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Research Article

Therapeutic Efficacy Evaluation of Metronidazole and Some Antifungal Agents with Meglumine Antimoniate on Visceral Leishmaniasis by Real-Time Light-Cycler (LC) PCR in BALB/c Mice

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Abstract

Purpose: *To develop a highly accurate molecular assay for evaluating the efficacy of metronidazole and some antifungal agents with meglumine antimoniate against L. infantum visceral leishmaniasis in different mouse tissues.*
Methods: *The assay was performed with the Light-Cycler system using SYBR Green I and primers amplifying ca. 120-bp fragment from minicircles of the kinetoplast DNA (kDNA).The mice were divided into two groups. Group I served to evaluate drug activity and parasite load while Group II was assigned to identify possible synergistic activity between meglumine (which is highly effective in the liver but less effective in the spleen) and drugs with significant activity against spleen infection.*
Results: *The assay was able to detect as little as 100 fragments of* L. major *DNA per reaction, which is equivalent to 0.1 parasites. The standard curve designed for quantitation of parasites showed linearity over at least 6-log DNA concentration range, corresponding to 0.1 to 10^4 parasites per reaction with a correlation coefficient of 0.979. Metronidazole, ketoconazole, fluconazole, itraconazole and terbinafine were less effective than antimonial agents in reducing hepatic parasite load while ketoconazole potentiated the effect of meglumine antimoniate reference therapy through its marked activity against spleen infection (L. infantum visceral leishmaniasis).*
Conclusion: *The assay technique is accurate, sensitive, and rapid for the detection of kDNA and would be of great help to scientists who use animals to monitor the efficacy of anti-leishmanial drugs or vaccines.*

Keywords: *Leishmaniasis, Molecular diagnosis, Kinetoplast DNA, Real-Time LightCycler(LC)-PCR, Spleen infection, Antifungal agents.*

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INTRODUCTION

Leishmaniases are a group of parasitic diseases of major and growing public health importance [1]**.** Leishmania species are intracellular protozoa that affect humans and dogs worldwide and are transmitted by the bite of hematophagous sand flies. About 21 *Leishmania* species have been reported to cause human infection [2]. They cause a large spectrum of diseases, ranging from spontaneously healing skin lesions to fatal visceral symptoms, if left untreated. Two million new human cases arise every year, and at least 350 million peoples are exposed to the risk of *Leishmania* parasite infection [3]. Experimental hosts, such as laboratory mice, are largely used to study the immunobiology of these parasites and to screen the efficacy of newly developed drugs and vaccines [4]. Most of those studies require detection and quantitation of the *Leishmania* burdens in different mouse tissues. This is still routinely performed by culture-based techniques [5]. These positive diagnostic methods for *Leishmaniasis* such as direct smear examination and culture, have major limitations. Direct examination requires some expertise and lacks sensitivity, culture is labor-intensive, and the result is not known for weeks [6].

By contrast, PCR assay provides results in one or two working days. PCR-based methods for detecting *Leishmania* species have been developed to amplify rRNA genes, miniexon genes, kinetoplast DNA (kDNA), and repetitive nuclear sequences [7]. Recently, a PCR-based assay to quantify the parasite load in mice infected with *L. major* using primers from the conserved sequences of kDNA [8] was reported. However, this technique is still cumbersome as it requires agarose gel image analysis. A more rapid alternative is real-time quantitative PCR, which quantifies DNA and has the potential for accurate microorganism enumeration in medical [9], environmental [10] and food samples [11].

Visceral leishmaniasis (VL) caused by *Leishmania infantum*, remains difficult to treat in patients with AIDS due to parasite resistance and high rates of relapse. There is a need for an alternative to antimonial agents and amphotercin B, especially for drugs that are effective by the oral route. Metronidazole and sterol biosynthesis inhibitors (ketoconazole, fluconazole, itraconazole and terbinafine) are well-tolerated drugs that are potentially active against *Leishmania* when given by mouth. It has been shown that *Leishmania donovani* experimentally resistant to amphotercin B is highly susceptible to ketoconazole. [12]. In this study, we tested the application of real-time LC-PCR by evaluating the efficacies of metronidazole and some antifungal agents with meglumine antimoniate against *L. infantum* visceral leishmaniasis in experimental BALB/c mice.

EXPERIMENTAL

Animals

Five- to 6-week-old mice, weighing 20 - 30 g. were purchased from Theodor Bilharz Research Institute TBRI, Egypt. Approval of the institutional animal ethical committee for the animal studies was obtained from the Office of Environmental Health and Radiation Safety, ACUC Protocol # 1096-5. The animals were maintained according to accepted standards of human care [13].

Materials

The following primers were used (forward, 5'-CCTATTTTACACCAACCCCCAGT-3' JW11; reverse, 5'-GGGTAGGGGCGTTCTGCGAAA -3' JW12) that amplify a ca. 120-bp fragment of the minicircles kDNA of *L. major,* ca. 10,000 copies of which are present in each parasite. These primers match the conserved sequences of the kinetoplast minicircle but do not match mouse frequent nucleic acid sequences according to the PCR-Rare software. The primers were obtained from Genset (Paris, France) as EasyOligos.

Leishmania strains and DNA extraction

L. major strain NIH173 (MHOM/IR/-/173), *L. donovani* LV9 (MHOM/ET/1967/Hu3:LV9), *L. infantum* 2176 (MHOM/FR/1991/LEM/2176), *L. amazonensis* LV79 (MNYC/BZ/1962/M1841), and *L. mexicana* M379 (MNYC/BZ/1962/M379) were cultured according to the method of [14]. Briefly, at 26 ^{0}C in Hosmem-ll medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 100 pg of penicillin/ml and 100 pg of streptomycin/ml, stationary-phase promastigotes of the different strains were harvested by centrifugation, washed twice with PBS, enumerated with a hemocytometer, pelleted and stored at -80 ^{0}C until DNA extraction. Genomic DNA was extracted from approximately 2 x 10^7 promastigotes with a DNeasy Tissue kit (Strata Gene Inc, USA) in accordance with the manufacturer's protocol.

Source of mouse tissue DNA

Mice were infected intradermally at the ear with 10^4 metacyclic promastigotes of *L. major* strain NIH 173 and killed at 6 or 12 months post-infection. Other mice were infected in the footpad with 2 x 10^6 amastigotes of *L. amazonensis* strain LV79 or *L. mexicana* strain M379 and killed at 12 or 32 weeks post-infection, respectively. Bone marrow and spleens were also collected from mice infected with *L. donovani* strain LV9 as previously described [15].

Real-time PCR with LC

A real-time hot-start PCR was performed with LC FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Meylan, France) in an LC (Roche Diagnostics). The 12-µl reaction mixture contained I x LC FastStart DNA Master SYBR Green I, 2 mM $MgCl_2$, 0.5 µM each primer and 1.2 µl of template. Times and temperatures are shown in Table 1. For fluorescence signal acquisition, channel F1 was used and the gain was set at 5. For normalization of fluorescent data, the Fl/1 ratio was applied.

Table 1: Times and temperatures used for PCR configuration with SYBR Green I and LightCycler

Parameter	emp (°C)	Time (second)	Slope (°C/s)	Acquisition mode
Denaturation	95	8	20	None
Amplification (40 cycles)	95	10	20	None
	56	10	20	None
	72	8	20	Single
	95	10	20	None
Melting	67	30	20	None
	95	10	0.1	Continue
Cooling	40	60	20	None

Application of anti-Leishmania chemotherapy

Group I

Drug screening to evaluate drug activity, parasite loads in the liver and spleen of mice treated from days 7 to 17 were determined on day 20 using real time LC-PCR. Parasite load was expressed as the $\log_{10}$ number of parasites per gram of tissue, and the mean (± SD) parasite load for four mice was calculated. Drug administration was daily, by mouth, for treatment with metronidazole (70 and 140 mg/kg), ketoconazole (50 and 100 mg/kg), fluconazole (50 and 100 mg/kg), itraconazole (50 and 100 mg/kg) and terbinafine (100 mg/kg) and daily by intraperitoneal injection, for treatment with meglumine antimoniate (200 mg/kg).

Group II

This was designed to identify possible synergistic activity between meglumine antimoniate which is highly effective in the liver but less effective in the spleen and for drugs with significant activity against spleen infection. The efficacies of ketoconazole and metronidazole alone and in combination with meglumine antimoniate were examined and compared with those in untreated mice and in mice-treated with meglumine antimoniate alone. Each group comprised 12 mice.

Statistical analysis

Assay results are shown as mean ± SD and reported as the coefficient of variation. Statistical and regression analyses were carried out with Sigma Plot software (SPSS Inc., Chicago, USA). Differences were considered statistically significant at $p < 0.001$.

RESULTS

LC PCR development

The JW 11 and JW12 primers, which amplify a ca 120-bp DNA fragment from *L. major* kinetoplast minicircles, were used. A 100-pg sample of DNA extracted from in vitro grown promastigotes of *L. major* NIH 173 was used as the template for the establishment of the LC-PCR assay, in particular, for the determination of the optimal annealing temperature and magnesium chloride concentration. Agarose gel electrophoresis of the PCR product confirmed the amplification of a ca 120-bp DNA fragment.

Detection of kDNA from other *Leishmania* species

Primers JW11 and JW12 were able to amplify a ca 120-bp DNA fragment from promastigotes of *L. donovani* LV9, *L. infantum* 2176, *L. amazonensis* LV 79 and *L. mexicana* M379 by conventional PCR. Therefore, the same primers were also assessed for amplification of kDNA in our assay. The sensitivity of the LC-PCR assay for detection of these strains was similar to that of *L. major* NIH 173, except for *L. mexicana* strain M379, for which the assay was approximately 100 times less sensitive (Table 2). The intra-assay variation coefficient was < 1.2 % showing good reproducibility of the assay for those *Leishmania* species as well.

Comparison of real-time PCR and conventional PCR for *Leishmania* detection

DNA was extracted from various tissues of BALB/c infected mice with various *Leishmania* strains (Table 3) and assayed with either a conventional PCR or the LC PCR. In the latter, the standard curve of the respective species was used to generate a relative *Leishmania* burden based on CT values. In all of the assays, the CT values of negative controls were always >36. Whatever the tissue and the strain, all of the samples that were positive by the conventional PCR were also positive by the LC assay and most of the CT values were far below the negative CT value threshold of 36. In addition, a few samples that were negative by conventional PCR were positive by real-time PCR.

Table 2: Mean CT values and intraassay SDs of dilution series of promastigote DNAs from different *leishmania* species

***Leishmania* species**	**Mean CT value ± SD**				
No. of parasites/reaction ➧	***1,000***	***100***	***10***	***1***	***0.1***
L. major NIH 173	21.06±0.260	25.51±0.057	29.46±0.160	33.05±0.446	35.85±0.153
L. donovani LV9	17.71±0.017	21.75±0.070	24.57±0.055	28.83±0.228	32.29±0.224
L. infantum 2176	19.95±0.011	24.36±0.272	28.90±0.006	33.39±0.051	[a]ND
L. amazonensis LV79	19.37±0.121	23.42±0.266	27.48±0.250	32.14±0.075	34.18±0.122
L. mexicana M379	25.84±0.095	30.09±0.121	35.01±0.627	[a]ND	[a]ND

[a]*ND = inconsistent quantification data.*

Table 3: Real-time and conventional PCR assay data for *leishmania* kDNA in mouse tissue

Sample	Species	Source	Conventional PCR	Real-time PCR CT	Qualitative data
1-6	*L. major*	Ear	-	>36	-
7	*L. major*	Ear	+	28.61	+
8	*L. major*	Ear	+	22.66	+
9	*L. major*	Ear	+	24.64	+
10	*L major*	Ear	+	34.94	+
11	*L. major*	Ear	-	35.14	+
12	*L. major*	Ear	-	>36	-
13	*L. major*	Lymph node	+	29.68	+
14	*L. major*	Lymph node	+	30.68	+
15	*L. major*	Lymph node	+	23.77	+
16-18	*L. major*	Tail skin	-	>36	-
19	*L. mexicana*	Blood	-	>36	-
20	*L. mexicana*	Blood	+	31.42	+
21	*L.* mexicana	Blood	-	33.05	+
22	*L. mexicana*	Bone marrow	+	28.80	+
23	*L mexicana*	Bone marrow	+	21.44	+
24	*L. mexicana*	Lesion	+	17.94	+
25	*L mexicana*	Lesion	+	18.16	+
26	*L. mexicana*	Lesion	+	16.46	+
27	*L. mexicana*	Liver	+	23.66	+
28	*L. mexicana*	Liver	+	24.46	+
29	*L mexicana*	Lymph node	+	19.81	+
30	*L. mexicana*	Lymph node	+	20.70	+
31	*L mexicana*	Spleen	+	24.61	+
32	*L. mexicana*	Spleen	+	20.96	+
33	*L. mexicana*	Tail skin	+	23.89	+
34	*L mexicana*	Tail skin	+	15.36	+
35	*L. mexicana*	Tail skin	+	18.67	+
36	*L. amazonensis*	Blood	-	>36	-
37	*L. amazonensis*	Lesion	+	15.81	+
38	*L. amazonensis*	Liver	-	>36	-
39	*L. amazonensis*	Lymph node	+	25.42	+
40	*L. amazonensis*	Spleen	-	>36	-
41	*L. amazonensis*	Tail skin	-	29.97	+
42	*L. donovani*	Bone marrow	+	22.73	+
43	*L. donovani*	Bone marrow	+	27.87	+
44	*L. donovani*	Bone marrow	+	28.44	+
45	*L. donovani*	Spleen	+	19.64	+
46	*L. donovani*	Spleen	+	19.88	+
47	*L. donovani*	Spleen	+	23.56	+

Table 4: Parasite load on day 20 in liver and spleen of *L. infantum*-infected mice treated from days 7 to 17

Group 1 treatment	Dose (mg/kg/day)	Day	Log_{10} parasites/g of tisuue[a] Liver	Spleen
Control		7	4.66±0.18	1.83±2.14
		20	4.99±0.31	3.82±0.30
Ketoconazole	50	20	4.66±0.21	0.86±1.76
	100	20	4.90±0.13	0*
Fluconazole	50	20	4.94±0.28	0.85±1.70*
	100	20	5.04±0.16	1.74±2.01
Itraconazole	50	20	4.72±0.30	2.51±2.19
	100	20	4.48±0.60	1.14±1.89
Terbinafine	100	20	4.57±0.49	2.29±1.10
Metronidazole	70	20	4.55±0.23	1.13±2.26*
	140	20	4.67±0.53	1.03±2.06*

[a]*Values are expressed as mean ± SD for six mice; * significantly different from value for untreated mice at p < 0.001*

Evaluation of 1st and 2nd line anti-leishmania chemotherapy

Group I treatment

None of the test compounds administered alone from days 7 to 17 significantly reduced parasite load in the liver by day 20, compared to that in infected untreated control mice (see Table 4). By contrast, the parasite load in the spleen of treated mice fell by 1 to 4 log_{10} parasite/g relative to the parasite load in the controls. Ketoconazole was the most effective of the drugs tested, since the parasite loads were markedly reduced at a dose of 50 mg/kg/day and were undetectable in mice treated with 100 mg/kg/day (p < 0.01), compared to results for untreated mice. Treatment with metronidazole or fluconazole at a dose of 50 mg/kg/day, also significantly reduced parasite load in spleen, when compared to that in infected untreated control mice. Surprisingly, treatment with fluconazole at 50 mg/kg/day seemed slightly more effective in spleen infection than that at 100 mg/kg/day. However, this effect is limited and individual variations between mice may partially explain such a difference.

Group II animals treatment

Meglumine antimoniate was highly effective in the liver, and treatment with ketoconazole resulted in the lowest spleen load. Interestingly, no parasites were detected on day 20 in the liver or spleen of mice treated with meglumine antimoniate plus ketoconazole or metronidazole. However, relapse was observed with both combinations at day 60, with parasite counts in the spleen comparable to those in mice treated with meglumine antimoniate alone (Table 5).

DISCUSSION

A new molecular real-time *PCR assay* for detection and quantification of *L. major* and several other *Leishmania* species of medical importance is described. This assay is based on the LC system with SYBR Green I. This quantitative LC PCR assay allows highly sensitive and reproducible detection and quantitation of parasite burden over a wide range, at least 6 logs, of parasite concentrations. The very high sensitivity (less than *0.1* parasites per reaction) is partly due to the high copy number of the target minicircle *kDNA,* which is present at ca. *10,000* copies per parasite. This avoids the use of internal molecular probes and therefore limits the cost of the assay. Including the DNA extraction step, the assay can be performed within 4 to 5 h without risk of contamination, as the reaction capillary remained closed.

Table 5: Parasite load on days 20 and 60 in the liver and spleen of *L. infantum*-infected mice treated from days 7 to 17

Group II treatment	Day	Log_{10} parasites/g of tissue[a]	
		Liver	***Spleen***
None (control)	7	3.87±0.07	1.45±1.74
	20	4.77±0.35	3.56±0.29
	60	4.57±0.27	4.79±0.51
Ketoconazole(50mg/kg/day)	20	4.69±0.26	0.68±1.66
	60	3.94±0.35	4.72±0.14
Metronidazole(70mg/kg/day)	*20*	4.89±0.32	2.13±1.56
	60	4.50±0.45	4.96±0.35
Meglumine antimoniate (200mg/kg/day) plus Ketoconazole (50mg/kg/day)	20	0*	0*
	60	0*	4.25±0.45
Meglumie antimoniate (200mg/kg/day) plus Metronidazole(70mg/kg/day)	20	0*	0*
	60	0*	3.17±1.62

[a]*Values are expressed as mean ± SEM for six mice*, significantly different from value for untreated mice at p < 0.001*

Application of real-time PCR for research and clinical diagnosis in parasitology is a useful technique and so far concerns mainly ***Toxoplasma gondii*** [16] and ***L. infantum*** [17]. With primers common to several ***Leishmania*** species, our assay technique can also be used to determine relative parasite burden in mouse tissues infected with ***L. amazonensis*** and ***L. donovani*** and to a lesser extent, in mouse tissues infected with ***L. mexicana*** based on CT values. In a previous study, preliminary assays have shown the PCR yield may be influenced by tissue DNA concentration above a threshold. Therefore, we are evaluating new developing internal standards based on housekeeping genes to determine parasite burden more accurately. Identification of a ***Leishmania*** infection for laboratory clinical diagnosis by culture or serological techniques requires a long time and has poor specificity.

With the development of a real-time PCR assay that can be improved for identification of *Leishmania* species with internal probes or different primers, as done previously with other pathogenic microorganisms such as *Campylobacter* spp [18], *Neisseria meningitides***,** *Haemophilus influenza* and *Streptococcus*

pneumonia [19], we hope the assay will enhance the current serology technique. In addition, a large field of application for the assay is monitoring of *Leishmania* infection, life cycle development, diagnosis, treatment with follow up and vaccine development in research experiments. Our studies indicate that LC-PCR improves the reliability of positive diagnosis and yields quantitative results for assessment of treatment efficacy. In an experimental mouse model previously used to test the efficacy of amphotercin B, lipid formulations of amphotercin B and aminosidine [20], the efficacies of metronidazole and antifugal agents against *L. infantum* (VL) were compared. Metronidazole, ketoconazole, fluconazole, itraconazole and terbinafine were less effective than antimonial agents in reducing hepatic parasite load. Ketoconazole potentiated the effect of antimoniate reference therapy through its marked activity against spleen infection.

CONCLUSION

The pentavalent derivatives of antimony are highly effective, since parasite burden were at an undetectable level in the liver. However, parasite foci persisted in the spleen, and this probably explains the occurrence of relapse. Despite being less effective than meglumine antimoniate in monotherapy, ketoconazole and metronidazole were the most effective second line treatments in this study. Furthermore, their combination with meglumine antimoniate resulted in marked decreases in parasite loads in both liver and spleen. However, the relapses that were observed in susceptible BALB/c mice treated with these combinations indicate the inefficacy of the host immune functions in clearing parasites when anti-leishmanial drug concentration decreased and/or the drug did not reach the parasitophorous vacuole. With regard to the use of marked drug carrier systems with meglumine antimoniate, ketoconazole, or metronidazole in order to reach sustained drug levels and to target the drugs toward infected tissues, the results obtained emphasize the need for multidrug therapy as well as maintenance therapy in the treatment of *L. infantum visceral leishmaniasis* with these drugs.

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