the Wasit Province, Iraq. This study involved 50 clinically suspected CL patients participating in this study; they were enrolled in Al-Zahra Teaching Hospital and Al-Karamah Teaching Hospital, during the time frame of November 2024 to February 2025. Samples of the skin lesion obtained in little samples were taken across the open body parts and subjected to the Nested-PCR amplification and genomic DNA isolation.

The results indicated that 20 samples out of the 50 (40 percent) were positive to the Leishmania DNA and 30 samples out of the 50 (60 percent) were negative. Among the positive cases, 15 (30) cases were Leishmania major and 5 cases (10) Leishmania tropica. Agarose gel electrophoresis revealed standard L. major and L. tropica bands of 560 bp and 750 bp respectively. The findings confirm the hypothesis that the L. major is the most widespread causative agent of CL in Wasit Province. The paper determines that the Nested-PCR is considerably sensitive and specific and would be reliable in the diagnosis of species of Leishmania and their differentiation and should be applied as a routine diagnosis tool as well as in epidemiological research in endemic regions.

Keywords: Cutaneous leishmaniasis (CL), Nested Polymerize Chain Reaction.

Introduction

Cutaneous leishmaniasis CL is a parasitological disease of vectors based on protozoa of genus Leishmania, which is contracted by humans after being bitten by the infected female phlebotomine sand flies. It is one of the most common leishmaniasis and a serious health problem to the people of majority areas of the tropical and subtropical world. Leishmaniasis is classified by the World Health Organization (WHO) as one of the most important of the neglected tropical diseases due to the fact that it is widely spread across a large geographical region, the prevalence of the disease is very high within the endemic areas and the disease is closely related to poverty, malnutrition, displacement of populations, and ineffective health systems and systems [1]. It remains that decades of research have left CL with numerous diagnostic, therapeutic, and epidemiological problems particularly in the developing countries.

It is also estimated that approximately 0.7-1.2 million cases of cutaneous leishmaniasis are contracted each year throughout the entire world, most of which are found in Middle East, North Africa, Central Asia, East Africa, and a small portion of Latin America [26], [27]. The disease has been endemic in more than 90 countries and the burden of disease is likely to be under-estimated due to underreporting, non-access to health services and poor systems of surveillance. Cutaneous leishmaniasis is not very fatal; skin lesions of this disease are usually persistent and could last a few months or even years and can result in permanent scarring. These scars can result in significant psychological unrest, interpersonal stigma and poor quality of life, especially in women

and children.

The clinical manifestations of CL are highly heterogeneous and they depend on a range of factors including infecting Leishmania species, the virulence of the parasite, host immune response, nutrition and presence of co-infection. The lesions typically begin with papules in the region of sand fly bite and most of them tend to be transformed into nodules, plaques or ulcer lesions which are accompanied by elevated indurated edges. There are few rare cases of atypical forms such as diffuse, disseminated and lupoid leishmaniasis that complicate clinical diagnosis [28]. As the manifestation might resemble other skin diseases such as bacterial or fungi, skin cancers and autoimmune diseases, laboratory diagnosis will be required.

The genus Leishmania is highly diverse taxonomically and more than 20 species of the genus have been known to infect human beings. Their distribution into the New World and the Old World species is traditional according to geographic distribution. In the Old World, the two species Leishmania major, Leishmania tropica and Leishmania infantum are the principal cause of CL and in the New World this is caused by species such as L. mexicana, L. amazonensis and L. braziliensis and L. panamensis [3], [27]. Each species is known to be associated with certain vectors, reservoir hosts and ecological niches, which result in distinct patterns of transmission and epidemiology.

The cutaneous leishmaniasis is a resurgent and endemic problem in the general population in the Middle East with Iraq being not an exemption. The occurrence of CL outbreaks in Iraq has been witnessed in a cyclical manner over the decades, and in fact the primary result of this has been political instability, armed conflict, population displacement, environmental degradation and disruption of the vectors control programmes. All of this has led to the favorable condition in the proliferation of the sand flies vectors and potential reservoir hosts and therefore, increased transmission [32]. Wast, a province in eastern Iraq, is considered as one of the foci of CL that has endemic cases annually in the rural and the urban sections.

Leishmania major and Leishmania tropica have remained the most significant causal agents of CL in epidemiological studies that have been conducted in Iraq [2], [4], [5]. The genus Leishmania major is typically associated with Zoonotic cycles of transmission of rodent reservoirs, and the primary one is Phlebotomus papatasi. This species has been frequently reported in the rural and peri-urban area where man intrudes on the habitat of rodents. Leishmania tropica on the other hand is mostly closely linked to anthroponotic mode which is characterized by human beings as the reservoir and Phlebotomus sergenti as the vector and is more prevalent in the overcrowded cities. The relative distribution of these species should be known in order to come up with effective control strategies.

Proper and timely diagnosis of cutaneous leishmaniasis is among the pillars of effective disease management and control. Traditionally diagnosis was done using direct parasitological methods, which involve microscopy of the Giemsa stained smears, histopathology of skin biopsies and in vitro parasitism culture. Even though the methods are not very expensive and are widely used in the endemic regions, they possess significant restrictions. Microscopy is highly operator-prone and less sensitive particularly in situations where the parasite loads in chronic lesions are low. The ways of culture are not only lengthy, but are also most likely to be contaminated, and cannot be performed in all the labs [7], [12].

The serological tests like indirect fluorescent antibody tests and enzyme-linked immunosorbent assays have a very limited diagnosis with CL since there are low or no levels of antibodies. Moreover, when cross-reaction with other pathogens occurs, a false-positive can be obtained. The immunological skin tests such as Montenegro test can help to show an exposure to Leishmania, but not distinguish between the active and the past infection or the infecting species [22]. These limitations serve to put more emphasis on the kind of importance of the more sensitive and specific diagnostic methods.

Diagnosis of leishmaniasis has been revolutionized by the application of molecular diagnostic techniques since it is fast, sensitive and specific in the detection of parasite DNA in clinical samples. One of the techniques is the so-called gold standard of the species identification which is the polymerase chain reaction (PCR)-based assays. Application of PCR technique has been done on various genetic targets like ribosomal RNA genes and internal transcribed spacer (ITS) regions and heat shock protein genes and kDNA minicircles [8], [13]. Each of the targets has its merits and demerits as far as the sensitivity, specificity and discriminatory power is concerned.

PCR has a specific attraction to the kinetoplast DNA due to its unique structure and large number of copies. The kinetoplast is a unique mitochondrial DNA net or DNA network comprising of thousands of minicircles and dozens of maxicircles. It is responsible that the mean Leishmania parasite has approximately 10,000 copies of minicircle per and thus, kDNA-based PCR tests are highly sensitive and are able to identify tiny amounts of parasite DNA [10], [17]. The latter feature is especially important when diagnosing cases of CL with a small parasite load or atypical clinical presentations.

Nested PCR is the advanced form of the conventional PCR and it involves two sequential amplification run in two sets of primers. The initial round of magnification enhances a bigger target region and the second round of magnification is a smaller and more specific fragment in the presence of internal primers. This technique is very sensitive and specific and possibility of non-specific amplification is minimal. Nested-PCR has been used widely in the case of diagnosis and as a method of species differentiation of Leishmania and has been demonstrated to be superior compared to single-round PCR and conventional diagnostic tests [9], [16].

Several studies that have been conducted in Iraq and other neighbouring countries have found the Nested-PCR to be useful in the diagnosis of cutaneous leishmaniasis. Researchers have suggested that the level of detection, and more accurate identification of species have been noted with the help of the molecular techniques compared to the use of microscopy and culture [11]-[13]. These implications are that the findings can be extensively used in epidemiological surveillance since the accurate identification of the species can allow understanding the dynamics of the transmission and reservoir hosts and the ecology of vectors. Moreover, the infecting species may also be familiar in order to guide treatment decisions because different Leishmania species may respond differently to antileishman drugs.

Even though a lot of molecular information has been acquired, the distribution of the Leishmania species continue to demand continuous monitoring in locations of the endemics of the same such as the Wasit Province. The distribution and spread patterns of vectors may alter over the years because of environmental fluctuation, urbanization and climate changes and human population migration. The new molecular epidemiological studies are therefore required to define the new trends, new point of focus of the transmission, and to guide the population health activities.

The cost, the availability of equipment, and technical expertise continue to limit assimilation of molecular diagnostics in the clinical practice in Iraq. However, other studies like that conducted by the present also show that Nested-PCR implementation in the local hospital laboratories is feasible and justified. The use of molecular tools can be applied to complement clinical techniques of identifying species and enhancing the effectiveness of the control program as it provides suitable and reliable species identifications.

This research was therefore meant to provide a comprehensive molecular characterization of Leishmania species that produce cutaneous leishmaniasis in Wasit Province, Iraq, by conducting Nested-PCR on kinetoplast DNA. The proposed study is expected to expand the horizons of the local study in the past and implement the existing molecular techniques, which would contribute to the cognizance of the epidemiology of CL in the eastern region of Iraq and advance the application of delicate molecular diagnostics in the endemic regions.

Materials and Methods

The study was conductedin the Al Zahra Teaching Hospital and Al-Karamah Teaching Hospital located in Wasit Province of Iraq in November 2024 to February 2025. A sample of 50 (50) patients that were clinically suspected to have cutaneous leishmaniasis was recruited. This was initially diagnosed through clinical presentation by the skilled dermatologists who incorporated the manifestation of common skin lesions in the form of ulcers, nodules and plaques in the opened parts of the body.

The skin lesions were sampled in an aseptic manner using active margins of lesions in the uncovered parts of the body like the face, arms and legs. The samples were harvested by mild scraping and stored under conditions that were favorable enough until the following processes. The lesion samples were made to undergo standard procedures of DNA extraction, concentration of the DNA, and purity ensured to ascertain suitability of PCR amplification of the genomic tissue.

The species of Leishmania major and Leishmania tropica were identified through Nested Polymerase Chain Reaction by targeting the kinetoplast DNA (kDNA) following the protocol of Noyes et al. [14] with minor adjustments. Two rounds of amplification were done to do this. The larger proportion of the kDNA was amplified in the first round (external PCR) and specificity was enhanced in the second round (internal PCR) by the use of species-specific primers.

A template DNA, primers, nucleotides, buffer, MgCl 2 and Taq DNA polymerase were placed together as a master mix. The best amplification cycles were done on a thermal cycler. PCR products were loaded on the gel in the electrophoresis on agarose gels with 1 x TBE buffer. Ethidium bromide staining was applied to it and amplified products were checked under ultraviolet light. The molecular size marker was 1002000 bp DNA ladder.

Results and Discussion

Table (1) shows that PCR method was capable of identifying the parasite as L. major and L. tropica in 15 (30 percent) and 5 (10 percent) respectively through Nested- PCR method, which analyzed 50 samples of which 20 (40 percent) were positive and 30 (60 percent) were negative to cutaneous leishmaniasis..

Table (1): Detection of CL by Nested-PCR

Method of detection and species	Total number of lesion	Positive		Negative	
		n	**%**	***n***	**%**
Nested- PCR	50	20	40.0	30	60.0
L. major (PCR)	50	15	30.0	35	70.0
L. tropica (PCR)	50	5	10.0	45	90.0

PCR results of this study showed the appearance of DNA bands of *L.major* (560) bp and *L.tropica* (750) bp, Figures (1, 2) where there is no other cause of cutaneous leishmaniasis in Iraq [2].

Figure (1): Agarose gel electrophoresis image that shows the Nested PCR product analysis of kDNA in CL positive isolates from skin lesion samples. Where M: marker (100-2000bp), lane (1-2,4,6,8,10, and 12) positive *L. major* at (560bp) PCR product size and lane (3, 5,9) positive *L. tropica* at (750bp) PCR product.

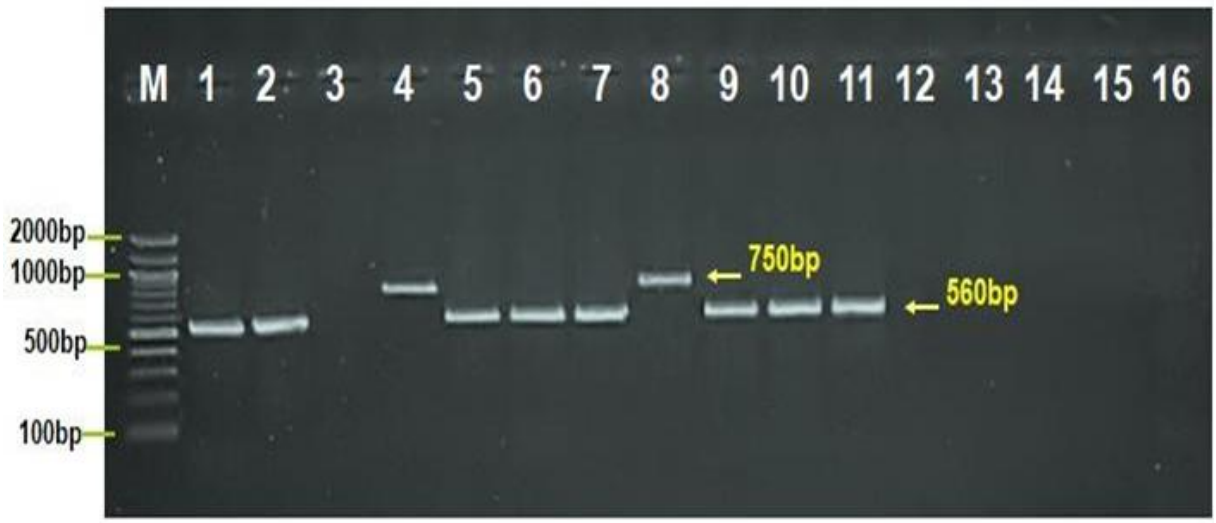

Figure (2): Agarose gel electrophoresis image that showed the Nested PCR product kDNA analysis of CL samples. Where (560bp): marker (100-2000bp), lane (1-2, 5-7 and 9-11) positive L. major at (560bp) PCR product and lane (4 and8) positive L. tropica at (750bp) PCR product. Where, neg, patient sample lane (3), neg, control sample lane (12-16).

Nested-PCR test was able to detect the DNA of Leishmania in 20/50 patients with clinical suspicions with a 40 percent positive rate with 30/50 samples giving negative results. Out of the positive samples, 15 cases (30 percent) had Leishmania major, with 5 cases (10 percent) having Leishmania tropica as shown in Table (1). Agarose gel electrophoresis was used to identify specific amplification that revealed distinct bands at 560 bp (L. major) and 750 bp (L. tropica).

Majority of L. was observed in the current study that concurs with the previous studies in the same area (Wasit Province) and other regions in Iraq [4], [5], [10]. L. major is rodent-borne and sand fly-borne resulting in zoonotic distribution, and this may be the explanation of its prevalence in the peri-urban and rural areas. Conversely, L. tropica is normally associated with anthroponotic and usually reported in regions with higher urbanization.

This research result is in agreement with the report of Al-Qurashi [11] who reported the same finding of the similarity in the distribution of the species by the application of Nested-PCR in

Wasit Province and those of Baraa [12] and Al-Khanaq [13]. The same results were also reported in the neighboring nations such as Iran where Nested-PCR has been identified to be a sensitive and precise method of species identification [15], [16].

The kDNA based Nested-PCR is extremely sensitive because a large number of copies of minicircles are present in each parasite and thus allows the detection of very low parasite loads as low as0.1 parasite/reaction [17]. This makes the technology particularly beneficial with respect to chronic or unusual cases in which the conventional microscopy might have not been useful.

In this connection, the traditional diagnostic methods do not manage to differentiate the species of Leishmania further and have low sensitivity, in most instances [18]–[20]. Molecular techniques avoid these limitations and provide proper determination of species, which is crucial in epidemiological studies, monitoring of transmission trends besides control measures.

Conclusions

The present study has demonstrated that the Nested-PCR targeting kDNA is highly sensitive and specific mode of diagnosing and identification of the species of cutaneous leishmaniasis. The results corroborate the fact that Leishmania major is the dominant causative agent of CL in the Wasit Province, Iraq followed by Leishmania tropica. Application of molecular diagnostics to the normal clinical practice can render the CL diagnosis much more precise and assist in developing more effective disease surveillance and control measures in the endemic areas.

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