Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Research article

Identification of *Leishmania donovani* in blood of experimentally infected rats by quantitative real-time PCR

Noor Idan Jarad Ghaidaa Abbas Jasim

Department of Microbiology and Parasitology, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq
Corresponding Author Email: Ghaidaa.Abass@qu.edu.iq

(Received 17/8/2017, Accepted 23/12/2017)

Abstract

The present study has been carried out at the Department of Microbiology and Parasitology, College of Veterinary Medicine/Al-Qadisiyah University to diagnose the visceral leishmaniasis molecular technique (Quantitative real time polymerase chain reaction). Experimental study include fourth Wistar female rats (weighted 250 ± 2 g.) were injected by blood from infected patient in the peritoneal cavity, after 8-10 days of experimental infection , blood samples had been collected directly from the heart in order to diagnose the infection by using quantitative real time polymerase chain reaction. Quantitative Real Time PCR qPCR technique was used for amplification of conserved region in GAPDH gene that was used for detection of Leishmania donovani in blood samples of rats. It show the Amplification of genomic DNA template concentrations of Leishmania donovani during reaction with syber dye inside the apparatus under threshold cycle, also shows the melt peak of Leishmania donovani genomic DNA template concentrations is demonstrate the specialization of Leishmania donovani genomic DNA amplification in single peak for all samples.

Keywords: Leishmania donovani, Rats, Experimental infection, Diagnosis, RT-PCR.

Introduction

Leishmania donovani is protozoan parasite belongs to Eukaryota, was discovered in parallel by Sir William Leishman in 1900 and by Charles Donovan in 1903 (1).The life cycle of Leishmania species are complex (2). These parasites remain in nature by transmission between mammalian hosts via the bite of infected female sand fly. Human becomes infected if he intrudes into this cycle (3). parasite Leishmania exist in two morphological stage, the promastigote (long flagellated form exist in the insect vector or in vitro culture at 25 C°) and amastigote (round, intracellular parasite, without free flagellum exist in mammalian host cell (4).Some studies found that the molecular basis for amastigote survival in the mammalian host, previously identified an amastigote stage-specific gene family termed ‘‘A2’’ whose corresponding mRNA and protein are abundant in amastigotes but are largely absent in promastigotes (5). Several lines of evidence have indicated that the A2 gene/protein family could be one of the most eligible factors of virulence in VL infections (6).There are at least seven members of the A2 gene family that encode a family of proteins ranging from 45 to 100 kDa and that are specific to the amastigote stage (7). A2 proteins are mainly comprised of a repetitive amino acid sequence; each repeat encodes a stretch of 10 amino acids (8). Diagnosis of Leishmaniasis is performed by direct visualization of amastigotes using microscopic examination of stained material

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

(9). In vitro culture techniques, more sensitive, they are susceptible to microbiological contamination, and are hampered by the particular growth requirements of different strains (10). Recently PCR based methods (Polymerase chain reaction) and DNA probes are highly sensitive and specific, compared with standard methods and are considered valuable for diagnosis (11). PCR, particularly quantitative real-time PCR, more sensitive and high specificity (12).

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 438

Experimental animals

Old female Wister rats (average weight was 250 ± 2g), born at the animal house of the College of Veterinary Medicine, Al-Qadisiyah University. The rats were reared under controlled ambient temperature (20-25C°) and fed on standard laboratory diet (19% protein ratio and 3000 kilocalories energy) and drinking water.

Collection of the Samples from Children

40 blood samples were collected from children who suffered from clinical symptoms of visceral leishmaniasis from the Educational Maternity and Pediatric Hospital in Al-Diwaniyah city within the period 2/10/2012 -22/ 3/2013. Blood samples (1ml) was collected from venous puncture by using sterile plastic syringe (2ml) after clinically diagnosed by a pediatrician, after that the blood was placed in anticoagulant test tubes for direct inoculation to the laboratory rats.

Laboratory Animals Inoculation

The blood have been taken from the patient and kept in a sterile test tube containing anti-clotting material (EDTA). 1ml of blood was injected in the peritoneal cavity of 40 rats .The injection was done without anesthetizing the rats; by raising the animal with the left hand from the back and lifting the head for the top, and then injected the blood into the peritoneal cavity by sterile syringe (1 ml). Each rat had been injected with different sample by the same way, and then each animal had been identified by special number for excellence (13).

Collection blood samples from infected rats

After 8-10 days from injection the laboratory rat by blood from infected children, the rats had been anesthetized using 0.2 Ketamine and 0.1 xylazine injected in the peritoneal cavity by using a sterile syringe (1ml) (14). Blood samples (1ml) had been collected directly from the heart of all rats by using a sterile syringe (3ml) and stored with anticoagulant test tube for DNA extraction to be diagnosed the experimental infection by Quantitative Real-Time PCR

Primers

Two primers were used in this study, first primer used for GAPDH gene as Housekeeping gene and second primer used for A2gene as target gene. These primers were designed by using NCBI- Gene Bank database and Primer 3 design online, the primers used in quantification of gene expression using qRT-PCR techniques based SYBER Green DNA binding dye, and supported from (Bioneer, Korea) company.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Table (1): The Primers and their sequences, GenBank accession number, and references

Primer	Sequences		Accession number
GGAPDH	F	CTCACAGTGCGTCTGGAGAA	AB608734.1
	R	TCGGTGTAGCCGAGAATACC	
A2 gene	F	CGCTGATGTGCTGACTTGTT	S69693.1
	R	CGGGGGCACTGAGAATAATA	

Diagnostic Real-Time PCR (qPCR)

qPCR technique was used for amplification of conserved region in GAPDH gene that used for detection of *Leishmania donovani* in blood samples of rats. This technique was done according to method described by (15). Genomic DNA was extracted from blood samples of rats by using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) and done according to the protocol described by the manufacturer instructions.

Real-Time PCR Master Mix Preparation

qPCR master mix was prepared by using (AccuPower® 2×Greenstar qPCR kit, Bioneer. Korea), and done according to kit instructions as following table:

Table(2): qPCR PreMix used in this study

qPCR PreMix		**Volume**
2×Greenstar Master Mix		25 µL
DNA template		10 µL
Primers	GAPDH-F	1.5µL
	GAPDH-R	1.5µL
DEPC water		12µL
Total		50µL

After that, qPCR master Mix were added into AccuPower 2× GreenStar qPCR PreMix tube. Then, real-time PCR tubes sealed by the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes centrifuged at 3,000 rpm, for 2 minutes, and then the tubes placed in Miniopticon Real-Time Thermocycler (Bio Rad, USA) and the following program was loaded according to kit instruction:

Table (3): Thermocycler condition for RT-PCR

Step	Temp.	Time	Cycle
Pre-Denaturation	95 °C	3 min	1
Denaturation	95 °C	10 sec	45
Annealing **Extension**	55 °C	30sec	
Detection(Scan)	72 °C	30sec	
Melting	65-95 °C	5 sec	1

Results

Quantitative Real Time PCR

The results that presented in figure (1) show the Amplification of genomic DNA template concentrations of *Leishmania donovani* during reaction with syber dye inside the apparatus under threshold cycle, figure (2) shows separation the links of genomic DNA template of *Leishmania donovani* in the Real-Time PCR melt curve during changing the temperature, figure (3) shows the melt peak of *Leishmania donovani* genomic DNA template concentrations which is demonstrate the specialization of *Leishmania donovani* genomic DNA amplification in single peak for all samples.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

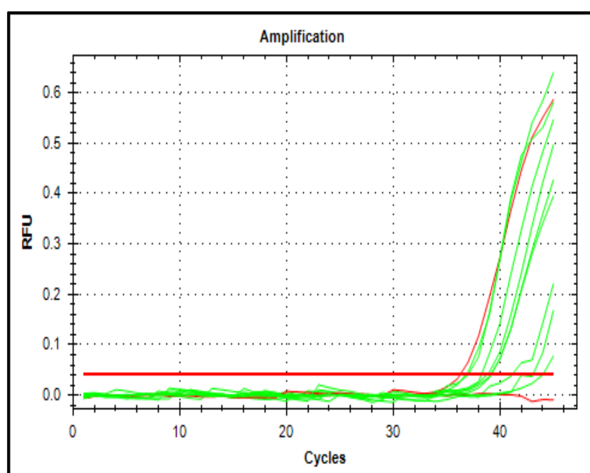

Figure (1): The Real -Time PCR Amplification plot of L. *donovani* genomic DNA template concentrations.

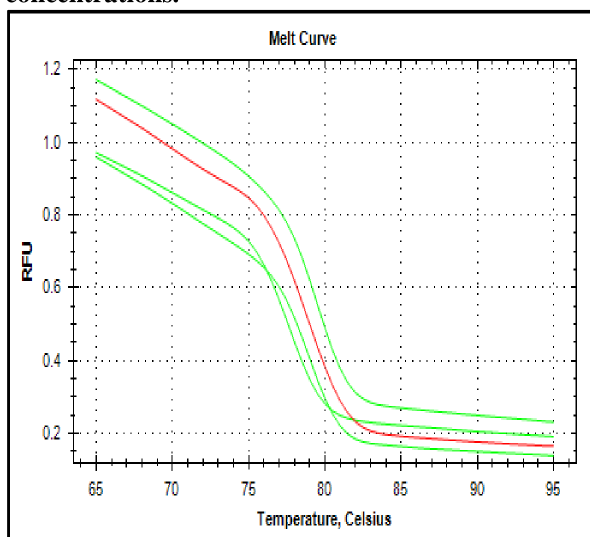

Figure (2): The Real -Time PCR melt curve of L. *donovani* genomic DNA template concentration

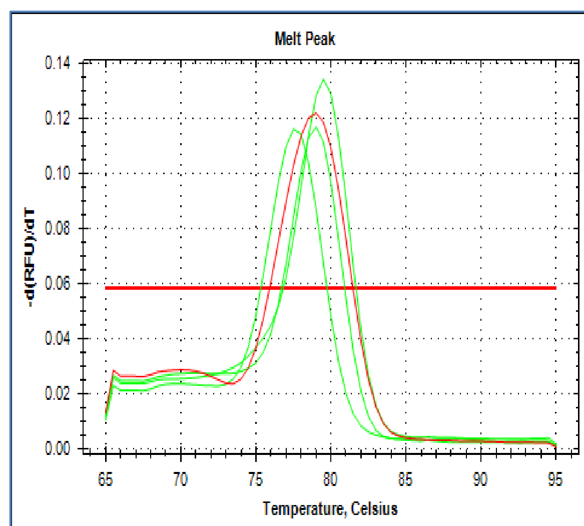

Figure (3): The Real -Time PCR Melt peak of L. *donovani* genomic DNA template concentrations.

Discussion

The use of molecular methods for diagnosis is warranted, among the methods available, real time PCR is particularly advantageous; it give its effectiveness for samples containing a small number of parasites and for cases in which differential diagnosis can be crucial in view of the wide range of clinical manifestation (16). The present study showed positive results for all the experimental infected rats .Similar results were documented by (17).Peripheral blood samples from 15 dogs (18). That were positive for VL, qPCR sensitivity was 100% when compared the conventional PCR with qPCR in bone marrow of the dogs, and they found 54 and 84% of positive results respectively for conventional PCR and qPCR (19). Also compared the conventional PCR with qPCR, using spleen and liver samples from *L. infantum*-infected mice, they found that qPCR was more sensitive than conventional (20). Described such a method, with a sensitivity of 0.625 parasites/ml (21). Developed a quantitative assay that was able to discriminate different *Leishmania* species, but the accuracy was weak when parasitemia was under 100 parasites/ml, as a consequence, such a test cannot be used for

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

follow-up testing of treated patients, while the qPCR allows the possibility of quantification at 1 parasite/ml (22). The qPCR is more sensitive than conventional PCR in diagnosis of *Leishmania* species; they found that the Real-Time PCR was sufficiently sensitive to detect as little as 0.001 parasite in blood samples (23). Also developed a real- time PCR for monitoring experimental *Leishmania* infections in mice, but sensitivity was too low for application to human disease(24).The present study revealed that the qPCR assay had sensitivity 100% for blood samples of all experimental infected rats.

Conclusion

In this study showed the experimental infection for all 40 Wister rats was give when injected the blood from infected children into the peritoneal cavity result. Also Quantitative Real time polymerase chain reaction was very accurate and fast in the diagnosis of experimental infection and the evaluation of A2 gene expression in samples of infected rats.

References

1-Schmidt GD, Roberts LS. Foundations of Parasitology. Third edition, Times Mirror/Mosby college publishing, USA. (1985).

2-Guerin PJ, Lliaro PO, Sundar S, Boelaert M, Croft SL, Desjeux P, Wasanna M, Bryceason ADM. Visceral Leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. The Lancet infectious disease. (2002); 2: 494-501.

3-Berman J. Recent Developments in *Leishmaniasis*: Epidemiology, Diagnosis, and Treatment,Current infectious Disease Reports, (2005);7:33-38.

4-World Health Organization, (1990). Control of Leishmaniasis. WHO Techn. Rep, Ser.793.

5-Krobitsch S, Brandau S, Hoyer C, Schmetz C, Hübel A, Clos J. *Leishmania donovani* heat shock protein 100. Characterization and function in amastigote stage differentiation.J. Biol.Chem. (1998); 273:6488-6494.

6-Zhang WW, Mendez S, Ghosh A, Myler P, Ivens A, Clos J. Comparison of the A2 gene locus in *Leishmania donovani* and *Leishmania major* and its control over cutaneous infection. J. Biol .Chem. (2003); 78: 35508–35515.

7-Zhang WW, Charest H, Ghedin E, Matlashewski G. Identification and overexpression of the A2 amastigote-specific protein in *Leishmania donovani*. Mol. Biochem. Parasitol. (1996); 78: 79-90.

8-Charest H, Matlashewski G. Developmental Gene Expression in *Leishmania donovani*: Differential Cloning and Analysis of an Amastigote-Stage-Specific Gene .Mol. Cell. Biol. (1994); 14, 2975–2984.

9-Culha G, Uzun S, Ozcan K, Memisoglu HR, Chang KP. Comparison of conventional and polymerase chain reaction diagnostic techniques for leishmaniasis in the endemic region of Adana, Turkey. Int. J. Dermatol. (2006); 45:569-572.

10-Chargui N, Bastien P, Kallel K, Haouas N, Akrout FM, Masmoud A. Usefulness of PCR in the diagnosis of cutaneous leishmaniasis in Tunisia. Trans. R .Soc. Trop. Med .Hyg. (2005); 99:762-768.

11-Fraga TL, Brustoloni YM, Lima RB, Dorval MEC, Oshiro ET, Oliveira J.Polymerase chain reaction of peripheral blood as a tool for the diagnosis of visceral leishmaniasis in children. Mem. Inst. Oswaldo. Cruz. (2010); 105(3): 310-313.

12-Quaresma PF, Murta SM, Ferreira Ede C, Rocha-Lima ACD, Xavier AA, Gontijo CM. Molecular diagnosis of canine visceral leishmaniasis: identification of *Leishmania* species by PCR-RFLP and quantification of parasite DNA by real-time PCR. Acta. Trop. (2009); 111(3): 289-294.

13-Jassim AMH (1998). Adirect agglutination test for the diagnosis and seroepidemiological survey of visceral leishmania in basrah Governorate, Iraq. Ph. D. thesis. Collage of medicine,University of AL-Mmustansiriya. Iraq.

14-Sharpe PE, La Regina MC.The Laboratory Rat .CRC. Press. Landon, New York. (1998); PP: 115.

15-Schulz AK, Mellenthin G, Scho¨nian B, Drosten C. Detection, differentiation, and quantitation of pathogenic Leishmania organisms by a fluorescence resonance energy transfer-based real-time PCR assay. J. Clin. Microbiol. (2003); 41, 1529–1535.

16-Mary C, Faraut F, Lascombe L, Dumon H. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. J. Clin. Microbiol. (2004); 42(11): 5249-5255.

17-Wortmann GW, Romero LI, Paz HM, Ortega-Barria E, Bayard V, Hochberg LP. Real-time polymerase chain reaction diagnosis of leishmaniasis in Panama from both fresh and frozen tissue. Trans. R .Soc. Trop. Med. Hyg. (2004);98(3): 148–51.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

18-Fichoux YL, Quaranta JF, Aufeuvre JP, Lelievre A, Marty P, Suffia I, Rousseau D. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. J. Clin. Microbiol. (1999); 37(6):1953-7.

19-Francino O, Altet L, Sanchez-Robert E, Rodriguez A, Solano-Gallego L. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Vet. Parasitol. (2006); 137: 214–221.

20-Rolão N, Cortes S, Rodrigues OR, Campino L. Quantification of Leishmania *infantum* parasites in tissue biopsies by real-time polymerase chain reaction and polymerase chain reaction-enzyme-linked immunosorbent assay. J. Parasitol. (2004); 90(5): 1150-1154.

21-Bossolasco S, Gaiera G, Olchini D, Gulletta M, Martello L, Bestetti L, Germagnoli A, Lazzarin A, Uberti-Foppa C, Cinque P. Real-time PCR assay for clinical management of human immune deficiency virus-infected patients with visceral leishmaniasis. J. Clin. Microbiol. (2003); 41: 5080-5084.

22-Zhang WW, Mendez S, Ghosh A, Myler P, Ivens A, Clos J. Comparison of the A2 gene locus in *Leishmania donovani* and *Leishmania major* and its control over cutaneous infection. J. Biol .Chem . (2003); 278: 35508-35515.

23-Mary C, Faraut F, Lascombe L, Dumon H. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. J. Clin. Microbiol. (2004); 42(11): 5249-5255.

24-Nicolas L, Prina E, Lang T, Milon G. Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. J. Clin. Microbiol. (2002); 40:1666–1669.