Molecular Study of Pediatric Visceral Leishmaniasis in Mid-Euphrates Area, Iraq

Rand Muhammed Abdul-Hussain Al-Hussaini, RashaAmer Nouri Al- Tufaili, Raed Ali Hussein

Abstract—This study was evaluated the effectiveness of molecular diagnostic assay for leishmaniasis by using PCR test targeting kinetoplastDNA (kDNA). Then it was assessed the efficacy of thIs diagnostic technique for detection the early stage of infection by determining the sensitivity and specificity for the test. In this study five hundred and eighty seven children aged 1- 60 months were admitted to paediatric ward of Pediatric and Maternal Hospitals of Al- Najaf, Babil, Karbala, and Al-Qadisiyyah provinces with confirmed or clinically suspected visceral leishmaniasis were included; most of them had clinical manifestation of fever, hepatosplenomegaly, weight loss, Leukopenia, and anemia. The Bone marrow aspiration was carried out for just 38 cases under the supervision of pediatricians at private hospitals in each province. Microscopic examination method applied and detected the *Leishmaniadonovani* in their Pathological Lab, and then the results of aspirated bone marrow were sent. Only 34 cases were positive of Kala-azar by the demonstration of the parasite -the amastigotes- from direct smear of bone marrow, while 4 cases were negative, *Leishmaniainfantum*kDNA was detected in 25.21% of patients, and the sensitivity and specificity of PCR test were 85.29 %, 100%, respectively.

Index Terms—: Leishmaniasis,kinetoplastDNA, *Leishmaniadonovani*, Mid-Euphrates Area, Iraq.

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1 INTRODUCTION

Leishmaniasis is one of the most important vector-borne diseases of humans, that caused by obligate intra-macrophage protozoa .This disease is characterized by both diversity and complexity: it is caused by more than 20 leishmanial species and is transmitted to humans by 30 different species of phlebotomine sandflies[1].

Leishmaniasis represents a major public health problem in the Eastern Mediterranean Region (EMR) of the World Health Organization (WHO). Visceral leishmaniasis is mainly seen in 14 of the 22 countries of the region [2][3].Iraq is a well-known area for endemicity with the Kala-azar disease, which is a long-lasting disease since 1954 [4].The Central Health Laboratories in Baghdad identified 14502 cases by indirect fluorescent antibody test (IFAT) suspected to be infected with Kala-azar from sixteen Province s in Iraq in 2002. It was seems that WasitProvince had the highest percentage of infections, and then it follows by Diala, Babil and Baghdad Provinces [5].Other studies reviewed cases with Kala-azar, recorded that the Kala-azar was one of the most important health problems of infants and young children and the majority of Kala-azar cases was from Baghdad [6], Babil Province [7][8][9], Basrah, [10][11] , and Thi-Qar[12]Provinces. Diagnosis of leishmaniasis is based on clinical, serological and parasitological identification. The confirmatory diagnosis of VL relies upon demonstration of parasite in tissue samples or tissue culture [13].In contrast to parasitological methods and routine serologic techniques, molecular assays offer the advantage that diagnosis and identification of the *Leishmania spp*. can be accomplished simultaneously.

Recently, PCR assays have been evaluated for the diagnosis of VL and have been shown to have excellent sensitivities and specificities, and they may prove to be useful for correlation of the findings at the time of diagnosis and the final outcome at the end of treatment because the serological test, in spite of the highly sensitive; but they remain positive well beyond the time of cure, and then cannot discriminate between past and current infections[14][15][16].Molecular diagnostic assays for leishmaniasis using PCR targeting multi-copy genes (e.g., genomic DNA, telomeric repeats, rRNA, kinetoplastDNA (kDNA) minicircles and gp63) have been developed for the detection of parasites directly from human tissues. Sensitivity and specificity of these tests depend

upon the region targeted. However, nucleic acid testing is currently difficult to perform in many clinical laboratories in the developing world [17][18].

Leishmania are members of the order Kinetoplastida; the distinguishing feature of this group of Protozoa is the kinetoplast organelle, a unique mitochondrial structure containing a concatenated DNA. This kinetoplast DNA (kDNA) consists of two components: the maxicircle, which is a DNA strand of 20,000-40,000 base pairs and is the genetic material encoding mitochondrial genes, and the minicircle, which is a small DNA molecule (an average length of 2,500 base pairs), which has no known coding function but is thought to be involved in the structure or replication (or both) of the kDNA. Previous analysis of the kDNA has demonstrated species-related heterogeneity in the sequence of the kDNA and such differences for new world species and others have demonstrated such differences for old world species of *Leishmania* and other kinetoplastida [19][20]. An excellent target for a sensitive and rapid detection method is the kinetoplastminicircle DNA, which is present at thousands of copies per cell. The mini-circles have been used as targets for selective amplification of parasite DNA in various studies [21].The identification of conserved sequence elements represented within the kinetoplast DNA (kDNA) of a given species of *Leishmania* would allow the design of oligonucleotide primers to be used for species-specific identification of parasites in clinical samples.Salotra and colleagues[22] had analyzed kDNA sequences from Old World Leishmanias and designed primers specific for *L. donovani* species to detect kDNA from a single parasite in the presence of huge excesses of human DNA. The utility of the primers designed for *L. donovani* had been examined in clinical samples from patients with KA and PKDL in India. The PCR test was found to be sensitive enough to detect parasite DNA from peripheral blood of patients with KA and from skin lesions of patients with PKDL. Furthermore, the test was specific for *L. donovani* species of the parasite, leading to simultaneous species identification of the parasite.

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2 METHODOLOGY

This study was carried out in the laboratories of Pediatric and Maternal Hospitals of Al-Najaf, Babil, Karbala, and Al-Qadisiyyah provinces and in the Department of Biology in the Faculty of Science – University of Kufa.

2.1 Sampling of Cases

a) Patients Group:including 587 children aged 1-60 months, admitted to pediatric ward of Pediatric and Maternal Hospitals of Al- Najaf, Babil, Karbala, and Al-Qadisiyyah provinces. Most cases had clinical manifestation of fever, hepatospleenomegally, weight loss, leukopenia, and anemia. Cough and vomiting were frequently reported accompanying symptoms. The diagnosis was under the supervision of pediatrician from each hospital. The Bone marrow aspiration was carried out for just 38 cases under the supervision of pediatricians at private hospitals in each province. Microscopic examination method applied and detected the *Leishmaniadonovani* in their Pathological Lab, and then the results of aspirated bone marrow were sent. Only 34 cases were positive of Kala-azar by the demonstration of the parasite -the amastigotes- from direct smear of bone marrow, while 4 cases were negative.

b) Control group:consists from 20 healthy children all were with no history of parasitic infection, and without clinical manifestation of any disease.

2.2Collection of Samples

Blood samples: Two ml of venous blood was collected from clinically suspected patients and control. Blood was collected in EDTA tubes and store at *-20°C*until used for PCR test.

2.3Molecular diagnostic assay

DNA extraction kitis a product of Geneaid Biotech. Ltd., Taiwan Company, and Cat. No. GB100, LOT. No. TJ21207. It is sufficient for 100 preps.Polymerase chain reaction (PCR) assay was performed using a sense *Leishmania* genus-specific oligonucleotides to amplify the conserved region of *Leishmaniadonovani*minicircle KDNA. Sequences were amplified using the primer-pairs:

The forward primer is: 5′-AAATCGGCTCCGAGGCGGGAAAC -3′, and the reverse primer is: 5′-GGTACACTCTATCAGTAGCAC -3′ .

These primers amplified a fragment of approximately600 bp that is seen on gels.

The primers (synthesized by AccuOligo® Bioneer Corporation .USA) were published previously (Salotra et al., 2001; Salotra et al., 2003; Maurya et al., 2005; Sharmand Kaur, 2013; Maurya et al., 2013) .

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Amplification was performed in a thermal cycler programmed for 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min, preceded by an initial denaturation of 5 min at 95°C. Final extension was for 7 min at 72°C.
The gel electrophoresis method was done according to Sambrook and Russell (2001) [23].

2.4Statistical Analysis

Statistical analyses of all results were carried out by the help of SPSS version 17 software statistical package using chi square (P value at level of significance less than 0.05).

3EXPERIMENTALRESULTS

The study population was included 587 patients confirmed or clinically suspected visceral leishmaniasis from four Iraqi provinces: 68 (11.58%) cases were from Al- Najaf, 202 (34.41%) cases were from Babil, 53 (9.02%) cases were from Karbala, and 264 (44.97%) was from Al-Qadisiyyah. Most cases had clinical manifestation of fever, hepatosplenomegaly, weight loss, Leukopenia, and anemia.The diagnosis was under the supervision of paediatrician from each hospital.

3.1 Molecular assay

*Leishmaniainfantum*kDNA was detected in 148 (25.21%) patients suspected of having the disease (the same cases that were positive for ICT) but was not detected in any healthy control (Figure 1), while 439 (74.78%) cases were negative (Table 1).

Table 1
The Results of the PCR Data

Test		Bone marrow aspirations		Total
		Positive	Negative	
PCR	Positive	29 True positive	0 False positive	29
	Negative	5 False negative	4 True negative	9
Total		34	4	38

*P< 0.05 significant

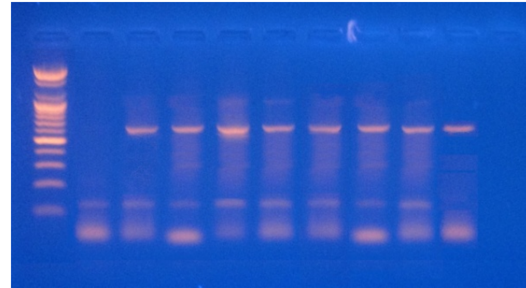

Fig.1.PCR Amplified 600 bp of *Leishmaniainfantum*kDNA Gene, 1: (100 bp) DNA Ladder, 2: Control, 3-10: *Leishmaniainfantum*kDNA.

3.2Evaluation the Accuracy of the Molecular test for Detecting VL to the Parasitological Confirmed Cases

Samples were selected for inclusion in the evaluations if they had parasitological confirmation of VL. Confirmed VL cases were defined as symptomatic individuals with positive bone marrow aspiration [24].
From 34 positive patients were confirmed by bone marrow aspirations, 29 patients were positive by PCR, so the sensitivity and specificity of PCR test were 85.29 %, 100%, respectively (Table 2).

Table 2
Accuracy of PCR for Diagnosis of VL.

Subjects	kDNA PCR results		Total
	Positive	Negative	
Healthy controls	0	20 100%*	20 3.29%
Disease suspects	148 25.21%	439 74.78%*	587 96.70%
Total	**148 24.38%**	**459 75.61 %***	**607 100%**

*P< 0.05 significant
Sensitivity = 29/34 x100 = 85.29 %
Specificity = 4/4 x 100 = 100 %
Accuracy rate = (29+4) /38 x 100 = 86.84 %

5 Conclusions

Visceral leishmaniasis is widely spread in different parts of Iraq, which regarded as an endemic place, especially in the middle and south parts, the main reasons is due to adaptation of the vector sand fly in these areas[25] .
Early and accurate diagnosis and treatment remain key components of VL control. As the clinical presentation of VL lacks specificity, confirmatory tests are required to decide which patients should

be treated. Such tests should be highly sensitive as VL is a fatal condition, but also highly specific because the current drugs used to treat VL are toxic. Ideally, a test should be able to make the distinction between acute disease and asymptomatic infection, because none of the drugs currently available is safe enough to treat asymptomatic infections. Moreover, such tests should be simple and affordable to identify treatment failures[1] .

In this study *Leishmaniainfantum*kDNA was detected in 25.21% of patients, and the sensitivity and specificity of PCR test were 85.29 %, 100%, respectively. This result almost agrees with many studies, which have reported different sensitivities and specificities for PCR assays with primers which amplify kinetoplast DNA (kDNA).

Salotra and colleagues (2001)[22] reported that the sensitivity was 96% while the specificity was 100%, Salotra and colleagues (2003) recorded that the estimated sensitivity was 88 % and the specificity was 100%.

The results of Maurya, et al. (2005) [16] revealed that the sensitivity was 99% while the specificity was 100%.

While Srivastava et al. (2011)[18] recorded less sensitivity and specificity than present results (87.8% and 84%, respectively)

Other recent study [26] reported that the sensitivity was 97.78 % while the specificity was 61.82 %.

In this study,the most likely reason for this finding is the probability of low circulation levels of parasites in the peripheral blood of *Leishmania* carriers, and the sensitivity of PCR is not enough to detect the parasites [27].

Also it was known that the sensitivity of PCR was expected to be higher for*Leishmania*-human immunodeficiency virus (HIV) asympto-matic coinfections. However, in a Spanish study, parasite existence was detected in only 28 of 92 HIV-infected patients (30.4%) that showed subclinical symptoms according to PCR analysis [28],[29].

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