Quantification of *Leishmania infantum* Kinetoplast DNA for Monitoring the Response to Meglumine Antimoniate Therapy in Visceral Leishmaniasis

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Abstract. Meglumine antimoniate (Glucantime) remains the therapeutic cornerstone of visceral leishmaniasis (VL). Twenty-one VL patients were treated with Glucantime, extending for 1 week after defervescence. For monitoring the response, *Leishmania infantum* kinetoplast DNA loads were evaluated using real-time polymerase chain reaction (PCR) assay in the blood. The maximum duration of treatment was 14 days. The loads before treatment ranged from 8 to 1,300,000 parasites/mL (mean = 73,095 parasites/mL), and the mean values on days 3, 7, 14, 28, and 90 were 4,902, 506, 6.33, 0.26, and 0.14, respectively. The loads decline to < 1 parasite/mL for 16 (76%) and 20 (95%) patients on days 14 and 28, respectively, and they decline for all patients by day 90. Results showed a dramatic decrease of the parasite loads, although complete clearance was not accomplished at the end of treatment. Only one relapse (4.5%) was observed. The parasite load can also serve as a dependable index for monitoring the response to Glucantime.

INTRODUCTION

Visceral leishmaniasis (VL; kala-azar) is a life-threatening parasitic infection that is endemic in 62 countries worldwide, including the Mediterranean region.¹ Based on regional reported incidences, approximately 0.2–0.4 million VL cases occur each year in the world.² *Leishmania infantum* is the most common cause of VL in Iran, where it mainly affects children, with an annual incidence rate of 300–600.^{2,3} VL is characterized by prolonged fever, weight loss, progressive anemia or pancytopenia, and hypergammaglobulinemia.¹

Antimony (sodium antimony gluconate and meglumine antimoniate) has been its therapeutic cornerstone throughout the world for more than 70 years.⁴ The World Health Organization (WHO) Expert Committee recommended that meglumine antimoniate (Glucantime) be administered in doses of 20 mg/kg per day up to a maximum of 850 mg for 28–30 days.¹ Importantly, cure rates exhibited by different drugs, including antimonials in the areas where they are still in routine use, are similarly high (95%) in immunocompetent patients. Data show that antimony resistance does not seem to be an emerging problem in Mediterranean countries, which is in contrast to the situation documented in Indian VL cases caused by *L. donovani*.⁵ In the endemic regions of southern Iran, treatment with meglumine antimoniate for a short duration (20 mg/kg per day intramuscularly, extending for 1 week after defervescence) remains the first-line treatment of VL patients.^{3,6–8}

In recent years, real-time polymerase chain reaction (PCR) has been developed and used successfully for the diagnosis and quantification of parasitic loads.^{9–12} Rapid and accurate methods for parasite detection and monitoring parasite loads in VL would greatly enhance the clinical management of the disease. Monitoring during therapy with amphotericin in immunocompromised patients has been promising, but therapeutic monitoring in immunocompetent patients treated with other regimens, such as Glucantime, has hardly ever been addressed in literature.¹⁰

In this study, *L. infantum* parasite loads in the blood were evaluated by the kinetoplast DNA (kDNA) leishmania quantitative real-time PCR assay for monitoring the response to

Glucantime in 21 VL patients, extending for 1 week after defervescence, in southern Iran.

MATERIALS AND METHODS

Patients. Two groups were enrolled in this study, namely patients and controls. The patient group consisted of 21 individuals (13 males and 8 females), with a mean age of 19 months (range = 4–72 months), admitted with a diagnosis of VL to Nemazi Hospital, a tertiary referral center affiliated with Shiraz University of Medical Sciences, Iran. The study was conducted between January of 2009 and July of 2010. The patients were referred from the southern provinces of Iran, where VL is endemic. Diagnosis of the disease was based on the clinical signs associated with the detection of a titer of 1:128 or higher of antibodies to *Leishmania* detected by the indirect immunofluorescence test (IFAT) and K39 strip tests. The 21 patients diagnosed with VL received treatment with meglumine antimoniate, 20 mg/kg per day intramuscularly, extending for 1 week after defervescence.^{3,6–8} Clinical response was defined as defervescence, and all of the patients who received the treatment were responsive to it.^{6,8} The day of defervescence was defined as the day at which fever dissipated and the patient subsequently maintained a temperature below 38°C. The second group (control) consisted of 40 individuals (31 males and 9 females), with a mean age of 7 years, similarly living in the leishmaniasis-endemic area of southern Iran with no history of clinical symptoms for VL or immunosuppression. This study was conducted with the approval of the Ethics Committee of Shiraz University of Medical Sciences, Iran, and informed consents were obtained from the individuals' guardians.

Samples. Two-milliliter peripheral blood samples from all of the participants, collected in EDTA-coated tubes, were aliquoted and frozen at –70°C for real-time PCR assay. At the time of sampling, the sera for IFAT and K39 strip tests were obtained from 2-mL clot blood samples. Additionally, 2-mL whole-blood samples were obtained from the patient group on days 3, 7, 14, 28, and 90 after the beginning of therapy and also, 2 or 3 days after defervescence. These samples were sent to the Professor Alborzi Clinical Microbiology Research Center (PACMRC) for determination of the parasite loads. Monthly follow-ups were carried out for all of the patients for a period of 14 months.

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Serological tests. The IFAT was made using antigens of *L. infantum* prepared from a reference human strain (strain MCAN/IR/96/LON-49). IFAT was determined from a serial dilution of 1:64 to 1:1,024 for each serum sample according to the WHO/LEISH/96.40 protocol. Human antiglobulin (Binding Site Group, Birmingham, UK) with a dilution of 1:300 was used. Control serum (titer = 1:1,024) was collected from the patients with VL. K39 strip test (InBios International, Seattle, WA) was used according to the manufacturer's instructions.

Quantitative detection of kDNA. DNA extraction was performed on 500- μ L whole-blood samples. To achieve maximum yield, digestion was performed on one volume blood in two volumes 0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH, 10 mM Tris (pH 7.2), and 320 μ g proteinase K per 1 mL (proteinase K lysis buffer) for 24 hours at 56°C and then boiled for 10 minutes.¹³ A simplified phenol-chloroform extraction was performed on this lysate followed by ethanol precipitation and resuspension in 50 μ L sterile distilled water and storage at -70°C.

Quantification of parasite load was performed according to a previously described technique with some modifications.^{10,11} An Applied Biosystem 7500 (Foster City, CA) was used for amplification and detection. Optimization experiments led us to use the TaqMan Gene Expression Master Mix (catalog number 4369016), 20 pmol direct primer (CTTTCTGGTC CTCCGGGTAGG), 20 pmol reverse primer (CCACCCGG CCCTATTTTACACCAA), and 50 pmol TaqMan probe (FAM-TTTTCGCGAGAACGCCCTACCCGC-TAMRA). Assays were performed with a 25 μ L final volume with 5 μ L sample DNA. The standard curve was established from *Leishmania* DNA extracted from 5×10^6 parasites; 5 μ L serial dilutions, ranging from 50,000 to 0.0005 parasites, were added to the reaction tubes. TaqMan Chemistry allowed two-step temperature (94°C and 60°C) cycling over 45 cycles. Primers were designed to hybridize to kDNA *L. infantum*, and the hybridization probe was specific for VL parasites. This assay had a sensitivity of 0.001 parasite/mL.

Statistical analysis. Data were analyzed using Spearman's correlation and the Mann-Whitney and paired-sign tests.

RESULTS

Parasite loads in the control group. Among the blood samples from 40 individuals living in the endemic area without any history of leishmaniasis, we detected *leishmania* kDNA in 55% of the cases (22 of 40). The parasite load was less than 1 parasite/mL, detected within a range of 0.02–0.9 parasite/mL, with a mean of 0.08 in all 22 individuals.

Parasite loads pre-treatment. *Leishmania* kDNA was found in all 21 patients with VL at the time of diagnosis, with a broad range (8–1,300,000 parasites/mL). The mean and median of the parasite loads were 74,357 and 280 parasites/mL, respectively. The load was not significantly correlated with age, duration of fever before treatment, or titer of anti-*Leishmania* antibodies at the time of diagnosis (Table 1).

Parasite load during treatment. Table 1 shows the parasite loads during treatment. The mean and median for defervescence were 5.14 and 5 days, respectively. The treatment with meglumine antimoniate extended for 1 week after defervescence, with a range of treatment durations from 10 to 14 days, with a mean of 11.8 days (Table 1).

A rapid decrease in parasitemia was observed during treatment with meglumine antimoniate, and the decrease of parasite load in each succeeding day was statistically significant ($P < 0.05$). The mean load of parasitemia was 4,902 on day 3, 506 on day 7, and 6.39 on day 14 after the treatment.

On the 14th day after the treatment, the parasite load was less than 1 parasite/mL in 16 patients (76%), and the mean parasite load in 5 patients was 26 parasites/mL (Table 1).

Parasite load post-treatment. On the 28th day after treatment, the parasite load was less than 1 parasite/mL in 20 patients (95%). One patient had a load of 2 parasites/mL. On the 90th day after treatment, the parasite load was less than 1 parasite/mL in all patients. The patient with 2 parasites/mL on the 28th day after

TABLE 1
Monitoring of kDNA loads in patients with VL treated with Glucantime in southern Iran

No.	Sex*	Age (months)	Duration of fever before treatment (days)	Anti- <i>Leishmania</i> Ab (titer)	Day of defervescence	<i>Leishmania</i> kDNA (parasites/mL)	<i>Leishmania</i> kDNA (parasites/mL) after starting treatment					
							3rd day	7th day	2–3 days after defervescence	14th day	28th day	90th day
1	M	6	28	1:256	5	8.00	1.60	0.00	0.00	0.10	0.00	0.00
2	M	7	14	1:256	4	8.00	0.40	0.10	0.10	0.00	0.00	0.00
3	F	30	14	1:128	3	32.00	7.00	0.60	0.50	0.90	0.40	0.05
4	F	36	21	1:128	5	1,330.00	6.00	1.00	0.00	0.00	0.00	0.00
5	M	7	28	1:128	4	80.00	16.00	0.00	0.00	0.00	0.00	0.00
6	M	36	14	1:512	5	227.00	200.00	0.00	0.00	0.00	0.00	0.00
7	M	24	21	1:256	4	10.00	4.00	1.20	1.20	0.80	0.00	0.00
8	F	6	28	1:128	5	9,600.00	520.00	0.90	0.20	0.90	0.10	0.40
9	M	30	14	1:128	5	49.00	9.00	4.00	4.00	0.00	0.50	0.00
10	M	10	21	1:128	5	280.00	40.00	4.00	4.00	0.30	0.00	0.00
11	M	13	21	1:128	5	350.00	400.00	8.00	8.00	0.20	0.30	0.04
12	F	9	21	1:512	4	20.00	10.00	10.00	2.00	0.00	0.00	0.08
13	M	18	28	1:128	4	44.00	10.00	12.00	12.00	0.02	0.00	0.00
14	F	24	21	1:256	5	1,000.00	28.00	16.00	2.00	0.50	0.05	0.20
15	F	4	14	1:128	4	6,400.00	1,560.00	80.00	80.00	0.30	0.00	0.10
16	M	72	14	1:512	5	5,600.00	3,000.00	180.00	40.00	0.90	0.10	0.30
17	M	12	28	1:256	6	34,900.00	6,400.00	1,000.00	50.00	4.00	0.80	0.80
18	M	24	14	1:256	5	254.00	290.00	130.00	82.00	22.00	0.90	0.03
19	M	36	21	1:128	5	1,320.00	292.00	108.00	108.00	12.00	0.40	0.60
20	F	7	21	1:512	6	1,300,000.00	5,040.00	160.00	160.00	14.00	0.00	0.00
21†	F	9	21	1:1,024	7	200,000.00	85,120.00	8,920.00	8,920.00	76.00	2.00	0.40

*F = female; M = male.

†Parasite load was 1,000 parasites/mL at the time of clinical relapse, which was observed 135 days after the end of treatment.

treatment developed clinical relapse, and 135 days after the end of treatment, the respective parasite load was 1,000 parasites/mL.

DISCUSSION

In this study, *L. infantum* kDNA parasite loads were determined by real-time PCR, because previous studies had shown that methods based on kDNA amplification were the most sensitive ones; this molecular target is present at ~10,000 copies in a single parasite and more suitable for detecting cases of less than 1 parasite/mL in the blood.^{10,11,14–16} Twenty-one VL patients were treated with meglumine antimoniate, 20 mg/kg per day intramuscularly, extending for 1 week after defervescence. This treatment method is routine in southern Iran.^{3,6–8}

At the time of diagnosis, the parasite load ranging from 8 to 1,300,000 parasites/mL showed individual variations that were not correlated with age, duration of fever before treatment, or IFAT antibodies (Table 1). A wide range of loads was previously reported by Mary and others¹¹ in immunocompetent patients (from 8 to 1,400,000 parasites/mL). This wide range of parasite loads may be explained by (1) the different parasite loads entered by sandfly bites, (2) the kDNA released from phagocytic cells to the blood in reticuloendothelial systems, and (3) the parasite replication controlled by host cellular immune responses.^{12,17,18}

In healthy individuals living in the endemic areas, parasitemia has been reported to be 7.4–50.8% using different PCR methods. This variation can be explained by the various levels of sensitivity of the PCR methods.^{11,16,19,20} In this study, parasite loads of less than 1 parasite/mL were circulated in the peripheral bloods of a high percentage (55%) of individuals in the control group. This point can serve as a cutoff point for the diagnosis of VL patients, which was shown in the works by Mary and others.^{10,11} Therefore, this point could be a target point for a successful treatment of VL patients receiving antimonial treatment. Being of high sensitivity, quantitative real-time PCR seems to be a reliable method to detect asymptomatic infected individuals.

A dramatic rapid decline was observed during treatment with meglumine antimonite (Figure 1). On day 14, when treatment was discontinued in all patients, the load ranged between 0 and 76 parasites/mL. At that time, the kDNA of parasites was cleared from peripheral blood in the majority (76%) of patients. On day 28, 2 weeks after the discontinuation of treatment, it was cleared in almost all (95%) patients, and finally, on day 90, it was cleared from all patients. Fourteen months after the discontinuation of treatment, no relapses were detected in any of the patients, except one case. The clearance of parasites after discontinuation of meglumine antimonite treatment could be because of the potentiation of the host immune function by up-regulating interferon- γ (IFN- γ) and interleukin-12 (IL-12) synchronized with a decline in IL-10 and transforming growth factor- β (TGF- β) levels.^{21–24} IL-12 helps the expansion of IFN- γ , which synergistically acts with tumor necrosis factor- β (TNF- β) to activate macrophages to kill leishmania parasites through the release of nitric oxide (NO).^{25–27}

In the current study, clinical relapse was observed in only one patient 135 days after the end of treatment (4.5%). This patient had a delay in the clearance of kDNA and was the only case that harbored *Leishmania* kDNA in the blood on the 28th day after the beginning of treatment. The same patient had less than 1 parasite/mL on day 90; therefore, we cannot be certain

FIGURE 1. Parasites' quantification in the peripheral blood of 21 patients with VL in the course of treatment with antimony and follow-ups in southern Iran. Dotted horizontal line indicates mean parasite level. The thick horizontal line indicates diagnostic cut off point.

that the disease will not relapse, even if the parasite load decreases to less than 1 parasite/mL.

In conclusion, treatment with meglumine antimoniate in a short duration, extending for 1 week after defervescence, could be associated with a dramatic decrease of parasite loads, although complete clearance was not accomplished at the end of treatment. Ultimately, parasitemia was < 1 parasite/mL within all of the patients, with only one relapse (4.5%). Quantification of *Leishmania* load by real-time PCR can also serve as a reliable, rapid, and non-invasive method for VL diagnosis and a dependable index of monitoring the response to meglumine antimoniate in immunocompetent patients.

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