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PEDIATRIC VISCERAL LEISHMANIASIS DIAGNOSIS IN TUNISIA: COMPARATIVE STUDY BETWEEN OPTIMISED PCR ASSAYS AND PARASITOLOGICAL METHODS

KAOUECH E.*, KALLEL K.*,TOUMI N.H.**, BELHADJ S.*, ANANE S.*, BABBA H.*** & CHAKER E.*

Summary:

There has been a steady increase of visceral leishmaniasis during the past 20 years in Tunisia. In this study, we assess the value of two optimised PCR *versus* those of classical methods for the diagnosis of human visceral leishmaniasis. 106 samples were collected from 53 cases of pediatric visceral leishmaniasis. Peripheral blood and bone marrow samples were analysed both by parasitological methods (direct examination, leukocytoconcentration (LCC) and culture) and by PCR methods with two primer pair (R221/R332 and Lei 70L/Lei 70R). We diagnosed visceral leishmaniasis in all patients: 44 cases were diagnosed by culture (83 %), 42 by direct examination of bone marrow (79 %), 17 by LCC (32 %), and 53 positive cases with both PCR assays (R221/R332 and/or Lei 70L/Lei 70R) (100 %). Regarding each PCR assay, for blood samples, the difference between the sensitivities of PCR Lei 70L/Lei 70R (86,8 %) and PCR R221/R332 (17 %) is statistically significant with p-value 0.025. For bone marrow, the sensitivities of the two PCR methods were respectively 96,2 % (Lei 70L/Lei 70R) and 75,5 % (R221/R332). On the whole, PCR Lei 70L/Lei 70R was more effective than PCR R221/R332 and conventional methods for the two biological samples. Moreover, the requirement of less invasive sample using blood has the advantage of being repeatable for screening and for post therapeutic monitoring.

KEY WORDS : visceral leishmaniasis, PCR, direct examination, leukocytoconcentration, culture, blood, bone marrow, Tunisia.

Résumé : DIAGNOSTIC DE LA LEISHMANIOSE VISCÉRALE DE L'ENFANT EN TUNISIE : COMPARAISON DE DEUX MÉTHODES PCR OPTIMISÉES AUX MÉTHODES CLASSIQUES

L'incidence de la leishmaniose viscérale en Tunisie est de plus en plus importante depuis la recrudescence des cas rapportés au cours de ces 20 dernières années. Pour une meilleure approche diagnostique, nous nous proposons d'appliquer deux méthodes PCR optimisées dans le diagnostic de la leishmaniose viscérale et de les comparer aux méthodes classiques. 106 échantillons ont été collectés chez 53 enfants hospitalisés atteints de leishmaniose viscérale. Sang périphérique et moelle osseuse ont été analysés par les méthodes parasitologiques (examen direct, leucocytoconcentration (LCC) et culture) et par les méthodes PCR moyennant deux couples d'amorces (R221/R332 et Lei70L/Lei70R). Tous les patients étaient atteints de la leishmaniose viscérale : 44 cas ont été diagnostiqués par la culture (83 %), 42 par l'examen direct de la moelle osseuse (79 %), 17 par la LCC (32 %) et 53 par les deux méthodes PCR (R221/R332 et/ou Lei70L/Lei70R) (100 %). En s'intéressant aux méthodes PCR, la sensibilité de la PCR Lei70L/Lei70R dans le sang périphérique est significativement plus élevée que celle de la PCR R221/R332 (respectivement 86,8 % et 17 %; p = 0,025). Pour les prélèvements de la moelle osseuse, les sensibilités des deux méthodes PCR sont respectivement 96,2 % (Lei70L/Lei70R) et 75,5 % (R221/R332). Ainsi, la PCR Lei70L/Lei70R est plus efficiente que la PCR R221/R332 et les méthodes classiques et ce, pour les deux sites de prélèvements. De plus, nous avons pu relever l'apport considérable du prélèvement du sang périphérique vu son caractère peu invasif et sa facilité de réalisation pour le diagnostic et le suivi post-thérapeutique.

MOTS CLÉS : *leishmaniose viscérale, PCR, examen direct, leucocytoconcentration, culture, sang, moelle osseuse, Tunisie.*

INTRODUCTION

Leishmaniasis is a parasitic disease caused by various species of *Leishmania*. genus. It presents various clinical forms (from benign cutaneous lesions to fatal visceral form). The first case of Mediterranean infantile visceral leishmaniasis was described in Tunisia by Laveran and Cathoire (1904). The area of transmission of visceral leishmaniasis in Tunisia is localized in the North and has noticeably extended towards the Centre and the South since 1980. (Ayadi *et al.*, 1991). During the past 20 years, there has been a steady increase in visceral leishmaniasis which affects children under the age of five years, with an incidence of 120 cases per year (*Direction des soins de santé de base*, 2000).

Visceral leishmaniasis in Tunisia was first reported to be caused by *Leishmania infantum* (Nicolle, 1908); in fact, the first *Leishmania infantum* Tunisian strain was identified by Lanotte *et al.* (1981). Actually, the isoenzymatic characterization revealed the existence of three zymodemes of the *Leishmania infantum* complex: *Leishmania infantum* MON-1, the most common (93.8 %),

* Laboratoire de Parasitologie-Mycologie (99-UR/08-05), Hôpital La Rabta, 1007 Tunis, Tunisia.
** Laboratoire d'hématologie, Hôpital d'enfants de Tunis, Tunisia.
*** Service de Parasitologie (99-UR/08-05), Faculté de Pharmacie de Monastir, Tunisia.
Correspondence: Emna Chaker. Tel.: / Fax: + 216 71 57 10 48.
E-mail: emna.chaker@rns.tn

followed by *Leishmania infantum* MON-24 (3.1 %) and *Leishmania infantum* MON-80 (3.1 %), and 72 % of the strains were obtained from children (Belhadj *et al.*, 2002).

Until the early 1990's, visceral leishmaniasis diagnosis relied on classical parasitologic methods such as Giemsa-stained bone marrow and in vitro culture of bone marrow and peripheral blood. Over the last decade, diagnosis has been established by molecular methods. PCR can be performed on any biological sample, bone marrow and blood, and has been applied to routine hospital diagnosis in many laboratories all over the world (Cascio *et al.*, 2002; Lachaud *et al.*, 2000, 2001; Spanakos *et al.*, 2002).

To assess the usefulness of PCR in amplifying *Leishmania Genome*, we report the results of two optimised PCR assays (R221/R332 and Lei70L/Lei70R) comparing them with classical methods, performed on biological samples, peripheral blood as a less invasive sample compared to bone marrow aspirates.

MATERIALS AND METHODS

SAMPLES

A prospective study was carried out in the parasitology laboratory at La Rabta's hospital in Tunis, Tunisia from June 2003 to December 2005. The study subjects were 53 pediatric patients, referred to from different hospitals of Tunisia: Children's Hospital of Tunis, the RABTA Teaching Hospital of Tunis and the Regional Hospital of Zaghouan. All patients presented fever, hepatosplenomegaly and pancytopenia. The mean age of the patients was three years: 27 males and 26 females. No child was HIV positive. On the other hand, 15 children presenting leukaemia (with fever, hepatosplenomegaly and pancytopenia) and recruited in the Pediatric Department of the Children's Hospital of Tunisia were included as negative controls.

106 clinical specimens (53 peripheral blood and 53 bone marrow) were collected from 53 study subjects, and 24 clinical specimens (11 peripheral blood and 13 bone marrow) were collected from 15 negative controls as follows: peripheral blood samples were collected in sodium citrated containing tubes for *in vitro* cultivation (5 ml) and EDTA containing tubes for leukocytoconcentration (LCC) and PCR (5 ml). Bone marrow samples (1.5 ml) were collected with an equal volume of 0,9 % NaCl containing benzyl penicillin at 200.000 IU/ml. All samples were subjected simultaneously to direct examination of bone marrow, *in vitro* cultivation, LCC and PCR analysis of both peripheral blood and bone marrow.

METHODS

1. DIRECT EXAMINATION

Smears were prepared with a drop of bone marrow and were stained with May Grünwald Giemsa.

2. *IN VITRO* CULTIVATION

For blood cultures, the buffy coat collected after simple centrifugation of 5 ml of peripheral blood was seeded in two blood agar NNN medium (Novy Mc Neal Nicolle) culture tubes. Bone marrow aspirates were seeded in two NNN tubes. The cultures were incubated at 24° C and were passaged every week. A culture was declared negative after four passages.

3. LEUKOCYTOCONCENTRATION

LCC was prepared in three steps, with 300 µl of whole blood, blood lysis with hemolysed solution (containing saponine, formaldehyde, glycerol, 0.9 % NaCl and distillated water), a cytoconcentration with a cytocentrifuge Cytospin 2 and staining with May Grünwald Giemsa.

4. DNA ISOLATION

For blood samples, 300 µl of buffy coat were collected after simple centrifugation. DNA isolation was carried out by Qiagen blood®. The DNA obtained was diluted in 200 µl buffer elution and stored at – 20° C until use. A 300 µl sample of whole human blood (the mean number of leukocytes in visceral cases was 2,000 leukocytes/µl) typically yields 3.6 µg of DNA in 200 µl elution buffer (18 ng/µl). Bone marrow was prepared by the same methods.

5. PCR AMPLIFICATION

Two couples of primers were used for PCR analysis:
- First couple of primers: R221/R332 (Van Eys *et al.*, 1992).

The DNA target for PCR amplification was the gene coding for 18 S RNA (20 to 40 fold repeated sequence). The primers used were 5'-GGTTCCTTTCCTGATTTACG-3' (R221) and 5'-GGCCGGTAAAGGCCGAATAG-3' (R332) which produce a 603 bp fragment upon amplification.
- Second couple of primers: Lei70L/Lei70R (Spanakos G. *et al.*, 2002)

Primers were designed from a conserved sequence of the ssu-r RNA *L. infantum* gene. Forward primer designated as Lei70L was 5'-CGCAACCTCGGTTCGGTGTG-3' and reverse designated as Lei70R was 5'-CGCGGTGCTGGACACAGGGTA-3'. This couple of primers amplifies a 345 bp DNA fragment specific for *L. infantum*.

• PCR optimisation

PCR optimisation was carried out with two *Leismania* reference strains: *Leishmania infantum* MON-1 (MHOM/FR/78/LEM75) and *Leishmania infantum* MON-24 (MHOM/

DZ/82/LIPA59) (Pratlong *et al.*, 1994; Belhadj *et al.*, 2002).

• *Leishmania* serial dilution assay
Promastigotes from a 4-day-old culture of the two strains were washed twice in 1 × PBS and counted on a Malassez cell. They were then diluted with 1 × PBS and the DNA was extracted by QIAGEN Blood®. The concentrations of parasites tested were: 2,45 10^4 to 2,45 10^{-2} *Leishmania*/µl corresponding to: 1,22 10^4 to 1,22 10^{-2} *Leishmania* genome/PCR tube. Two PCR methods were optimised by using different concentrations of $MgCl_2$ and Taq polymerase, and testing *Leishmania* serial dilution. After optimisation, the first PCR reaction with R221/R332 was performed in a final volume of 50 µl containing 1 × buffer, deoxynucleoside triphosphate at a concentration of 200 µM, 4 mM of $MgCl_2$, 0.6 µM of each primer (R221 and R332) and 1 U of Taq polymerase including 5 µl of sample DNA. PCR was performed in a Biometra thermal cycler, under the following conditions: initial denaturation at 94° C for 3 min, 40 cycles at 94° C for 30 s, 54° C for 30 s and 72° C for 1 min 30 s, and a final elongation step at 72° C for 10 min.
The second method used primers Lei70L/Lei70R optimised in a final volume of 50 µl containing 1 × buffer, deoxynucleoside triphosphate at a concentration of 200 µM, 2.5 mM of $MgCl_2$, 0.5 µM of each primer (Lei 70L and Lei 70R) and 1.25 U of Taq polymerase including 5 µl of sample DNA. PCR was performed in a Biometra thermal cycler, under the following conditions: initial denaturation at 94° C for 5 min, 40 cycles at 94° C for 1 min, 65° C for 1 min and 72° C for 1 min 30 s, and a final elongation step at 72° C for 10 min.

• PCR product analysis
The reaction products were visualized under UV light after electrophoresis of the 20 µl of the reaction solution in a 2 % agarose gel containing ethidium bromide. To avoid contamination, all precautions were taken: DNA isolation, PCR assays and electrophoresis were carried out in separate areas using dedicated pipettes and aliquoted reagents; as well as decontamination (UV exposure and laminar flow hood).

• PCR analysis
Each sample was tested using two PCR methods R221/R332 and Lei70L/Lei70R. For each experiment, positive and negative controls were used; positive control contained DNA of *Leishmania* and negative control contained distilled water. For negative samples, PCR amplifications were performed with diluted DNA extracts (1/5 and 1/10).

• Internal control
The DNA extraction control procedure consisted of the amplification of a fragment (120 pb) of the human b globin gene with the primers BG1 and BG2 described by Schaffer *et al.*, 1995.

6. STATISTICAL ANALYSIS

The sensitivity and the specificity of PCR R221/R332 and PCR Lei70L/Lei70R were calculated according to the results of the culture and/or direct examination. Statistical comparisons were performed using Fisher's exact test.

RESULTS

PERFORMANCE OF THE OPTIMIZED PCR ASSAYS

After the optimisation, the first method using primers R221/R332 can detect 0.122 *Leishmania*/tube PCR whereas the second method using primers Lei70L/Lei70R can detect 0.0122 *Leishmania*/tube PCR.

APPLICATION OF THE OPTIMIZED PCR ASSAYS AND CLASSIC METHODS FOR THE DIAGNOSIS OF VISCERAL LEISHMANIASIS

Leishmania infection was considered definite when the clinical diagnosis was confirmed by positive bone marrow smears and/or *in vitro* culture of bone marrow and/or peripheral blood (Table I).
53 cases were diagnosed, 44 cases by culture (83 %), 42 by direct examination of bone marrow (79 %), 17 by LCC (32 %), and 53 positive cases with both PCR methods (PCR R221/R332 and/or PCR Lei70L/Lei70R) (100 %).
Biological samples (53 peripheral blood and 53 bone marrow) were used for the diagnosis of visceral leishmaniasis. PCR amplification with the first couple of primers (R221/R332) and/or the second one Lei70L/Lei70R was positive in 98 of 106 samples (92.4 %); whereas direct examination of bone marrow had a sensitivity of 79 % (42 of 53 samples), parasite culture had a sensitivity of 55.6 % (59 of 106) and leukocytoconcentration was positive in 21 of 106 samples (19.8 %) (Table II).
In vitro cultivation, LCC and PCR assays (R221/R332 and Lei70L/Lei70R) were performed in peripheral blood for all patients; 47 samples gave positive results: 18 samples with both methods (*in vitro* cultivation and PCR assays). For 29 samples, only PCR assays detected parasitemia (Table III)
For two samples, *in vitro* cultivation and PCR assays remained negative; the diagnosis was confirmed by direct examination of bone marrow.
Concerning bone marrow aspirates, 51 samples were positive: 36 samples by NNN culture and PCR assays. For 15 samples, *Leishmania* DNA was detected in bone marrow by PCR (Table III).
For two samples, only classic methods were positive (a positive direct examination and a positive culture) whereas PCR assays remained negative.

Overall, for peripheral blood, the sensitivity of PCR was 88.7 % (47/53), *in vitro* cultivation had a sensitivity of 41,5 % (22/53) and LCC a sensitivity of 7.5 % (4/53) (Table II).

For bone marrow, the sensitivity of both PCR methods was 96.2 % (51/53) *versus* that of direct examination 79 % (42/53) whereas culture and leukocytoconcentration had respectively a sensitivity of 69.8 % (37/53) and 32 % (17/53) (Table II).

Comparison of the two PCR methods for visceral leishmaniasis diagnosis

Concerning PCR methods, visceral leishmaniasis diagnosis was established by PCR (Lei70L/Lei70R) for all

	Peripheral blood				Bone marrow				
Patients	**LCC**	**Culture**	**PCR R221/R332**	**PCR Lei70L/Lei70R**	**LCC**	**Culture**	**Direct examination**	**PCR R221/R332**	**PCR Lei70L/Lei70R**
1	–	+	+	+	–	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3	–	–	–	+	+	+	+	+	+
4	–	+	–	+	–	–	+	+	+
5	–	–	–	+	–	+	+	+	+
6	–	+	+	–	–	+	+	+	+
7	–	+	–	+	–	–	+	–	+
8	–	–	–	+	–	+	–	–	+
9	–	–	–	+	+	–	+	+	+
10	–	–	–	+	–	+	–	+	+
11	–	–	–	+	–	+	–	+	+
12	–	–	–	+	+	+	+	+	+
13	–	–	–	+	+	+	+	+	+
14	–	+	–	–	+	+	+	+	+
15	–	+	–	+	+	+	+	+	+
16	–	–	–	+	+	+	+	+	+
17	–	–	–	+	–	+	–	–	+
18	–	+	–	+	+	–	+	+	+
19	–	–	–	+	–	+	+	+	+
20	+	+	+	+	+	+	+	+	+
21	–	–	–	+	+	+	+	+	+
22	–	–	–	+	+	+	–	+	+
23	–	+	–	+	+	+	–	+	+
24	–	+	–	+	–	+	+	+	+
25	–	+	–	–	–	–	–	–	+
26	+	+	–	–	+	–	+	+	+
27	–	–	–	–	–	+	–	–	+
28	–	+	–	+	–	–	+	–	–
29	–	–	–	–	–	+	+	–	+
30	–	–	–	+	–	–	+	+	+
31	–	–	+	+	–	–	+	+	+
32	–	+	–	+	–	+	–	–	–
33	–	+	–	–	–	+	+	+	+
34	–	–	–	+	–	+	+	+	+
35	–	+	+	+	–	+	+	+	+
36	–	–	+	+	–	+	+	+	+
37	–	+	+	+	–	+	+	+	+
38	–	–	–	+	–	–	+	–	+
39	–	–	–	+	–	+	+	+	+
40	–	–	–	+	–	+	+	+	+
41	–	–	–	+	–	–	+	–	+
42	–	–	–	+	–	–	+	+	+
43	–	–	–	+	–	+	+	+	+
44	+	+	–	+	+	+	+	+	+
45	–	–	–	+	–	–	+	–	+
46	–	–	–	+	–	–	+	–	+
47	–	+	–	+	–	+	–	+	+
48	–	–	–	+	–	+	+	+	+
49	–	–	–	+	+	+	+	+	+
50	–	–	–	+	–	–	+	–	+
51	–	+	–	+	+	–	–	+	+
52	–	+	+	+	–	+	+	+	+
53	–	–	–	+	–	+	+	+	+

Table I. – Results of PCR assays and classical methods for peditaric visceral leishmaniasis diagnosis.

Methods	Bone marrow	Peripheral blood
Smears	42/53 (79.2 %)	–
Culture	37/53 (69.8 %)	22/53 (41.5 %)
LCC	17/53 (32 %)	4/53 (7.5 %)
PCR Lei70L/Lei70R	51/53 (96.2 %)	46/53 (86.8 %)
PCR R221/R332	40/53 (75.5 %)	9/53 (17 %)
Both PCR	51/53 (96.2 %)	47/53 (88.7 %)

Table II. – Performances of PCR methods and classical methods for visceral leishmaniasis diagnosis according to the nature of samples.

	Peripheral blood		
Methods	PCR positive	PCR negative	**Total**
Culture positive	18	4	22
Culture négative	29	2	31
Total	47	6	53
	Bone marrow		
Methods	PCR positive	PCR negative	**Total**
Culture positive	36	1	37
Culture négative	15	1	16
Total	51	2	53

Table III. – Comparison of PCR assays (R221/R332 and/or Lei70L/Lei70R) and *in vitro* cultivation performed on peripheral blood and bone marrow for VL diagnosis.

patients (100 %) whereas PCR (R221/ R332) was positive in 40 cases (75,5 %); therefore the PCR Lei70L/Lei70R performance is better than that of PCR R221/R332 with a significative fisher test ($p < 0.0001$).

Regarding each biological sample, for peripheral blood, the difference between the sensitivities of the two PCR methods PCR (Lei70L/Lei70R) (sensitivity: 86.8 %) and PCR (R221/ R332) (sensitivity: 17 %) is statistically significant with p-value: 0.025. (Table II).

For bone marrow samples, the sensitivities of the two PCR assays were respectively 75.5 % with R221/R332 and 96.2 % with Lei70L/Lei70R. The specificity of PCR R221/R332 *versus* that of PCR Lei 70L/Lei 70R was 100 % *versus* 100 % with peripheral blood and 100 % *versus* 92,3 % with bone marrow (only one false positive result was observed among the 13 bone marrow specimens collected from negative control patients tested with PCR Lei70L/Lei70R).

Complete inhibition was not observed for all samples; nevertheless, the ß-globine gene was not sensitive enough to detect low grade PCR inhibitors.

Only partial inhibition was observed for two peripheral blood and one bone marrow of the total samples tested with PCR R221/R332 and for five peripheral blood of the total specimens tested with PCR Lei70L/Lei70R; inhibition was solved by dilution of DNA samples to 1/5 or 1/10.

No PCR contamination was ever observed. Overall, all negative control test tubes remained negative over the period of the study.

DISCUSSION

Further to optimisation of PCR assays with a serial dilution of *Leishmania* reference strain *L. infantum* Mon-1 (MHOM/FR/78/LEM75), PCR with primer pair R221/R332 can detect 0.12 genome *Leishmania*/PCR tube, a limit detection that was not satisfactory compared to the value reported by authors using the same primer pair 0.05 (Lachaud *et al.*, 2000), 0.07 (Chargui *et al.*, 2005).

Regarding the second PCR assay with Lei70L/Lei70R, the lowest detection threshold was 0.01, a result that is in agreement with the best values reported by authors using the same method 0.01 (Spanakos *et al.*, 2002) or different methods 0.01 (Lachaud *et al.*, 2002); then it was considered highly satisfactory compared to the first PCR assay used (R221/R332) and to those of other studies reported (Lachaud *et al.*, 2000).

In this study, we assess the value of two optimised PCR methods (R221/R332 and Lei70L/Lei70R) compared with classical methods for the diagnosis of pediatric visceral leishmaniasis.

The mean age, sex ratio and clinical features of the present patients are similar to those reported by other studies on pediatric Mediterranean VL (Minodier & Garnier, 2000; Cascio *et al.*, 2002; Tanir *et al.*, 2006; Cruz *et al.*, 2006).

For all cases, diagnosis was established by PCR assays whereas classic methods remained positive for 44 cases by culture and for 42 cases by direct examination.

Both PCR analysis of peripheral blood yielded better results (88,7 %) than NNN culture (41,5 %) and LCC (7,5 %). Therefore, PCR assays performed on bone marrow were capable of detecting *Leishmania* DNA in 51 of 53 samples (96,2 %), whereas parasites were isolated by NNN culture in 37 of 53 samples (69,8 %) and amastigotes forms were observed in 42 out of 53 smears (79,2 %) and in 17 out of 53 LCC slides (32 %).

VL diagnosis with NNN culture and microscopic examination was established exclusively by bone marrow samples that require an invasive procedure. Direct examination has the advantage of being rapid and simple but it requires an efficient microscopic practice (Berman, 1997), and time-consuming microscopic examination (Da Silva *et al.*, 2005).

If bone marrow samples are obtained, culture generally yields positive results from 70 % to 81 % (Lachaud *et al.*, 2000; Costa *et al.*, 1996; Berman, 1997; Belhadj *et al.*, 2002, 2005).

Therefore, the limitation of culture performed on bone marrow and/or blood is that it requires a much longer time (four weeks) before declaring negative results.

Regardless of LCC, it is less sensitive, due to the maximum speed of the cytospin which is probably not enough to complete the sedimentation of elements of such a small size as *Leishmania*, moreover the use of hemolized solution may damage the morphology of the parasite. Due to its lack of efficiency, it has rarely been used in the search for *Leishmania* in kala-azar cases from the Mediterranean region (Petithory *et al.*, 1997; Ben Said *et al.*, 1998).

Over all, higher sensitivities were obtained with PCR analysis performed on peripheral blood and/or bone marrow samples.

These present results are in agreement with those reported by other authors. In fact, Cruz *et al.*, 2006 reported that *Leishmania* DNA was detected in peripheral blood in 19 of 24 patients (79 %) and in bone marrow for all patients studied (100 %) and considered that PCR analysis was the most sensitive method compared to NNN culture (44 %) and bone marrow smears (67 %). Sensitivities of PCR performed in blood and bone marrow found by Antinori *et al.*, 2007 were respectively 97.1 % and 96.4 %, and were superior to the conventional techniques (sensitivities of culture and microscopic examination were 76.6 % and 88.8 %).

Brustoloni *et al.*, 2007 reported that among 91 pediatric VL, PCR analysis performed in Giemsa-stained slides was positive in 84, yielding a sensitivity of 92.3 %; whereas bone marrow aspirates microscopy yielded a sensitivity of 79.1 % and culture a sensitivity of 59 %. Regarding PCR assays, we have to prove the efficacy of the PCR with primer pair Lei70L/Lei70R *versus* that of PCR R221/R332.

We found that PCR Lei70L/Lei70R (Spanakos *et al.*, 2002) amplifying a 345 pb DNA fragment was positive in all cases (53 children) and concordant with microscopy and/or culture whereas PCR R221/R332 (Van Eys *et al.*, 1992); producing a 603 pb DNA fragment was positive in only 40 cases.

PCR Lei70L/Lei70R performed on bone marrow had an excellent sensitivity 96.2 % compared to the sensitivity of 75.5 % for the first primer pair R221/R332. Then, the first PCR assay was highly satisfactory compared to the results reported (Cruz *et al.*, 2006; Antinori *et al.*, 2007; Brustoloni *et al.*, 2007; Cascio *et al.*, 2002; Lachaud *et al.*, 2000; Mathis & Deplazes, 1995; Costa *et al.*, 1996; Katakura *et al.*, 1998; Minodier *et al.*, 1997; Nuzum *et al.*, 1995; Piarroux R. *et al.*, 1994). Perhaps, its specificity was 92,3 % *versus* that of the PCR R221/R332 100 %. Regarding peripheral blood, the difference between the sensitivities of the two PCR methods is statistically significant (sensitivities: Lei70L/Lei70R 86.8 % *versus* PCR R221/R332 17 % with p-value 0.025). Specificities of both methods were 100 % (no false-positive result was observed in peripheral blood specimens from the 15 negative control patients tested either with PCR Lei70L/Lei70R or PCR R221/R332).

These results can be explained by the ability of PCR Lei70L/Lei70R to detect very small numbers of parasites (0.012) demonstrated in experiments using serial dilution of *Leishmania* whereas the limit detection of PCR R221/R332 was 0.12. In fact, Spanakos G. *et al.*, 2002 considered that the low detection limit of this PCR-method (Lei70L/Lei70R) can be attributed mainly to the design of the primers (high melting temperature was selected for the primers, resulting in less primer-target dissociation during the transition from annealing to elongation temperature and less primer-dimmer formation).

Furthermore, less sensitivity of PCR R221/R332 was not due to the few or no circulating *Leishmania* parasites in pediatric patients with Mediterranean visceral leishmaniasis (Antinori *et al.*, 2007; Lachaud *et al.*, 2000, 2001, 2002; Minodier *et al.*, 1997; Le Fichoux *et al.*, 1999) nor to the presence of residual amounts of hemoglobin (Cascio *et al.*, 2002; Adhya *et al.*, 1995) (Spanakos *et al.*, 2002 confirm that inhibition is a rare phenomenon), since 37 samples were scored negative with this assay whereas the parasite was detected with the primer pair Lei70L/Lei70R.

On the whole, the PCR Lei70L/Lei70R detection of pediatric visceral leishmaniasis using blood presents considerable advantages; in particular, its high sensitivity compared with PCR R221/R332 and with conventional methods. Moreover, the requirement of less invasive sample using peripheral blood has the advantages of being straight forward and easily repeatable compared with bone marrow for screening and for post therapeutic monitoring.

CONCLUSION

We showed PCR Lei70L/Lei70R to be both specific and more sensitive than PCR R221/R332 and classical methods, moreover, it can be performed on any biological sample especially on peripheral blood. We suggested that visceral leishmaniasis diagnosis should be applied firstly by PCR Lei70L/Lei70R in peripheral blood and if it is not established, sternal puncture can be used.

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