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Genetic characterization of *Leishmania* species causing cutaneous leishmaniasis in Iraq

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Abstract: *Sixty-four of skin scraping samples were collected from suspected cutaneous leishmaniasis (CL) patients during the period from October, 2014 to February, 2015 at Wasit province-Iraq. All samples were detected for Leishmania amastigotes by Giemsa-stained smear were gave 94% positive; however the Novy Macneal Nicolle (NNN) and RPMI$_{1640}$ cultures led to the growth of promastigotes in all samples (81% and 75% respectively). The incidence rate with CLwas 100% by using Conventional PCR and RFLP-PCR methods. Twelve (60 %) of twenty isolates from different patients were typed as L. major and 8 (40 %) of isolates were typed as L.tropica by using Conventional PCR while 8 (40 %) of cases were typed A and 12 (60%) were typed B by using RFLP-PCR. Also,our study appeared that ulcerative wet type lesions were present in 71.9 %, while the nodule dry type lesions were present in 28.1% and the incidence rate of single lesions in CL patients was higher (67.2%) than of the multiple lesions 32.8 % . The statistical analyses were carried out with Minitab version.*

Keywords : Cutaneous leishmaniasis, RFLP-PCR,Culture, Human

1. INTRODUCTION:

Leishmaniasis is one of the most important vector-borne diseases of humans. This parasitic disease can be caused by many species of *Leishmania*, most of which are zoonotic. In humans, different species of the parasite are associated with different forms of the disease. Many *Leishmania* spp. cause skin ulcers and nodules. The protozoan Leishmania is an obligatory intracellular parasite which exists in two distinctive forms. In man and other hosts it occurs as a non-flagellar amastigote form, while in culture and gut of sandflies the flagellar or the promastigote form is seen. They are neither found in the peripheral blood nor in any visceral organ . The amastigote are small, round to oval, bodies which measure about 2-5µm and found only in the macrophages of infected vertebrate hosts, The flagellar or the promastigote forms are seen in the culture media and in the gut of the sandfly [1].

In all areas of Iraq there had also been cases of cutaneous leishmaniasis. The course of the disease is much more gentle than that of kala-azar (VL). In 2001 there were 625 cases of cutaneous leishmaniasis, 955 cases in 2000 and as many as 8779 cases in the peak year 1992 (45 cases for every 100 thousand citizens). Cases of cutaneous leishmaniasis caused by *L. tropica* mostly occur in the suburbs of big cities (Baghdad,Mosul) among large conglomerations of people where the sanitary conditions are unsatisfactory. Incidences caused by *L. major* are much more common; they appear primarily in rural areas, especially in the northern and southern provinces of the country [2,3].

The diagnosis of CL in Iraq is based on clinical signs of the disease, microscopic observation of parasites in stained skin biopsies and cuture [4]. Specific and sensitive molecular diagnostic tools been implemented and information about disease distribution, parasite life cycle and combining risk factors is confined [5]. The present study aimed to compare among giemsa-smeared, culture, conventional PCR and PCR-RFLP in the diagnosis of *Leishmania* species on clinical samples in Iraq.

2. MATERIALS & METHODS:

2.1. Collection of samples

This study was performed in the dermatology clinic of AL-Karamah Teaching Hospital in Kut city from October 2014 to the February 2015. A total of 64 cases of suspected cutaneous leishmaniasis were included in this study.

2.2. Microscopical Examination

Small quantities of tissue obtained by skin scrapings were smeared on glass slides, air dried and fixed with methanol for a few seconds. Giemsa stain was filtered and diluted 1:20 with phosphate buffer (pH 7.2). After 20 minutes of staining

the slides were washed with tap water and air dried. The stained smears were examined under the microscope with

a 40 x lens and with a 100 x oil immersion lens. If at least one intra- or extra-cellular amastigote with a distinctive kinetoplast was found the smear was declared positive. When no amastigotes were seen after 15 minutes of inspection, the smear was declared negative. Many of the patient smears were double checked, the observations were in concordance.

2.3. Culture

The lesions and the adjacent normal-looking skin around them were cleaned, sterilized with 70% ethanol, and allowed to dry. Similar to the preparation of the slide smears, a small amount of the scraped tissue was inoculated on the liquid phase of Novy-McNeal-Nicolle (NNN) medium (10% of rabbit blood) and RPMI1640 medium. The cultures were incubated at 25°C and examined for parasite growth by the inverted microscope and also light microscope every 4 days until promastigotes were seen or up to one month before being discarded as negative. The cultures were made at least in duplicates for each case [6].

2.4. Genomic DNA Extraction

Genomic DNA was extracted from frozen skin lesion samples by using (Genomic DNA Mini Kit, Geneaid. USA). The extraction was done according to company instructions by using frozen Blood extraction Protocol method with Proteinase K. After that, the extracted DNA was checked by Nanodrop spectrophotometer, and then stored at -20 °C at freeze until used in PCR amplification.

2.5. PCR amplification

PCR assay was carried out by using specific primer for small subunit (SSU) ribosomal RNA (rRNA) and 5.8S rRNA regions that are related to ribosomal ITS1, the primers forward primer (CTGGATCATTTTCCGATG) and reverse primer (TGATACCACT TATCGCACTT) were used to amplify approximately (350 bp PCR product) in *L. major* and *L. tropica*. The primers were provided by (Bioneer company, Korea). Then PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea).

The PCR premix tube contains freeze-dried pellet of [Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye] and the PCR master mix reaction was prepared according to kit instructions in 20µl total volume by added 5µl of purified genomic DNA and 1.5µl of 10 p mole of forward primer and 1.5µl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea).

The reaction was performed in a thermocycler (Techne TC-3000. USA) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 50 °C for 30 s, and extension 72 °C for 30 s and then final extension at 72 °C for 7 min. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

2.6. RFLP-PCR

RFLP-PCR were used in genotyping of *L. major* type (LmA & LmB) as well *as L. tropica* type (LtA & LtB) by using HaeII (Promega, USA) that digested the PCR products into 140-bp and 220-bp fragments corresponding with *L. major*, and 60-bp and 200-bp fragments corresponding with *L. tropica* in buffer at 37°C for 1 hours. Restriction fragments were separated in 2% agarose gels in 1×TBE buffer and visualized by staining with ethidium bromide on a UV Transilluminator.

2.7. Statistical Analysis

The suitable statistical method was used in order to analyze and assess the results by using T-test in Minitab version [7].The comparison of significant (P-value) in any test were: S= Significant difference ($P<0.05$), HS= Highly Significant difference ($P<0.01$), and NS= Non Significant difference ($P>0.05$).

3. RESULTS & DISCUSSION:

RESULTS

Table 1. show the prevalence of positive cases of CL by using different diagnostic methods. The highest infection (100 %) appeared by using PCR while the lowest infection (75%) appeared by culture on $RPMI_{1640}$ media.

Table 2. appear the distribution of CL cases in relation to the clinical features. The wet type lesions represent (71.9%) and the dry lesions were (28.1%). Also, the single lesions recorded (67.2%) than mutiple lesions (32.8%).

Table 3. represents the diagnosis of two species of *Leishmania* by using PCR method. The present study was revealed that the highest infection (60 %) caused by *L.major* than *L.tropica* (40 %).

Table 4. show the diagnosis of two species of *Leishmania* by using RFLP-PCR method. The present study was appeared that there were two type of *L.major* and *L.tropica* ; type A (40 %) and type B (60 %).

Table 1. Distribution of CL cases by different diagnostic methods

Result	Giemsa-smeared (%)	Culture on NNN (%)	Culture on $RPMI_{1640}$ (%)	PCR (%)
Positive	60/64 (94%)	52/64 (81%)	48/64 (75%)	20/20(100%)
Negative	4/64 (6%)	12/64 (19%)	16/64 (25%)	0

Table 2. Distribution of CL cases in Relation to the Clinical Features

No. of Lesions	Types of Lesions		Total
	Dry (%)	Wet (%)	
Single	11/64 (17.2%)	32/64 (50%)	43/64 (67.2%)
Multiple	7/64 (10.9)	14/64 (21.9%)	21/64 (32.8%)
Total	18/64 (28.1)	46/64 (71.9%)	64/64 (100%)

Table 3. PCR results

Leishmania sp.	PCR result (%)
L.major	12 positive (60%)
L.tropica	8 positive (40%)

Table 4. RFLP-PCR results

Leishmania sp.	RFLP-PCR result	
	Type A	Type B
L.major	5	7
L.tropica	3	5

Figure 1. Promastigote forms of *Leishmania* spp.

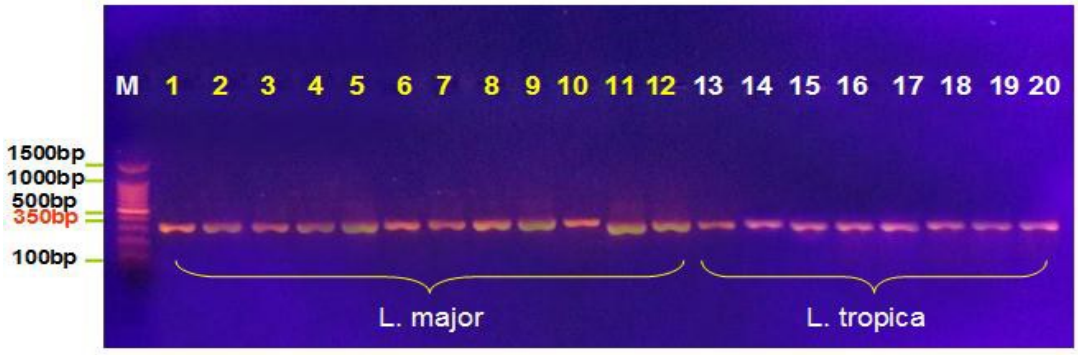

Figure 2. PCR amplification results

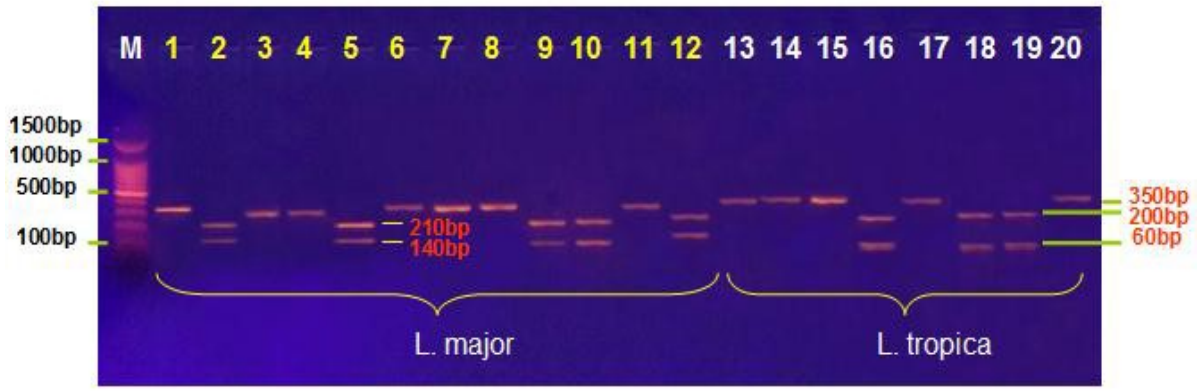

Figure 3. RFLP-PCR analysis results

DISCUSSION:

A round shape *Leishmania* parasite without flagellum was grown in culture media with biphasic NNN and RPMI1640 media as demonstrated by light microscopy examination. The change of promastigotes to amastigotes did take place completely in culture. These finding again emphasize that optimal condition for propagation of axenic amastigotes vary and have to be determined for each *Leishmania* species isolates . DNA isolated from promastigote forms obtained from in vitro culture of *Leishmania* allowed for optimization of PCR reaction [8] .

The diagnosis of CL classically relies on microscopic examination and in vitro cultivation. These classical methods require the presence of a relatively high number of viable or morphologically intact parasites; this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low. In contrast, the molecular approach is both sensitive and specific [9]. In this study we set up a well documented, genus-specific PCR to detect *Leishmania* species in clinical cutaneous samples and compared this method with classical methods.

Ulcerative wet type lesions were present in 71.9 %, while the nodule dry type lesions were present in 28.1 %. These observations are in agreement with those reported from Iraq [2], Iran [10], Colombia [11], Pakistan [12], and Afghanistan [13]. The present study indicated that the incidence rate of multiple lesions in CL patients was 32.8 % . This result could be due to long periods of exposure to Plebotomine sandflies and the high population density of sandflies in this area.

PCR methods using either genomic or kinetoplast DNA(kDNA) are now frequently cast in this role.When the amplicon is digested with restriction enzymes, it is possible to identify almost all pathogenic *Leishmania* species by RFLP, allowing direct, rapid characterization and identification of the infecting parasite [14,15].

Several DNA targets were used for DNA amplification, such as the SSU rRNA gene [16], the ITS regions [15], the microsatellite DNA [14] or extrachromosomal DNA, such as the repetitive kinetoplast DNA (kDNA) minicircles[17]. Related to the sequence variation in the first part of the spacer, the ITS1-DNA target allows the identification of almost all medically relevant *Leishmania* parasites with the use of only one restriction enzyme (HaeIII) for amplicon digestion [18].

As a result of digestion with HaeIII, ITS1-PCR products yielded 140-bp and 220-bp fragments corresponding with *L. major*, and 60-bp and 200-bp fragments corresponding with *L. tropica*. In this study, we applied ITS1-RFLP as a tool for identification of *Leishmania* species. For a further characterization of DNA polymorphisms within *L. major* and *L. tropica* isolates from different areas of Iraq, we used sequencing of the amplified ITS1 region of representative strains of each RFLP pattern .Through PCR-RFLP,a genetic polymorphism was determined for *L. major* as LmA and LmB and for *L. tropica* as LtA and LtB for a number of samples. This may be related to either strain heterozygosity or mixed strains, as isolates were not cloned. Also, the Giemsa-stained slides were examined by both microscopy and ITS1-PCR. Most of the slides that were high scored amastigote numbers as microscopy- positive were also positive by PCR-RFLP. Although the costs for PCR-RFLP diagnosis are higher and its concordance is lower than microscopic examination, but this method can identify *Leishmania* species without the need for cultivating them [14,19].

CONCLUSIONS:

Characterization of *Leishmania* isolates collected from different parts of Iraq showed that *L.major* and *L.tropica* are the agents of CL . Moreover, this study revealed that the genetic properties of a species can play an important role in the clinical manifestations, pathogenesis, epidemiology, and classification of the parasites.

REFERENCES:

[1] Chang KP, Fong D, Bray RS. Biology of leishmania and leishmaniasis. In: Chang KP, Bray RS, eds. Leishmaniasis: Human Parasitic Diseases, Vol 1. Philadelphia: Elsevier; 1985. p. 1-30.

[2] Korzeniewski K; Health hazards in Iraq, Lekarz Wojskowy. 2005; 81(3): 176-180.

[3] World Health Organization. Communicable disease profile for Iraq. 2003.

[4] Rahi AA, Ali MA, Valian HK, Mohebali M, Khamesipour A; Seroepidemiological studies of visceral leishmaniasis in Iraq. Sch J App Med Sci., 2013; 1(6): 985-989.

[5] Kumar R, Bumb RA, Ansari N, Metha R; Cutaneous leishmaniasis caused by *L. tropica* in Bikaner, India: Parasite identification and characterization using molecular and immunologic tools. Am J Trop Med Hyg., 2007; 76(5): 896-901.

[6] Herwaldt, B.L. Leishmaniasis. comprehensive review covering parasitology, epidemiology, clinical manifestations with variations,diagnosis and treatment. Lancet.1999; 354 : 1191-1199.

[7] Ruth KM, Krueger DD; A Minitab Guide to Statistics. 3rd edition, Upper Saddle River, NJ: Prentice-Hall Publishing, 2004.

[8] Pan AA, Duboise SM, Eperon S, Rivas L, Hodgkinson V, Traub-Cseko Y, Mc Mahon-Pratt D. Developmental life cycle of *Leishmania* cultivation and characterization of cultured exteracellular amastigotes. J Eurkaryot Microbiol., 1993; 40 (2): 213-23.

[9] Laskay, T., Miko, T.L., Negesse,Y., Solbach, W., Rollinghoff, M. & Frommel, D. Detection of cutaneous *Leishmania* infection in parafin-embedded skin biopsies using the polymerase chain reaction. Transactions of the Royal Society of Tropical Medicine and Hygiene, 1995; 89: 273–275.

[10] Talari SA, Shajari G, Talaei R; Clinical finding of cutaneous leishmaniasis as a new focus of Iran. Internet J Infec Dis., 2006; 5(2).

[11] Ramírez JR, Agudelo S, Muskus C, Alzate JF, Berberich C, Barker D . Diagnosis of cutaneous leishmaniasis in Colombia: the sampling site within lesions influences sensitivity of parasitologic diagnosis.J Clin Microbiol.,2000; 38(10): 3768-3773.

[12] ul Bari A1, ber Rahman S; Correlation of clinical, histopathological, and microbiological finding in 60 cases of cutaneous leishmaniasis. Indian J Dermatol Venereol Leprol., 2006; 72(1): 28-32.

[13] Faulde M, Schrader J, Heyl G, Amirih M; Differences in transmission seasons as an epidemiological tool for characterization of anthroponotic and zoonotic cutaneous leishmaniasis in northern Afghanistan. Acta Trop., 2008; 105(2):131-138.

[14] Schönian G, Nasereddin A, Dinse N,Schweynoch C, Schallig HD, Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis 2003;47:349-358.

[15] Serin MS, Daglioglu K, Bagirova M, Allahverdiyev A, Uzun S, Vural Z, et al. Rapid diagnosis and genotyping of *Leishmania* isolates from cutaneous and visceral leishmaniasis by microcapillary cultivation and PCR restriction fragment length polymorphism of miniexon region. Diagn Microbiol Infect Dis. 2005; 53: 209-214.

[16] Van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol 1992;51:133–42.

[17] Nicolas L, Milon G, Prina N. Rapid differentiation of Old World Leishmania species by LightCycler polymerase chain reaction and melting curve analysis. J Microbiol Methods 2002;51:295–9.

[18] Russell R, Iribar MP, Lambson B, Brewster S, Blackwell JM, Dye C, et al. Intra and inter-specific microsatellite variation in the *Leishmania* subgenus. Viannia Mol Biochem Parasitol 1999;103:71–7.

[19] Al-Jawabreh A, Schönian G, Hamarsheh O,Presber W . Clinical diagnosis of CL: A comparison study between standardized graded direct microscopy and ITS1-PCR of Giemsa-stained smears. Acta Trop, 2006; 99(1):55-61.

