DonnishJournals 2041-1180

Donnish Journal of Medicine and Medical Sciences
Vol 2(3) pp. 026-031 April, 2015.
http://www.donnishjournals.org/djmms

Original Research Article

Species Typing with PCR–RFLP from Cutaneous Leishmaniasis Patients in Iraq

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Accepted 4th April, 2015.

A total of 20 skin lesion samples collected from patients with cutaneous leishmaniasis (CL) attended AL-Karamah Teaching Hospital at Wasit province. Dermal scrapings were analysed by examination of Giemsa-stained smears, by Conventional PCR and RFLP-PCR methods. The incidence rate with CL was 80% by using Giemsa-stained and 100% for both PCR and RFLP-PCR. Twelve (60%) of isolates were typed as *L. major* (LmA & LmB) and 8 (40%) were typed as *L.tropica* (LtA & LtB) by using HaeIII enzyme. The higher infection (45%) appeared in age group (1-12) years old and the wet type was predominance (65%).

Keywords: CL, RFLP-PCR, Giemsa

INTRODUCTION

Leishmaniasis, a vector - borne disease caused by obligate intramacrophage protozoa, is characterized by their diversity and complexity [(1)]. Generally, leishmaniasis is divided into three clinical syndromes: cutaneous, mucosal, and visceral infection. Cutaneous leishmaniasis is localized to the skin. Mucosal leishmaniasis occurs mainly as a late complication in newworld CL and is associated particularly with infection by *Leishmania* (*Viannia*) species. Visceral leishmaniasis is caused predominately by *L. donovani* and *L. infantum- chagasi*, though in the Arabian Gulf War, *L. tropica* caused a mild form of visceral disease referred to as viscerotropic leishmaniasis [(2)].

Cutaneous leishmaniasis (CL) occurs throughout Africa, Asia, South America, the Middle East, and the Mediterranean regions. It is a disease with diverse clinical manifestations, which poses a public health problem in endemic countries [(3)]. Cutaneous leishmaniasis (CL) caused by *Leishmania major*, *L. tropica* and visceral leishmaniasis (VL) caused by *L. infantum,* are major health problems in Iraq. The majority of VL cases are reported from south of Iraq and CL is also endemic in different parts of Iraq [(4)].

Identification of the infected *Leishmania* species based on clinical signs and symptoms can be problematic because several species cause both visceral and cutaneous involvement [(5)]. With the advent of the PCR technology, several PCR based assays such as the SSU rRNA gene [(6)], repetitive sequences [(7)], the gp63 gene locus [(8)], kinetoplast minicircle sequences [(9, 10)], mini-exon gene sequences [(11)] for *Leishmania* species differentiation, were developed. Culture techniques require a sophisticated laboratory setup, time-consuming and increase risk of contamination [(12)]. The present study aimed to evaluate PCR-RFLP in the diagnosis of *Leishmania* species on clinical samples.

MATERIALS AND METHODS

Samples collection

A total of 20 skin lesion samples collected from patients with cutaneous leishmaniasis attended AL-Karamah Teaching Hospital at Wasit province and placed in anticoagulant tubes,

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then transported to laboratory and stored in freezer until genomic DNA extraction step.

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.

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, $MgCl_2$ 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.

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.

RESULTS

Epidemiological results

Table (1) shows the comparison between Giemsa-smeared stain and PCR-RFLP in the diagnosis of Cutaneous Leishmaniasis. Out of 20 CL samples, 16 samples (80%) gave positive results while all samples gave positive by PCR-RFLP (100%).

The types of CL lesions according to the age groups are shown in table (2). The higher infection (45%) appeared in age group (1-12) years old and the wet type was predominant (65%).

Table (3) showed the prevalence of CL cases according to the age groups and gender. The higher infection (45%) appeared in age group(1-12) years old and the male (60%) was predominant.

Molecular Results

See PCR amplification and RFLP-PCR analysis results below.

DISCUSSION

Characterization of *Leishmania* species in clinical infections is important, as different species may require distinct treatment regimens [13, 14]. 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 [15-18]. Several studies reported the efficiency of PCR-RFLP assay in identifying *Leishmania* species directly from clinical material [19-23].

Several DNA targets were used for DNA amplification, such as the SSU rRNA gene [24], the ITS regions[15], the microsatellite DNA[14] or extrachromosomal DNA, such as the repetitive kinetoplast DNA (kDNA) minicircles [25]. 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 [26].

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, 27].

There was a strong tendency for cases to be more prevalent, significantly in male than in female ($P<0.05$), but there is no clear explanation for such a gender distribution. It might be due to behavioural and individual risk factors [28]. Also, the high prevalence of CL in male might be explained by the fact that males in this group are more exposed to insect bites than female in the same group. This finding is found by others [29,30]. On the contrary of other studies that found the higher incidence of infection among female than male [31,32]. Moreover, the highest proportion of infections (45%) was recorded in 1-12 years age group, and the lowest (10 %) was in the age group above 60 years, which is in agreement with previous reports indicating more exposure as a result of educational and occupational situations [33-35]. Ulcerative wet type lesions were present in 65%, while the nodule dry type lesions were present in 35%.

Table 1. Positivity of diagnostic procedures in patients with CL

Diagnostic test	Examined samples	No of CL positive
Giemsa-smeared stain	20	16 (80%)
PCR-RFLP	20	20 (100%)

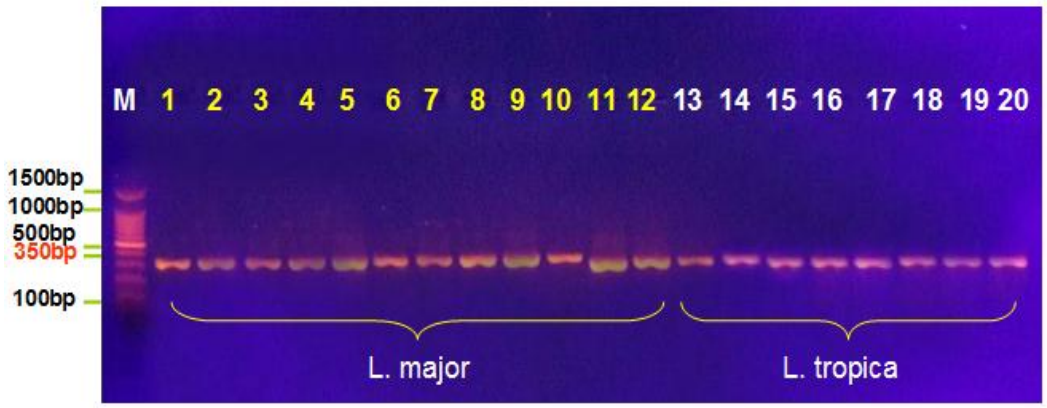
Table 2. Distribution of cases of Cutaneous Leishmaniasis in different age groups

Age groups / Years	+Ve cases	Wet lesions	Dry lesions
Children (1-12)	9(45%)	6(30%)	3(15%)
Young adults (13-30)	5(25%)	3(15%)	2(10%)
Adults (31-60)	4(20%)	3(15%)	1(5%)
Old (above 60)	2(10%)	1(5%)	1(5%)
Total +Ve cases	20(100%)	13(65%)	7(35%)

Table 3. Relationship between age groups and Gender of CL patients

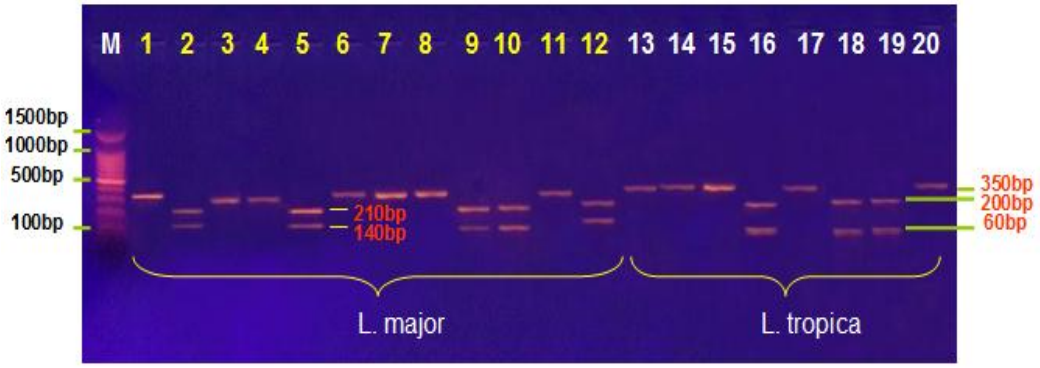
Age groups / Years	+Ve cases	Male	Female
Children (1-12)	9(45%)	5(25%)	4(20%)
Young adults (13-30)	5(25%)	3(15%)	2(10%)
Adults (31-60)	4(20%)	2(10%)	2(10%)
Old (above 60)	2(10%)	2(10%)	0(0)
Total +Ve cases	20(100%)	12(60%)	8(40%)

A. PCR amplification results



Species	PCR results
L. major	**12 positive**
L. tropica	**8 positive**

B. RFLP-PCR analysis results



Species	RFLP-PCR results	
	Type A	Type B
L. major	5	7
L. tropica	3	5

Sample No.	RFLP-PCR results
	Genotype
1	LmB
2	LmA
3	LmB
4	LmB
5	LmA
6	LmB
7	LmB
8	LmB
9	LmA
10	LmA
11	LmB
12	LmA
13	LtB
14	LtB
15	LtB
18	LtA
17	LtB
18	LtA
19	LtA
20	LtB

These observations are in agreement with those reported from Iraq [31], Iran [36], Colombia [37], Pakistan [38], and Afghanistan [39]. In the current study, the ulcers were observed among all age groups.

CONCLUSIONS

The genetic properties of a species can play an important role in the clinical manifestations, pathogenesis, epidemiology, and classification of the parasites.

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