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Detection of *Leishmania infantum* Minicircle Kinetoplast DNA in Bone Marrow and Peripheral Blood Samples of Paediatric Patients from Children's Hospital of Tabriz Medical University

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Abstract: Visceral leishmaniasis is a life-threatening disease caused by the multiplication of the protozoan parasite *Leishmania infantum* in the phagocytes of the reticuloendothelial (RE) system. In Northeast Azerbaijan Province of Iran, childhood Visceral Leishmaniasis (VL) is endemic particularly in Kalaibar and Meshkin Shahr region, the areas which lack adequate diagnostic facilities. The disease manifests as the Mediterranean type of Kala-azar, which mainly affect children. Early diagnosis and prompt treatment could prevent its high mortality. In order to study the frequency of this disease in the region and also to examine the applicability and sensitivity of various diagnostic methods in identifying the childhood Visceral Leishmaniasis, a total of 30 blood and bone marrow samples were collected from patients admitted to Children's Hospital of Tabriz Medical University over a 12 months period. These patients showed predominant signs of the disease such as fever and splenomegaly. They were screened for visceral leishmaniasis using various methods such as direct examination, culture isolation, Direct Agglutination Test (DAT) and PCR. Median age of the patients was 32.13 months and the prevalence of the infection was 46.7%. According to these results, PCR which was used to detect *L. infantum* kinetoplast DNA (kDNA) had the highest sensitivity and specificity using bone marrow aspirate. Other diagnostic protocols were also useful in identifying the disease even though they had a lower sensitivity.

Key words: *Leishmania infantum*, PCR, Visceral Leishmaniasis, laboratorial diagnosis, Iran

INTRODUCTION

The protozoan parasites of the genus *Leishmania* are the causative agents of a group of diseases called Leishmaniasis, prevalent in more than 80 countries worldwide (Pirmez *et al*., 1999). Considerable morbidity and mortality occur in the visceral infections termed Visceral Leishmaniasis (VL), which is a symptomatic infection of the liver, spleen and bone marrow. In Iran, paediatric visceral Leishmaniasis is also endemic in the Northwest region (WHO, 2005), where it is caused by *Leishmania infantum* and it is transmitted by the bite of hematophagous sand fly belonging to *Phlebotomus* sp. dog constitutes the main reservoir of the infection.

In Northeast Azerbaijan Province of Iran, which is situated in Northwest of Iran, childhood Visceral Leishmaniasis (VL) is endemic particularly in Kalaibar and Meshkin Shahr region (Shamsizadeh *et al*., 2006), the areas which lack adequate diagnostic facilities. The disease manifests as the Mediterranean type of Kala-azar, which mainly affects children and early diagnosis and prompt treatment could prevent its high mortality.

The similarities among the clinical manifestations of pediatric visceral leishmaniasis and other diseases require the performance of laboratorial exams for quick beginning of treatment (Sabaini Venazzi *et al*., 2006). The diagnosis of the visceral leishmaniasis is customarily made by physicians after the symptoms are worsen as the serological and histological tests can not give the correct results. Consequently, unavailability of more sensitive and improved techniques to detect and identify the causal parasite usually results in late diagnosis, which could lead to death.

Microscopic examination, mostly based on Bone Marrow Aspirates (BMA) and serological tests such as Direct Agglutination Test (DAT) have been used on a large scale for diagnosis and sero-epidemiological studies of VL. However, the disadvantages of these techniques is

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that these protocols still need assessment, as they are not very sensitive to detect asymptomatic infectious children due to low antibody titer. Moreover, DAT remains positive for a long period in treated patients (Jeromino *et al*., 2005).

In the other hand, the use of PCR performance on peripheral blood and bone marrow samples has been reported to be highly sensitive for the diagnosis. In this study, PCR has been applied as an analytical method to amplify a short sequence in the minicircles and to reveal the presence of small numbers of parasites directly in samples (Adhya *et al*., 1995; Belli *et al*., 1998; Lainson and Shaw, 1979). It is known that the *L. infantum* genome comprises 36 chromosomes and a variably sized DNA maxicircle (20 to 40 kb) and minicircle (1 to 2 kb) named kinetoplast DNA (kDNA) (Acedo *et al*., 1996). Here, the new approach using *Leishmania* specific oligonucleotides to amplify the conserved region of minicirles kDNA was employed.

In order to examine the applicability and sensitivity of various diagnostic methods in identifying the childhood visceral Leishmaniasis, a total of 30 blood and bone marrow samples were collected from patients admitted to Children's hospital of Tabriz medical university over a 12 months period (from March 2006-March 2007). This hospital is the only central medical facility in the region with special unit for Patients infected by *Leishmani*.

Furthermore, the use of PCR was examined as a diagnostic tool for pediatric visceral leishmaniasis. Then, correlation between direct examination, culture isolation, serological tests and PCR results was made.

MATERIALS AND METHODS

Patients: Thirty children with VL hailing from East Azerbaijan province of Iran and reporting to Children's Hospital of Tabriz Medical University were included in this study at the pre-treatment stage. The patients presented with characteristic symptoms such as fever, anemia and leukopenia. Blood and bone marrow samples were taken from all 30 patients.

Direct examination of bone marrow aspirates: Slides were stained with Giemsa and examined with an optical microscope using a 100xoil objective. Two series of observation were performed and approximately 20 microscopic fields were examined for each sample.

Parasite cultures: The culture media was prepared by dissolving agar powder and NaCl in distilled water, which was then autoclaved for 15 min. Then, 15% rabbit blood, 5% fetal bovine serum, 250 μg of gentamicin mL^{-1} and 500 μg of 5-fluorocitosine mL^{-1} was added to the media. Peripheral blood and bone marrow samples were added separately to the media and the cultures were incubated at 25°C for 7 days. In case of a negative culture result, 1 mL of the culture sample was subcultured in the medium for another 10 days to confirm the absence of the parasite.

Serology: Direct agglutination assay (DAT) was carried out on the series of plasma samples collected from patients. *L. infantum* LON49 (Edrissian, 1990) was used for the preparation of DA antigens. Titers of 1:3200 were considered as seropositive (Mengistu *et al*., 1990).

PCR: Bone marrow and blood were collected in NET buffer (150 mM NaCl, 15 mM Tris-HCl [pH 8.30], 1 mM EDTA). Blood was collected in heparinized tubes. Samples were transported to the laboratory at ambient temperature; Samples were then transferred to 4°C. Blood (0.2 to 1 mL) was treated with RBC lysis buffer (114 mM sodium phosphate [pH 8.0], 1 mM NH_4Cl) and the buffycoat was isolated. DNA from parasite cultures as well as from clinical samples (bone marrow and blood) was isolated by overnight lysis in NET buffer with proteinase-K (100 μg mL^{-1}) and 1% sodium dodecyl sulfate. DNA was extracted by phenol-chloroform extraction and ethanol precipitation.

The kinetoplast mini-circle sequence of *L. infantum* was analyzed using PC-Gene software and appropriate primers were identified. Four sets of primers used, which are as follow:

5' CCC AAA CTT TTC TGG TCC TTC G 3	LH2
5' GAG GGT TGG GCT TGG ATA CA 3	LH3
5' AAA CCG AAA AAT GGG TGC AGA A 3	LH4
5' TCG CAG AAC GCC CCT ACC 3	LC2
5' CCA CGA CGC ATC CAA TCC AA 3	LC3

DNA from cultured parasites (1 ng) and from clinical samples (100 ng) was taken for amplification using four sets of primers (LH2+LC3, LH3+LC2, LH4+LC3, LH4+LC2) described above. The reaction mixture (50 μL) contained 10 mM Tris-HCl (pH 8.3) 50 mM KCl, 1.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, 50 ng of each primer and 1.25 U of Taq DNA polymerase (cinagene). Each reaction mixture was overlaid with mineral oil and amplification was performed in a thermal cycler (model?) programmed for 35 cycles of denaturation at 94°C for 4 min, annealing at 65°C for 30 sec and extension at 72°C for 10 min. Products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide (0.5 μg mL^{-1}) in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) and photographed under UV illumination. Ethical considerations. All patients provided with formal consent form and it was signed before participating in the study.

RESULTS

The median age of the patients participated in this study, over a period of 12 months, were 32.13, with Std. of 7.681 and with minimum and maximum age of 4 and 180 months, respectively (Table 1). The number of samples in both sex groups was the same value of 15 cases (Table 2). All of the children were from rural endemic areas and the disease commonly observed during March-May.

Of the 30 samples taken from children who were admitted to Children's Hospital of Tabriz Medical University from March 2006 till March 2007, with early symptoms of visceral Leishmaniasis, increased plasma cells, active RE cells as well as Leishman bodies and extra cellular bodies were found in 36.7% of patients using direct microscopic examination of Bone Marrow Aspirates (BMA). Therefore, 63.3% of the patients showed negative results according to the results (Table 3). Of the 11 patients (36.7%) with positive findings, 8 were female and 3 were male paediatric patients (Table 4). The independency test (Chi-square tests) on the basis of two variables of sex and BMA test results based on the information provided in Table 5 at 0.05 level, indicated the level of independency of the BMA test results from sex of the patients.

Furthermore, 43.3% of patients showed anti-*Leishmania* antibodies using DAT technique (Table 6). The total number of the patients with positive results were 13, which comprised of 9 female and 4 males (20 and 36% of the total patients relatively) and these findings are demonstrated in Table 7. Chi-Square test was also repeated for the results of the DAT procedure and the sex of the patients and as a result there was no dependency between those variables (Table 8).

Table 1: General statistics

Age (N)	Statistics
Valid	30.00
Missing	0.00
Mean	32.13
SD	42.07
Range	176.00
Minimum	4.00
Maximum	180.00

Table 2: No. of samples on both sex groups

		Gender			
Parameters		Frequency	(%)	Valid (%)	Cumulative (%)
Valid	F	15	50	50	50
	M	15	50	50	100
	Total	30	100	100	

Moreover, 46.7% of samples showed positive results using culture isolation protocol and a PCR technique using a bone marrow samples. In both of these tests, 14 patients out of 30 showed positive results, which comprised of 9 female and 5 males and the independency test indicated no correlation between the culture isolation and PCR (using BMA) results and gender of the patients (Table 9-14).

Table 3: Results of microscopic analysis of BMA

		BMA			
Parameters		Frequency	(%)	Valid (%)	Cumulative (%)
Valid	-	19	63.3	63.3	63.3
	+	11	36.7	36.7	100.0
	Total	30	100.0	100.0	

Table 4: Gender * BMA test crosstabulation

		Gender * BMA test crosstabulation			
			BMA		
			-	+	Total
Parameters			7	8	15
Gender	F	Count			
		% of total	23.3	26.7	50.0
	M	Count	12.0	3.0	15.0
		% of total	40.0	10.0	50.0
	Total	Count	19.0	11.0	30.0
		% of total	63.3	36.7	100.0

Table 5: Independency test between the results of BMA test and the sex of patients (two variables)

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	3.589[b]	1	0.058		
Continuity correction	2.297	1	0.130		
Likelihood ratio	3.690	1	0.550		
Fisher's exact test				0.128	0.064
No. of cases	30.00				

a: Computed only for a 2×2 table; b: 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.50

Table 6: Results of DAT procedure

		DAT			
Parameters		Frequency	(%)	Valid (%)	Cumulative (%)
Valid	-	17	56.7	56.7	56.7
	+	13	43.3	43.3	100.0
	Total	30	100.0	100.0	

Table 7: Gender * DAT crosstabulation

			DAT		
Parameters			-	+	Total
Gender	F	Count	6.0	9.0	15.0
		% of total	20.0	30.0	50.0
	M	Count	11.0	4.0	15.0
		% of total	36.7	13.3	50.0
	Total	Count	17.0	13.0	30.0
		% of total	56.7	43.3	100.0

Table 8: Independency test between the results of DAT and the sex of patients

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	3.394[b]	1	0.065		
Continuity correction[a]	2.297	1	0.141		
Likelihood ratio	3.690	1	0.063		
Fisher's exact test				0.139	0.070
No. of cases	30.00				

a: Computed only for a 2×2 table; b: 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.50

Table 9: Culture Isolation (Cu_ISO) results

		Cu-ISO			
Parameters		Frequency	(%)	Valid (%)	Cumulative (%)
Valid	-	16	53.3	53.3	53.3
	+	14	46.7	46.7	100.0
	Total	30	100.0	100.0	

Table 10: Gender * culture isolation results crosstabulation

			CU_ISO		
Parameters			-	+	Total
Gender	F	Count	6.0	9.0	15.0
		% of total	20.0	30.0	50.0
	M	Count	10.0	5.0	15.0
		% of total	33.3	16.7	50.0
	Total	Count	16.0	14.0	30.0
		% of total	53.3	46.7	100.0

Table 11: Independency test between the results of culture isolation and the sex of patients

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	2.143[b]	1	0.143		
Continuity correction[a]	2.297	1	0.272		
Likelihood ratio	3.690	1	0.141		
Fisher's exact test				0.272	0.136
No. of cases	30.00				

a: Computed only for a 2×2 table; [b]: 0 cells (0.0%) have expected count less than 5. The minimum expected count is 7.60

Table 12: The results of PCR using Bon marrow samples (PCR_BON)

		PCR_BON			
Parameters		Frequency	(%)	Valid (%)	Cumulative (%)
Valid	-	16	53.3	53.3	53.3
	+	14	46.7	46.7	100.0
	Total	30	100.0	100.0	

Table 13: Gender * PCR (using bon marrow samples) crosstabulation

			PCR_BON		
Parameters			-	+	Total
Gender	F	Count	6.0	9.0	15.0
		% of total	20.0	30.0	50.0
	M	Count	12.0	3.0	15.0
		% of total	33.3	16.7	50.0
	Total	Count	19.0	11.0	30.0
		% of total	53.3	46.7	100.0

Table 14: Independency test between the results of PCR_BON and the sex of patients

	Chi-Square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	2.143[b]	1	0.143		
Continuity correction[a]	1.205	1	0.272		
Likelihood ratio	2.170	1	0.141		
Fisher's exact test				0.272	0.136
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.0%) have expected count less than 5. The minimum expected count is 7.50

Table 15: PCR results using peripheral blood samples (PCR_BL)

		PCR_BL			
Parameters		Frequency	(%)	Valid (%)	Cumulative (%)
Valid	-	25	83.3	83.3	83.3
	+	5	16.7	16.7	100.0
	Total	30	100.0	100.0	

Table 16: Gender * PCR_BL crosstabulation

			PCR_BL		
Parameters			-	+	Total
Gender	F	Count	11.0	4.0	15.0
		% of total	36.7	13.3	50.0
	M	Count	14.0	1.0	15.0
		% of total	46.7	3.3	50.0
	Total	Count	25.0	5.0	30.0
		% of total	83.3	16.7	100.0

Table 17: Independency test between the results of PCR_BL and the sex of patients

	Chi-Square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	2.160[b]	1	0.142		
Continuity correction[a]	0.960	1	0.372		
Likelihood ratio	2.288	1	0.130		
Fisher's exact test				0.330	0.165
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.5%) have expected count less than 5. The minimum expected count is 2.50

Table 18: The results of Chi-square test for analyzing the correlation between the results of BMA test and culture isolation (CU_ISO)

	Chi-Square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	19850[b]	1	0.00		
Continuity correction[a]	16.610	1	0.00		
Likelihood ratio	24.881	1	0.00		
Fisher's exact test				0.00	0.00
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.5%) have expected count less than 5. The minimum expected count is 13

and 1 was male (Table 16) and the Chi-Square-Test indicated no dependency between the PCR results using blood samples and gender of the patients (Table 17).

Furthermore, to compare the results between the diagnostic tests, Chi-Square test was used. According to these findings, the test of independency between all variables at the level of 0.05 was rejected, which indicates the dependency and correlation between all variables. In another sense, there was a correlation between the findings of various diagnostic protocols used in this study. All these data analysis are presented in Table 18-27.

Table 19: The results of Chi-square test for analyzing the correlation between different variables

	Chi-Square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	22.713[b]	1	0.00		
Continuity correction[a]	19.215	1	0.00		
Likelihood ratio	28.267	1	0.00		
Fisher's exact test				0.00	0.00
No. of cases	30.00				

a. Computed only for a 2×2 table; b. 0 cells (25.0%) have expected count less than 5. The minimum expected count is 77

Table 20: The results of Chi-square test for analyzing the correlation between the results of BMA test and PCR_BON

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	19.850[b]	1	0.00		
Continuity correction[a]	16.610	1	0.00		
Likelihood ratio	24.881	1	0.00		
Fisher's exact test				0.00	0.00
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.13

Table 21: The results of Chi-square test for analyzing the correlation between the results of BMA test and PCR_BL

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-square	10.364[b]	1	0.001		
Continuity correction[a]	7.349	1	0.007		
Likelihood ratio	11.875	1	0.001		
Fisher's exact test				0.003	0.003
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (50.0%) have expected count less than 5. The minimum expected count is 1.83

Table 22: The results of Chi-square test for analyzing the correlation between the results of CU_ISO and DAT

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-square	26.218[b]	1	0.00		
Continuity correction[a]	22.573	1	0.00		
Likelihood ratio	33.849	1	0.00		
Fisher's exact test				0.00	0.00
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.07

Table 23: Chi-square test for analyzing the correlation between the results of CU_ISO and PCR _BON

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-square	30.000[b]	1	0.00		
Continuity correction[a]	26.117	1	0.00		
Likelihood ratio	41.455	1	0.00		
Fisher's exact test				0.00	0.00
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.53

Table 24: Chi-square test for analyzing the correlation between the results of CU_ISO and PCR_BL

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-square	6.857[b]	1	0.009		
Continuity correction[a]	4.527	1	0.033		
Likelihood ratio	8.784	1	0.003		
Fisher's exact test				0.014	0.014
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.5%) have expected count less than 5. The minimum expected count is 2.33

Table 25: The results of Chi-square test for analyzing the correlation between the results of DAT and PCR_BON

	Chi-Square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-Square	26.218[b]	1	0.00		
Continuity correction[a]	22.573	1	0.00		
Likelihood ratio	3.849	1	0.00		
Fisher's exact test				0.00	0.00
No. of cases	30.00				

a. Computed only for a 2×2 table; b. 0 cells (0.5%) have expected count less than 5. The minimum expected count is 6.07

Table 26: The results of Chi-square test for analyzing the correlation between the results of DAT and PCR_BL

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-square	7.846[b]	1	0.005		
Continuity correction[a]	5.321	1	0.021		
Likelihood ratio	9.710	1	0.002		
Fisher's exact test				0.009	0.009
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.5%) have expected count less than 5. The minimum expected count is 2.17

Table 27: The results of Chi-square test for analyzing the correlation between the results of PCR_BON and PCR_BL

	Chi-square tests				
Parameters	Value	df	Asymp. Sig. (2-sided)	Exact. Sig. (2-sided)	Exact. Sig. (1-sided)
Pearson Chi-square	6.857[b]	1	0.009		
Continuity correction[a]	4.527	1	0.033		
Likelihood ratio	8.784	1	0.003		
Fisher's exact test				0.014	0.014
No. of cases	30.00				

[a]: Computed only for a 2×2 table; [b]: 0 cells (0.5%) have expected count less than 5. The minimum expected count is 2.33

DISCUSSION

In this study, various diagnostic methods were used on both samples (blood and bone marrow) for identifying the visceral leishmaniasis amongst children admitted to Children's Hospital of Tabriz Medical University who showed early symptoms of the disease. Of 30 patients that were studied here, 14 (46.7%) were positive for visceral leishmaniasis. All the patients were mostly under 7 years old and their predominant clinical features were fever and splenomegaly.

However, the initial aim of the study was to define a set of PCR primers based on kDNA sequences, which would allow sensitive and specific detection of *L. infantum*. In order to do this, both blood and bone marrow samples were used for PCR application. According to present findings, 14 cases showed a positive band using bone marrow samples. In contrast, blood samples were quite difficult to detect *L. infantum* and only 5 samples showed a positive *Leishmania* kDNA band and these samples were those with high anti-leshimania antibody titer (>5000).

The comparison of PCR results of both type of samples from same patients, together with cultural isolation, microscopic examination and serological tests (DAT) permitted us to establish that bone marrow aspirates were best matrices for PCR diagnosis during the infection period.

However, blood sampling is less invasive and easily performed. In this study, blood samples only gave a positive result on samples from patients with more advanced forms of the disease. Consequently, those samples that showed a very high antibody titer were those that gave a positive band for the PCR technique using a blood samples.

Moreover, serology also proved to be quite useful and applicable assay in the diagnosis of visceral leishmaniasis (Jelinek *et al*., 1999; Sacks and Perkins, 1985). Nearly all of patients showing a serum antibody titer of over 3200 had *L. infantum* kDNA in their blood, which is probably indicative of an active infection.

Other methods such a direct analysis of bone marrow aspirates also appeared to be valuable as a diagnostic procedure, although it can not be used for assessment of asymptomatic leishmaniasis. On the other hand, Culture isolation was a functional protocol with high sensitivity rate. But, it cannot be used as diagnostic method because it is very time consuming and it takes up to week or two to establish the final results. Furthermore, cultures are susceptible to contamination. In cases of double infections one strain usually outgrows the other, so that misdiagnosis can occur (Edrissian, 1988).

Our results also address the question of whether it is worth aiming at the maximal sensitivity for diagnosing leishmaniasis. With our PCR system, we can confidently diagnose most of VL cases using a bone marrow aspirates.

The PCR method is rapid, it requires less time from sampling to detection of the infection (Belli, 1998). Moreover, it may be possible to use this test in epidemiological studies aimed to determine the prevalence in areas in which the disease has not been controlled (Le Fichoux *et al*., 1999; Rodríguez *et al*., 1994). According to the results of this study, serological data are not enough to make correct diagnosis of leishmaniasis and that using PCR could help reveal further cases of infection in children (Ramírez *et al*., 2000).

In East Azerbaijan province of Iran, Visceral Lieshmaniasis is prevalent amongst children mainly under fifteen years old and is caused by *Leishmania infantum*. This study was mainly focused on gathering some data about the prevalence of the disease as Children's Hospital of Tabriz Medical University is the only central medical facility in the region that these children are referred. Applicability of different diagnostic techniques emphasizes the importance of prevention strategies, as most of these procedures are quite invasive to perform.

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