Original Article

Identification of *Leishmania tropica* from Pediatric Visceral Leishmaniasis in Southern Mediterranean Region of Turkey

Derya Alabaz[1], Fadime Eroğlu[2], Hüseyin Elçi[3] and Ümmühan Çay[1].

[1] Department of Pediatrics, Pediatric Infectious Diseases, Faculty of Medicine, Cukurova University, Adana, Turkey.
[2] Aksaray University, Basic Sciences Medical Parasitology Departman, Aksaray, Turkey.
[3] Department of Pediatrics, Kızıltepe State Hospital, Kızıltepe, Mardin.

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Abstract. *Background and Objective:* **Protozoa of the genus *Leishmania* are obligate intracellular parasites, and *Leishmania* species cause a spectrum of species-specific clinical symptoms known as cutaneous, mucocutaneous, and visceral leishmaniasis. For example, *Leishmania major* and *Leishmania tropica* cause cutaneous leishmaniasis, while *Leishmania infantum* and *Leishmania donovani* cause visceral leishmaniasis (VL). However, molecular studies in recent years have shown that *Leishmania* species cause different clinical symptoms.**

Objectives: **Our aim was to evaluate the relationship between the clinical and molecular characterization of leishmania isolates in children with VL defined in Turkey, an intercontinental transitional region.**

Methods: **The clinical diagnosis of VL was confirmed by detecting amastigotes in the bone marrow aspirate and/or the rK39 test and/or molecular methods (genus-specific PCR, Real-Time PCR, ITS1 PCR-RFLP, DNA sequencing).**

Results: **Most of the VL patients were referred from the districts of Adana (53.3%) and others from neighboring provinces; Hatay (16.6%), Osmaniye (3%), Gaziantep (3%), Adıyaman (3%), and 20% case were Syrian immigrants A clinical diagnosis of VL was confirmed in 30 patients with different diagnostic methods. 93% was found positive with microscopic examination, 79.1% with rK39 dipstick test, and 60% with genus-specific PCR assay in clinical samples. The *Leishmania* isolates were identified as *L. infantum* (40%), *L. donovani* (26.7%), and *L. tropica* (23.3%) using Real-Time PCR, ITS1 PCR-RFLP, and DNA sequencing. There was no cutaneous finding in any case in clinical examination.**

The most common clinical findings were fever (93.3%) and splenomegaly (90%), followed by hepatomegaly (76.6%). The most common laboratory finding was thrombocytopenia (86.6%), followed by anemia (70%). In addition, hemophagocytic lymphohistiocytosis was detected in bone marrow aspiration in two of our patients. Since pentavalent antimony salts treatment initially failed in four patients, it was necessary to switch to Liposomal-Amphotericin B with treatment success.

Conclusions: **The presence of *L. tropica* in VL patients, despite the absence of cutaneous findings in any of the cases, shows that this strain can cause VL, contrary to conventional knowledge. In the Adana province, where this study was carried out, *L. infantum* from CL cases in previous studies should be taken into account, and visceral spread in CL cases and accompanying cutaneous lesions in VL cases should be investigated in detail.**

Keywords: Visceral leishmaniasis; DNA Sequencing, *Leishmania infantum; Leishmania donovani; Leishmania tropica*.

Correspondence to: Derya ALABAZ Mailing address: Cukurova University, The Faculty of Medicine, Pediatric Infection Department Adana, Turkey. Tel: 05326534166. E-mail: deryaalabaz@yahoo.com, Orcid/0000-0003-4809-2883

Introduction. Leishmaniasis, a chronic and persistent intracellular protozoal infection caused by many different species in the genus *Leishmania*, according to the World Health Organization (WHO), is one of the most neglected tropical diseases in the world.[1] It is a poverty-associated parasitic disease that is endemic in over 90 countries and transmitted by sandflies and is associated with malnutrition, displacement, inadequate housing, illiteracy, weakened immune systems, and lack of resources. Leishmaniasis is also related to geographic changes, and the geographic distribution of leishmaniasis cases reflects the mobility of the population in displacement.[2]

There are three main forms of the disease: Cutaneous leishmaniasis (CL), Visceral leishmaniasis (VL), also known as kala-azar (KA), and Mucocutaneous leishmaniasis (MCL).[3,4] It is known that approximately twenty *Leishmania* species are the causative agents of leishmaniasis, of which *Leishmania tropica* (*L. tropica*) is the causative agent of CL, while *Leishmania donovani* (*L. donovani*) and *Leishmania infantum* (*L. infantum*) are the causative agents of VL in the literature to date. However, in molecular studies on *Leishmania* species in recent years, it has been reported that *L. donovani* and *L. infantum* can also cause CL in some cases.[5,6,7] Global warming and changes in climatic conditions may affect the distribution of the causative species of this disease. In addition, the molecular epidemiology of leishmaniasis may change with the increase in migration due to war in regions where leishmaniasis is endemic and the rise in business and touristic travels with the developing technology. Therefore, up-to-date information on this disease's molecular epidemiology is needed in regions where leishmaniasis is endemic. The aim of this study was to identify the causative *Leishmania* species in children with a clinical diagnosis of VL and to investigate the effects of these species on the disease.

Materials and Methods.
Study Area and Clinical Analysis. Turkey is located at 36° to 42° North latitude and is described as one of the endemic countries for leishmaniasis. Turkey is divided into seven regions covering a surface area of 783.562 km². Adana province, where the study was conducted, is located at 37° north latitude and 35.32° east longitude in the eastern Mediterranean region. Adana province borders Osmaniye, Kahramanmaras, Gaziantep in the east, Icel in the west, Nigde in the northwest, Hatay in the southeast, the Mediterranean Sea in the south, and Syria in the southeast. Due to its location, Adana province receives a lot of immigration, especially from Syria. In addition, the Adana has the characteristics of the Mediterranean climate, the median relative humidity is approximately 90%, and the median temperature is 28°C. The geographical and climatic conditions of Adana province are suitable for the development of *Phlebotomus* species, which are the causative agents of leishmaniasis.

This study characterizes pediatric patients admitted for investigation and confirmed diagnosis of VL by Çukurova University Hospital, Pediatric Infectious Diseases Department, Adana, in the south of Turkey. It serves as a research hospital and is an important center where patients come for treatment from the south/southeast regions of Turkey and Syrian immigrants. In addition, other family members of the case were questioned and examined in terms of clinical findings, and simple hematological tests were performed.

In the clinical examination of the patients included in this study, permanent systemic infections such as fever, fatigue, anorexia, weight loss, and sizes of liver and spleen were investigated. In addition, demographic characteristics of the patients, such as age and gender, were examined in this study. Body mass index (BMI) was determined by dividing weight (kg) by height (m) squared. On WHO charts, the normal range is generally defined as between -2 SD and +2 SD (i.e., Z-scores between -2.0 and +2.0), corresponding to approximately the 2nd and 98th percentiles. In addition, non-specific laboratory data such as full blood count, gamma-globulin concentration, and erythrocyte sedimentation rate (ESR) were reported.

Laboratory Methods. Clinical examination of VL was confirmed by detection of amastigotes in bone-marrow aspirate, and/or rK39, and/or molecular tests (genus-specific PCR, Real-Time PCR, ITS1 PCR-RFLP, DNA sequencing, and Phylogenetic analysis).
Microscopic Examination. The bone marrow sample was smeared onto a glass slide. The prepared bone marrow samples were fixed with absolute methanol, stained with 10% Giemsa, and examined under the light microscope with magnification oil 100X objective. The preparations with amastigote were considered positive, and those without amastigote were considered negative.
The Recombinant Antigen Dipstick Test (rK39). The rapid immune-chromatographic test is based on a recombinant 39-amino acid repeat antigen. The dipstick test was performed according to the manufacturers' instructions (InBios International, Inc. Seattle, WA). Twenty microliters of serum taken from the patients were mixed with two drops of buffer in the rK39 dipstick test.

After 10 minutes of incubation, serum samples with two bands on the cellulose strip were considered positive, and serum samples without were considered negative only if the control band appeared.

Molecular tests.[8-10]
Genus-Specific PCR Assay. DNA was extracted from bone marrow samples with an Agencourt DNA kit (Beckman Coulter, Beverly, USA), according to the manufacturer's protocol. The kDNA using the primers 13A (5'-GTG GGG GAG GGG CGT TCT-3') and 13B (5'-ATT TTC CAC CAA CCC CCA GTT-3') was investigated for the presence of Leishmania in clinical samples.[8] A reaction mix with a final volume of 25 µL consisted of 0.2 mM of dNTP, 1.5 mM $MgCl_2$, 1 unit of Taq polymerase, 1 µM of each primer, and 5 µL DNA samples for use PCR analyses. The DNA was amplified for 35 cycles in a thermocycler, set to run at 94 °C for 1 min, 54 °C for 1 min, and 72°C for 1 min for each one.

The amplification reactions were analyzed by bromide staining and visualization under UV light. The PCR products with a fragment image of approximately 120 bp under UV light were considered positive.

Real-Time PCR Assay. The kDNA minicircle gene region was targeted to identify *Leishmania* species, and *Leishmania* isolates were amplified by melting real-time curve PCR (Real-Time PCR) using primers JW13 and JW14.[9] The Real-Time PCR was performed by hot-start PCR using the LightCycler FastStart DNA Master SYBR Green I Kit in a LightCycler™ (Roche Diagnostics). The 20 µL reaction mixture contained 1 X LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Meylan, France), 2 mM $MgCl_2$, 10 µM of each primer and 5 µL of DNA of the parasite.

The Real-Time PCR Reaction and DNA samples were amplified using a 35 cycles thermal cycler program consisting of 10 s at 95°C, 10 s at 53°C, and 10 s at 72°C. The melting program consisted of one cycle of 95°C for 10 s, 67°C for 10 s, and heating at 0.1°C per 1 second to 98°C. The transition rates were 20°C/s except for the extension step and the final step, which had a temperature transition rate of 1°C/s and 0.1°C, respectively. Fluorescence was measured through the slow heating phase. For improved visualization of the melting temperatures (Tm), melting peaks were derived from the initial melting curves (fluorescence [*F*] versus temperature [*T*] by plotting the negative derivative of fluorescence over-temperature –dF/dT versus dT). The melting temperatures were 88.5±0.4°C for *L. donovani,* 89.3±0.2°C for *L. tropica,* and 90.5±0.2°C for *L. infantum,* respectively.

ITS1 PCR-RFLP Assay. In this study, to identify Leishmania species according to the study of Schönian et al., the ITS1 gene region was targeted, and LITSR-L5.8S primers were designed.[10] The 40 µL PCR reaction mixture consisted of PCR buffer 1 X (75 mM KCl, pH

8.3 and 20 mM Tris-HCl, 1.5 mM $MgCl_2$, 1U Taq polymerase), 0.2 mM dNTPs, 0.5 pmol of each primer and 5 µL of DNA sample. After the initial denaturation (5 min at 94 °C), 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 54°C, and elongation for 1 min at 72°C were carried out, and a final extension terminated the PCR at 72°C for 10 min. The PCR products were analyzed in 1% agarose gel by electrophoresis at 100 V in 1 X Tris-boric-EDTA buffer (0.04 mM Tris-boric and 1mM EDTA, pH 8) and visualized by UV light after being stained with ethidium bromide.

All PCR samples with approximately 350 bp fragment length bands were considered positive according to the agarose gel electrophoresis. ITS1 PCR products (10 µL) were digested with the Hae III restriction enzyme (Promega, Wisconsin, USA) according to the manufacturer's instructions, and the restriction fragments were analyzed by 2% agarose gel electrophoresis. The restriction products were identified as *L. donovani, L. infantum*, and *L. tropica,* according to Schönian et al.[10]

DNA Sequencing. ITS1 PCR products were purified for DNA sequencing using a SentroPure DNA purification kit (Sentromer DNA, Istanbul, Turkey). Purified PCR products were sequenced with LITSR-L5.8S primers using BigDye Terminator V 3.1 cycle sequencing kit (Applied Biosystems). This reaction was analyzed according to the instructions for the 3730 DNA analyzer (Applied Biosystems). The ITS1 PCR products of *Leishmania* isolate sequences were approximately 320 bp in length. The reference strains of *Leishmania* species (*L. donovani* ATC 50212, WHOM/IN/80/DD8; *L. infantum* ATCC 501340, WHOM/TN/80/IPT-1; *L. tropica* ATCC 50129, WHOM/SU/74/K27) were used in this analysis. The obtained sequences were processed with the available GenBank and checked using basic local alignment search tool (BLAST) analysis software (www.ncbi.nlm.nih.gov/BLAST.

Treatment. The conventional treatment of VL/kala-azar consists of pentavalent antimony salts (PAS- Sbv) - sodium stibogluconate (SSG (Pentostam)) and meglumine antimoniate (MA, (Glucantime®)). In addition, liposomal amphotericin B (L-AMB (AmBisome®)) was used intravenously at a dosage of 3 mg/kg on days 1-5, 10, and 21 (for a cumulative dose of 21 mg/kg). The therapy used in our department was one of the following in **Table 1** (choice made according to a supplied drug that day, and/or the patient's suitability, and/or the physician's preference).

Follow-up. Post-treatment follow-up (at least 1 year, every month for the first 3 months, then every 2 months, and every 3 months after 6 months) included clinical examination, blood cell count, and ESR.

Table 1. Used antileishmanial drugs in this study and their prescribed dosage.

Drug	Dose	Admistration site	Action Mechanism	Side effects
Pentavalent antimony salts (PAS) • **Sodium stilgluconate-SSG (Pentostam)** • **Meglumine antimonite -MA (Glucantime®)**	20 mg/kg daily for 28-30 d	IV or IM	Inhibits glycolysin and other metabolic pathways	Musculoskeletal pain, gastrointestinal disturbances, headache, and increase in liver and pancreatic enzymes
Liposomal amphotericin B (L-AMB)	3 mg/kg on 1-5, 14, and 21 d	IV	Inhibits cell membrane synthesis	Elevation of LFT, cytopenia, nephrotoxicity, pancreatitis

Statistical analysis. The medications used and/or management modalities were collected on a spreadsheet. The Statistical Package for the Social Sciences (SPSS) version 23.0 (IBM, Somers, NY, USA) was used for statistical analysis. Categorical measurements were summarized as numbers and percentages, while continuous measurements were summarized as mean, deviation, and minimum-maximum.

Ethics Statement. Confidentiality and patient privacy were maintained throughout the process. Ethical approval was obtained from Çukurova University Scientific Research Ethics Committee (decision no: 03.04.2022/120) and was performed in accordance with the ethical standards of the Declaration of Helsinki.

Results. In this study, the VL clinical diagnosis of 30 patients was confirmed by different diagnostic methods. According to the results of microscopic examination, 28 (93%) of 30 patients who were clinically diagnosed with VL were found to be positive, while 2 patients (7%) were negative. Of the patients with VL, 19 (79.1%) were positive, and 5 (20.9%) were negative using the rK39 dipstick test. However, the rK39 dipstick test could not be studied in 6 (20%) patients for technical reasons. The leishmania parasite was detected by genus-specific PCR assay in 27 (90%) VL patients. Still, genus-specific PCR assay could not be studied in 3 (10%) of the VL patients due to technical reasons in the laboratory. The Real-Time PCR method was used to identify *Leishmania* species in VL patients, and 12 (40%) *L. infantum*, 8 (26.7%) *L. donovani,* and 7 (23.3%) *L. tropica* were identified in the study; according to this method. ITS1 PCR-RFLP assay and DNA sequencing were analyzed to confirm the *Leishmania* species in this study. The results of the ITS1 PCR-RFLP assay confirmed the Real-Time PCR results in this study. In addition, each of the samples and reference strains of *Leishmania* species (*L. donovani* ATC 50212, WHOM/IN/80/DD8; *L. infantum* ATCC 501340, WHOM/TN/80/IPT-1; *L. tropica* ATCC 50129, WHOM/SU/74/K27) was identical to the sequence of *Leishmania* species reported in GenBank. Compared with the previous records in GenBank, we determined 100% similarity between sequences of leishmania isolates from VL patients and *Leishmania* strains in GenBank. All of the diagnostic results used in this study have been shown in **Table 2** and **Figure 1**. The Leishmania species and the patients' clinical symptoms were compared, and no significant relationship was found between them.

In addition, the epidemiological findings, clinical features, treatment, and outcome of the patients with VL have been shown in **Table 2**. Most of the VL patients were referred from the districts of Adana (53.3%) and others from neighboring provinces; Hatay (16.6%), Osmaniye (3%), Gaziantep (3%), Adıyaman (3%), and 20% case were Syrian immigrants (**Table 3**). Therefore, it was determined that 76.6% of the patients in this study lived in rural areas, and 23.4% lived in urban areas.

According to family history, a sibling of Case 3 was previously diagnosed with VL, while the father of Case 11 had just died due to VL before the boy applied. The median age of the 30 patients [20 male (66.7%)] was 37 months (range 12 months – 12 years, mean ± SD, 47.96 ± 32.87 months).

Duration of symptoms before admission ranged from 3 days to 6 months (mean 37.5 ± 46.3, median 20 days), while a Syrian boy (Case 23) complained for 3 years of abdominal distention and pallor. In the study, the body mass index values of the patients were 14,9 (12,1-21,15) on average, and malnutrition was detected in 34.48 % of our patients (**Figure 2**); 10 cases were below the BMI -2 score. No association was found between BMI and parasite type. Fever was present in all patients except 2 (93.3%) (**Table 3**). The duration of fever persists between 3 and 21 days (mean 10.9± 5.3, median 10 days) after beginning treatment. In the study, splenomegaly (90%) and hepatomegaly (76.6%) were found to be the most common clinical findings after fever (93.3%). No relationship was found between symptoms and parasite types. The most common laboratory finding was thrombocytopenia (86.6%), followed by anemia (70%) (**Table 4**). In two of our patients, we detected hemophagocytic lymphohistiocytosis (HLH) in bone marrow aspiration (Case 18 and Case 2). HIV was not detected in any patient. In this study, although the PAS regimens were effective in the treatment, some disadvantages were experienced like prolonged

Table 2. Demographic findings of the patients diagnosed VL.

Case /gender /age	Time to diagnosis	Symptoms			Physican findings		Hematologic findings				Parasitological findings			Treatment	Outcome
		Fever	BMI (Z-score)	Others	Liver (cm)	Spleen (cm)	HCT	WBC	Trombocit	Response time	BM mic	IC	Species		
1 /EK / m / 3 y	90 d	+	15.7 (-3.11)	Abd pain	9	11	21	5100	138.000	25 d	+	Nd	L. tropica	MA	Cure
2 /TG /f /5 y 3 m	7 d	+	12.1 (-3.11)	Fatigue	3	0	28	4800	112.000	10 d	+	Neg	L. tropica	MA	Cure
3 /YAA /m / 2 y	20 d	+	14.8 (-1.17)	Fatigue, vomiting	3	6	23	4000	92.000	5 d	+	+	L. infantum	YMA	Cure
4 /YK /m /5 y 9 m	30 d	+	15.5 (0.11)	Jaundice	0	10	22	3200	113.000	10 d	+	+	L. infantum	MA	Cure
5 /OK /m /3 y 3 m	10 d	+	16.3 (0.56)	-	2	0	32	5200	210.000	1 d	+	+	L. tropica	MA	Cure
6 /AK /m /2 y 8 m	15 d	+	19.8 (2.18)	-	7	12	19	2000	44.000	15 d	+	Nd	L. donovani	SSG	Cure
7 /EK /m /2 y	10 d	+	16.5 (-1.06)	Fatigue, anorexia	2	4	28	5000	100.000	5 d	+	+	L. infantum	MA	Reccurens (3 m later) Cure with AmB
8 /KS /f /1 y	30 d	+	14.07 (-2.15)	Pallor	5	6	27	4900	32.000	10 d	+	+	L. donovani	AmB	Cure
9 /RÇ /m / 7 y	60 d	Neg	21.15 (-0.01)	Abd distantion, pallor	8	12	23	2500	99.000	Uk	+	+	L. tropica	SSG	Unresponsiveness Cure with AmB
10 /AS /f /5 y Father death of VL	90 d	+	12.58 (-2.56)	Weigh loss	2	5	19	3700	190.000	15 d	+	+	L. donovani	SSG	Cure
11 /İÖ /m /11 y	20 d	+	15.20 (-0.99)	Abd pain, weight loss	5	4	28	3500	160.000	10 d	+	+	L. tropica	SSG	Cure
12 /YET /f /1 y 6 m	30 d	+	16.8 (0.9)	Abd pain	7	8	24	8300	117.000	10 d	Neg	+	L. donovani	SSG	Cure
13 /AA /m /2 y 2 m	30 d	+	17.58 (0.87)	Abd pain	10	9	21	2600	77.000	25 d	+	+	L. infantum	SSG	Unresponsiveness Cure with AmB
14 /SA /f /6 y	180 d	+	13.65 (-1.35)	Fatigue, abd distantion	4	10	28	5500	97.000	25 d	+	+	L. infantum	SSG	Unresponsiveness Cure with AmB+MA
15 /SK /f /2 y	10 d	+	13.2 (-2.26)	Weigh loss	5	5	27	3400	123.000	30 d	+	+	L. donovani	L-AmB	Unresponsiveness Cure with MA
16 /CY /m /4 y	10 d	+	14.6 (-0.84)	-	5	5	23	5900	97.000	5 d	+	+	L. infantum	MA	Cure
17/ FD /m /4 y 6 m	10 d	+	14.79 (-0.64)	-	3	4	18	2700	63.000	15 d	+	+	L. donovani	MA	Cure
18 /ZK /f /2 y 9 m	30 d	+	16.19 (0.51)	Abd pain, HLH	2	2	15	1130	11.000	40 d	+	Nd	Nd	L-AmB	Cure
19 /SG /f /1 y	15 d	+	14.9 (-1.29)	-	4	6	23	1270	43.000	15 d	+	Neg	L. tropica	L-AmB	Cure
20 /İS /m /2 y 8 m	30 d	+	16.19 (0.51)	Abd pain	5	7	15	2970	32.000	15 d	+	+	L. donovani	L-AmB	Cure
21 /MA /f /1 y	3 d	+	19.39 (1.65)	Pallor	2	4	19	3370	60.000	15 d	+	+	L. infantum	MA	Cure
22 /SA /f /7 y	15 d	+	Uk	Fatigue	2	4	22	1500	94.000	10 d	+	Nd	L. infantum	L-AmB	Cure
23 [a] /HC /m /5 y	3 years	Neg	15.33(-0.12)	Abd distantion, pallor	5	10	33	4100	110.000	Uk	+	+	L. infantum	L-AmB	Cure
24 [a] /MŞ /m /3 y 4 m	180 d	+	13.74 (-2.09)	Fatigue, abddistantion	8	5	24	2600	47.000	5 d	+	Nd	L. tropica	L-AmB	Cure

No/Initials/Sex/Age				Symptoms										Species	Drug	Outcome
25 [a] /MH /m /3 y 2 m	60 d	+	14.58 (0.53)	Abd distantion	4	6	27	2200	14.000	10 d	+	+		L. infantum	L-AmB	Cure
26 [a] /AR /m / 7 y	7 d	+	13.38 (-2.77)	Fatigue	5	8	24	1240	13.000	10 d	+	+		L. infantum	L-AmB	Cure
27 [a] /DA /m / 7 y	15 d	+	14.35 (-2.59)	Fatigue, abd pain, pallor	10	10	16	41900	43.000	Out of follow	+	Neg		Nd	L-AmB	Out of follow [b]
28 [a] /AH/m/3 y 6 Chronic Granulomatous Disease	Uk	+	17.0 (1.24)	Fatigue, abd distantion, vomiting	10	12	24	2700	222.000	5 d	+	Neg		L. infantum	L-AmB	Cure
29 /FY /m /12 y	Uk	+	14.2 (-2.16)	Abd distantion, HLH	4	6	18	2700	62.000	20 d	Neg	Neg		L. donovani	L-AmB	Cure
30 /GÇ /f /1 y 4 m Glycogen Store Disease	7 d	+	14.0 (-3)	Fatigue, edema	3	0	20	2400	44.000	15 d	+	Nd		Nd	L-AmB	Cure

*: Syrian patient; Uk: Unknown, Nd : not done HLH: hemophagositosis; BM: bone marrow; IC: rk39- based immunochromatographic test; -: negative; +: positive.
SSG (Sodium stilgluconate- Pentostam), MA- Meglumine antimonite (Glucantime®) L-AmB: Lipozomal amphoterisin B
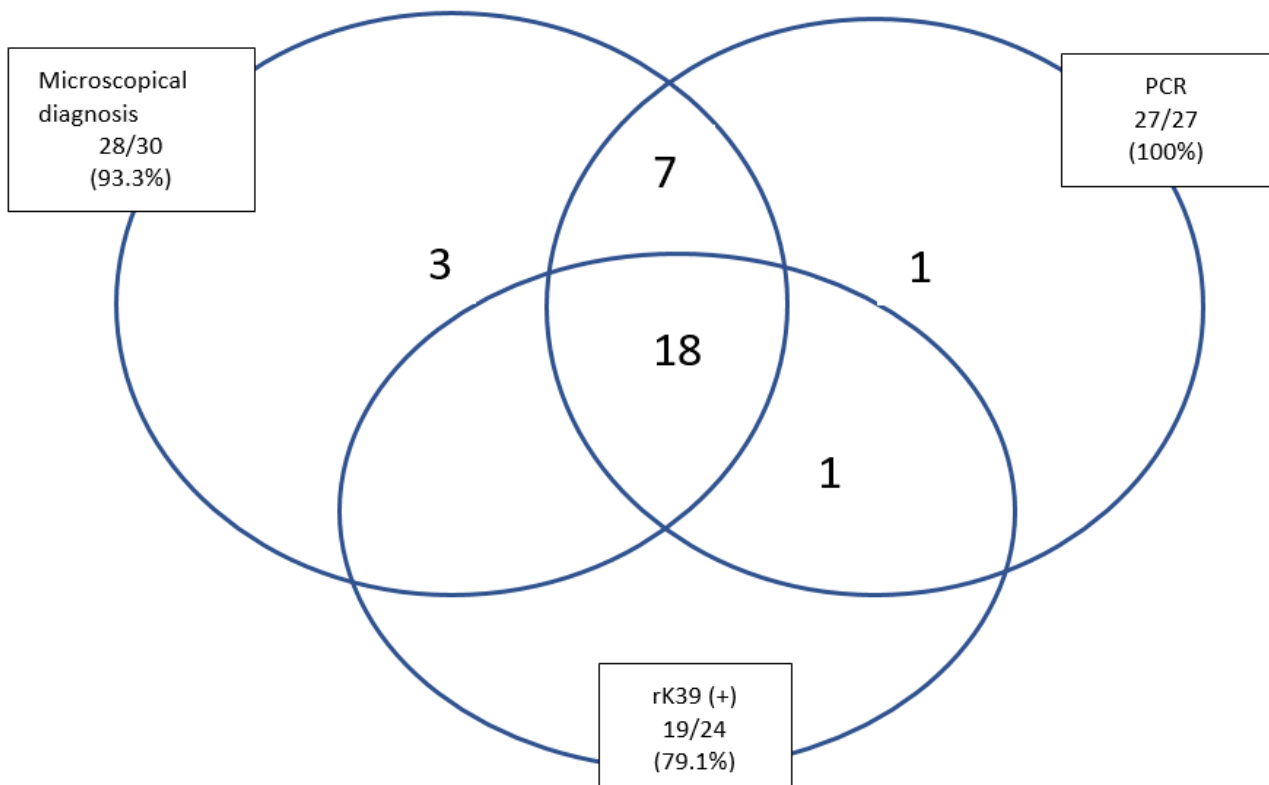
Figure 1. Venn diagram with data about PCR, r K39 and microscopic examination positivity regarding VL patients (N: 30 VL pediatric case).

Table 3. Distribution of Leishmania strains by age and provinces.

	L. infantum 12 case (40%)	*L. tropica* 7 case (23.3%)	*L. donovani* 8 case (26.7%)	ND 3 case (10%)	Unresponsive/reccurens 5 case (16.6%)
0-12 month (3 case, 10%)	1	1	1		
13 month- 2 years (5 case, 16.6%)	2*	-	2*	-	2
2 years-6 years (17 case, 56.6%)	7**	5	4		2
7 years-12 years (5 case, 16.6%)	2	1*	1		1
Province					
Adana (16)	6	4	5	1	3
Hatay (5)	-	1	-	-	-
Gaziantep (1)	2	1	2	-	2
Osmaniye (1)	-	-	1	-	-
Adıyaman (1)	-	-	-	1	-
Syrian refugees (6)	4	1	1	1	-

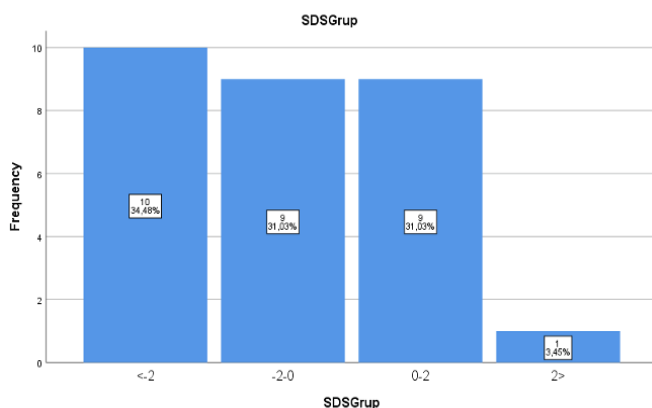* : for each case that was non-responsive to first treatment.

Figure 2. Distribution of patients according to body mass index. *SDS = standard deviation scores.*

hospitalizations and side effects, such as malaise, arthralgia, abdominal pain, increased levels of liver transaminases, amylase, and lipase, vomiting, renal effects, and T-wave changes in the electrocardiogram.

The treatment outcome of only one of our patients remained unknown. This patient was the child of a Syrian immigrant family, and they had returned to their hometown; the patient's family discharged the patient before the treatment was finished. Therefore, the patient's treatment outcome could not be followed up. In this study, it was observed that 5 (17%) of VL patients did not respond to the initial PAS treatment. One of these had anorexia, and the VL infection recurred. While four

Table 4. Clinical findings of the VL diagnosed pediatric patients.

Symptoms/findings	Number of cases (%) (Total 30 case)
Fever	28 (93.3)
Fatigue	10 (33.3)
Abdominal distention	7 (23.3)
Abdominal pain	7 (23.3)
Pallor	5 (16.6)
Weight loss	3 (10)
Vomiting	2 (6)
HLH	2 (6)
Jaundice	1 (3)
Edema	1 (3)
Trombocytopenia	26 (86.6)
Anemia	21 (70)
Neutropenia	19 (63.3)
Splenomegaly	27 (90)
Hepatomegaly	23 (76.6)

unresponsive patients were shifted to L-AMB treatment, and a full cure was achieved. Finally, a single patient who did not respond while receiving L-AMB treatment was moved to PAS treatment, which was concluded successfully (**Table 2**). No patient died.

Discussion. According to WHO data, more than 1 billion people live in leishmaniasis endemic areas and are at risk for this disease. More than 90% of new cases reported to WHO occurred in 10 countries: Brazil, China, Ethiopia, Eritrea, India, Kenya, Somalia, South Sudan, Sudan, and Yemen.[2] Leishmania is also widespread in most countries in the Mediterranean basin, including Turkey and our neighbor countries, Syria, Iran, and Iraq.[11] Turkey is an endemic area for CL, according to the data of WHO. New outbreaks of CL cases in the southeast of Turkey are concurrent with the start of the war in Syria (now a day, one of the top countries in the world for CL) and large migration to Turkey;[12] in addition, Turkey is a geographical transition region that receives immigrants from many other countries (e.g., Afghanistan, Pakistan). Besides this, many people go to endemic areas outside of Turkey for work and military duty (e.g., Iraq, Syria, Afghanistan). Leishmaniasis is an important public health problem for Turkey due to its geographical location, climatic conditions, and internal and external migration prevalence.

It is emphasized that the development of infection with *Leishmania* is a complex structure under the influence of different factors. Individual factors (age group, nutritional status) and infecting parasite species/virulence factors, as well as the host's immune status, play a major role in the disease formation of this agent. Macrophages and neutrophils are the primary cells interacting with *Leishmania* parasites after host infection.

Macrophages and neutrophils are considered the primary cells that react to Leishmania infection. Still, several other immune cells, such as monocytes, dendritic cells (DCs), natural killer (NK) cells, and CD4+ and CD8+ T cells, as well as effector molecules, such as cytokines like interferon (IFN)-γ and interleukin (IL)-12 and nitric oxide (NO) produced by inducible NO synthase (iNOS), play distinct roles in the host response. The development of an immune response against *Leishmania* is associated with the parasite's activation of CD4+ T cells and the differentiation of these cells into T-helper (Th) 1 or Th2 effector cells. In addition, interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β), which are defined as immune response regulators, play a key role in VL formation by inducing parasite proliferation.[13,14]

The prevalence of leishmaniasis is increasing rapidly in the pediatric population in many parts of the world. The mechanisms underlying its higher incidence in children still contain many speculations, and the exact mechanism that causes VL sensitivity is unknown. Still, it is thought to be associated with immunodeficiency in particular. In addition, studies in South America and Africa have reported malnutrition may increase the risk of VL infection in children.[14] Kumar et al. have examined the effect of malnutrition on the profile of innate immune functions of polymorphonuclear neutrophil granulocytes (PMNs) and monocytes by comparing well-nourished and malnourished Indian patients with VL. They have reported that malnourished VL patients were observed with severely dysfunctional PMNs and monocytes in terms of ROS activity that could not be recovered by stimulation with *Leishmania* parasites.[15] Other studies have been conducted on the relationship between malnutrition and VL infection and have suggested that in many cases, malnutrition plays a critical role in the progression of VL to its severe form.[16,17] In our study, malnutrition was detected in 34.48% of patients, and VL infection recurred in a patient during anorexia.

The clinical presentation, combined with the epidemiological context and non-specific biological parameters,[18] raises suspicion of VL, but laboratory confirmation of the diagnosis before deciding on treatment is required. The gold standard for VL diagnosis is direct *Leishmania* amastigotes visualization by microscopic examination in the Giemsa-stained slides of tissue aspirate samples (spleen, bone marrow, or lymph node).[19] Although our success in the direct microscopic evaluation of the bone marrow in the diagnosis was high (93.3%), two children were diagnosed only by molecular testing, and these results made us think that many cases may have been missed. The rK39 dipstick test positivity in the current study was 79.1% in all VL patients. The PCR assays are a rapid tool with high sensitivity (99%), allowing species-specific diagnosis, therapeutic approach, and clinical progress.[20]

The literature shows that *L. tropica* is the causative agent of CL, while *L. donovani* and *L. infantum* are the causative agents of VL. However, it has been reported with the development of molecular methods that leishmaniasis causative species vary. Recent studies have reported the presence of *L. infantum* in CL cases without a VL history and that of *L. tropica* in VL cases without a CL history.[21,22] The *L. infantum, L. major, L. tropica,* and *L. donovani* from leishmaniasis agents have been reported in the southern/southeast Anatolia region of Turkey.[23,24] In the present study, as causative agents of VL in pediatric cases, *L. infantum, L. donovani,* and *L. tropica* were as follows; 40%, 26.7%, and 23.3%, respectively. In the Persian Gulf War (1990-1991), *L. tropica* was the causative agent for leishmaniasis in American soldiers and veterans at bases in Bahrain and Northern Saudi Arabia,[6,25] and several other reports from various parts of the world suggest that *L. tropica* can cause VL.[26-28] Southern and southeast Anatolia has a geography similar to the settlements where these notifications are made. In our study, *L. tropica* was isolated in VL patients without CL symptoms. Therefore, *L. tropica* may be the causative agent for VL more than expected in the area of Turkey. *L. donovani* and *L. infantum* species have also been detected in VL patients. When the relationship between *Leishmania* species and clinical symptoms was compared, no significant association was found between them.

The VL is the most severe form of leishmaniasis and represents a spectrum of chronic infections. The incubation period ranges from weeks to months (even years); thus, due to slow progression, most patients remain subclinical and are correctly diagnosed only very late in the course of the disease. While WHO estimates that the illness in Syria is 2-5 times higher than the actual reported figures, this is frightening considering the risk posed by asymptomatic *Leishmania* carriers to the Turkish population, especially due to the massive influx of immigrants from Syria in recent years.[2] Due to the prolonged presence of asymptomatic and subclinical people in the community, the VL infection can be transmitted to many people. In addition, the asymptomatic VL patients may serve as a silent reservoir of parasites, presenting a risk to public health through infection of the phlebotomine vector.[29] Although most of our patients in our study had a history of being in rural areas and having contact with sandflies, some lived in the city and whose risks were not defined.

WHO has established a useful and suspicious clinical case definition for a diagnostic algorithm: being in an endemic area, prolonged fever (more than two weeks), splenomegaly (mostly), and weight loss, also with pancytopenia, VL is suspected in any case.[30] Including VL in the differential diagnosis of leukemia-like syndromes in infants living in/traveling (even in the past) to endemic areas such as the Mediterranean basin[31]. VL is an infection not commonly managed by Turkish practitioners and even more by the European physicians of Mediterranean and non-Mediterranean countries, where immigrants can bring the disease. This situation can lead to delayed recognition of undiagnosed reactivated VL, inappropriate treatment in VL, and poor clinical outcomes.

Turkey is an endemic country for CL, with the main causative agent as *L. tropica*.[32] Although about a quarter of our VL cases, the cause was *L. tropica*, cutaneous lesions were not found in any of them. Concomitant cutaneous lesions should be investigated, and each patient with cutaneous lesions should be examined in detail regarding visceral spread. In southeast Anatolia, where CL is known to be endemic, the possibility of VL in a patient diagnosed with leukemia should not be forgotten.

Few anti-leishmanial formulas have been proven worthy; PAS, L-AMB, pentamidine, miltefosine, and paromomycin. Socio-economic and pharmaceutical conditions (production, distribution) have a tremendous impact on the choice of therapeutic option.[33] Although only PAS is given free of charge by the government in Turkey, L-AMB has recently become the first choice drug instead of PAS due to its faster effect (7 days' hospitalization versus 28-30 days. Approximately 17% of the patients were reported to fail to respond to the current study's initial therapy; four failed to respond to PAS therapy, resulting in a complete cure with L-AMB. Considering the cost advantage of shortening the hospital stay, despite the high cost of L-AMB treatment, it seems to be a cost-effective regimen compared to antimonial agents with its high success rate and being the most effective treatment.[34,35] In addition, the less toxic effect of L-AMB confirms its preference.

Conclusions. The present study reveals that all three isolate *L. infantum, L. donovani* and *L. tropica* are causative agents of pediatric VL in Turkey's south/southeast Mediterranean region. However, this study must be supported by studies with more examples. Furthermore, determining possible animal reservoir hosts for effective control should be explored using molecular methods. While it is obvious that the cost spent for the diagnosis and treatment of leishmaniasis, an important public health problem, is higher than the measures to prevent this disease, it is very sad that not enough attention is paid to this issue in the world.

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