Diagnosis of Visceral leishmaniasis by conventional PCR to DNA extracted from Giemsa's solution-stained slides.

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ABSTRCT

Visceral leishmaniasis (VL), or kala-azar, is an infection of the reticuloendothelial system, usually with *L. donovani* and *L. infantum*, both old world species of the parasite; or *L. chagasi*, a New World species. Fatalities due to leishmaniasis are associated with visceral disease. The aim of this study was to evaluate the sensitivity and specificity of polymerase chain reaction (PCR) in the detection of *Leishmania* DNA in archived Giemsa-stained bone marrow slides for diagnosis of visceral leishmaniasis (VL), and to compare PCR with conventional diagnostic techniques, such as direct microscopy. Furthermore PCR diagnosis DNA extracted from Giemsas solution-stained bone marrow slides is a suitable tool to confirm diagnosis in patients with VL and is useful in the diagnosis of difficult cases in addition to the easily of bone marrow smears to stored and sent to research center where PCR diagnosis can be already achieved. Twenty archived Giemsa-stained bone marrow slides were used in the present study,10 were negative and 10 positive by direct microscopy test while 8 were negative and 12 were positive by PCR test. PCR showed the highest

sensitivity and specificity (100%), while the direct examination gave (85.7% sensitivity) and (100% specificity), in addition PCR was able to detect VL in 10% of blood samples which were negative by microscopy.

Key words: visceral leishmaniasis, PCR diagnosis, DNA extracted, giemsas solution-stained bone marrow slide

Introduction

The leishmaniases are parasitic diseases which are endemic in many countries in the tropics and subtropics. Approximately 350 million people are considered to be at risk of contracting the disease. Visceral leishmaniasis (VL), also known as kala azar, accounts for an estimated 75,000 deaths annually (Wijeyaratne *et al.,* 1994) Conventional methods for diagnosis of visceral leishmaniasis . VL, such as parasitological or serological tests, still have limitations in their use (DaSilva *et al.* 2004).The sensitivity of microscopical examination tends to be low and can be highly variable (Reithinger and Dujardin, 2007) depending on the number and dispersion of parasites in biopsy samples, the sampling procedure, and most of all the technical skills of the personnel. The serological testis unable to differentiate between recent and old infection (Schalling *et al*., 2002).So, improved tools for the diagnosis of VL are needed . Molecular methods including Polymerase Chain Reaction (PCR)-based techniques have proven to be highly sensitive and specific and may be used with the a variety of clinically samples(Reithinger and Dujardin, 2007). Giemsa's solution- stained bone marrow smear on glass slides are potentially suitable sources of DNA for molecular diagnosis. PCR diagnosis methods using DNA extracted from Giemsa's solution stained bone marrow

slides is useful in the diagnosis of difficult cases in patients with VL cases in addition to the easily of bone marrow smears to stored and sent to research center where PCR diagnosis can be already achieved. The aim of this study was to evaluate the use of PCR in VL diagnosis using archived giemsa stained Bone marrow aspirate (BMA) slides, evaluating its sensitivity and specificity, and to compare between molecular diagnosis and direct microscopy.

Materials and Methods

20 slides of bone marrow smear to suspected visceral leishmaniasis patient were included in the study. A Bone marrow sample were obtained only from patient group about 0.5ml of marrow is drawn by aspiration of iliac crest by professional pathologist. A few drops are smeared directly on slides. The smears are stained and then examined under the micro-scope by the assistant of hemat-ologist.Culture for parasite *Leish-maniadonovani* was obtained from *leishmania* center follow to AL-Nahren University (Moham /IQ/2005/MRCIO)was used as positive control. Detached cell from the flask and transfer the appropriate number of cells to 1.5ml micro-centrifuge tube and centrifuge for 5min at 3000r.p.m removed the supernatant completely and resus-pend cell pellet in PBS to a final volume of 200 µl.The extraction of DNA was done by DNeasy Qiagen kit and the same protocol of extraction from blood and body fluid explain in method of DNA extraction according to (Qiagen) mini Kit Protocol (DNA Purifica-tion from Blood or Body Fluids (Spin Protocol) :.All the samples were examined duplicates for each test.

The DNA target for PCR amplification was the gene coding for 18s rRNA(Lachaud*et al.*, 2001)

which is specific for *Leishmania Spp*.

The primers used were (5-GGT-TCC-TTT-CCT-GAT-TTA-CG3)(R221) as forward and (5-GGC-CGG-TAA-AGG-CCG-AAT-AG-3)(R332) as reverse, which produce a 603-bp fragment upon amplification , thermal cycler using the following conditions: 94°C for 4 min and 40 cycles of 94°C for30 s, 54°C for 30 s, and 72°C for 90 followed by 72°C for 10 min,(Laurence *et al*., 2002).DNAExtraction from bone marrow on hematologica slides were done according to Qiagen (2010).

The PCR products from amplification of each primer by thermo cycler were then electrophoresis on an ethidium bromide-stained 2%agaros gel.100v, 20A for 30second at least. The presence of specific bands was indicative of the infections (positive).The bands victualed under UV light and Gel documentation.

The chi-square ($\chi 2$) and t tests were used as tests of significance. The differences were recorded as significant whenever probability (p) was less than 0.01by using SPSS analysis programs version 17.

Results and Discussion

Twenty bone marrow slides belong to 20patients were used. Ten of them were positive and ten were negative by microscopical examination(Table.1)

Table1: Microscopical test to bone marrow slides smear

B.M test	**NO**	**%**
Positive	10	50
Negative	10	50
Total	20	100

$\chi 2$= 0.00 ,df= 1, p <0.01t=4.359 ,df= 19, p <0.01

The result of these slides by PCR test (Table 2Fig .2) were 12 positive (60%) and 8 negative (40%).

Table 2. Result of PCR test in B.M .of Giemsa stained slides

PCR test of B.M slides	**NO**	**%**
Positive	12	60
Negative	8	40
Total	20	100

$\chi 2$= 0.800 ,df= 1, p <0.01

t=5.339 , df= 19, p <0.01

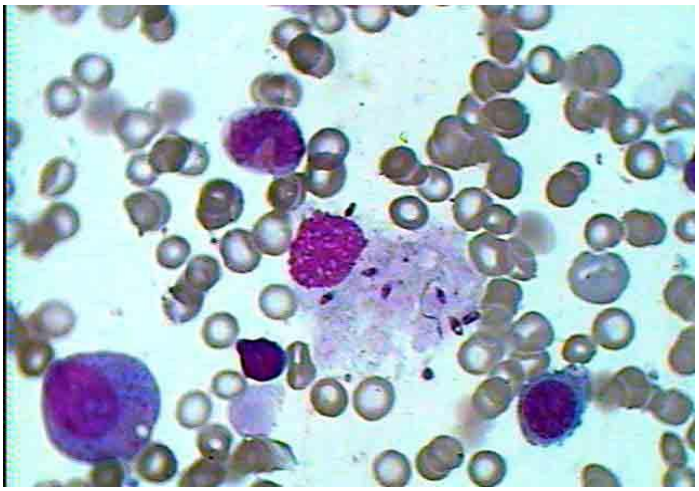
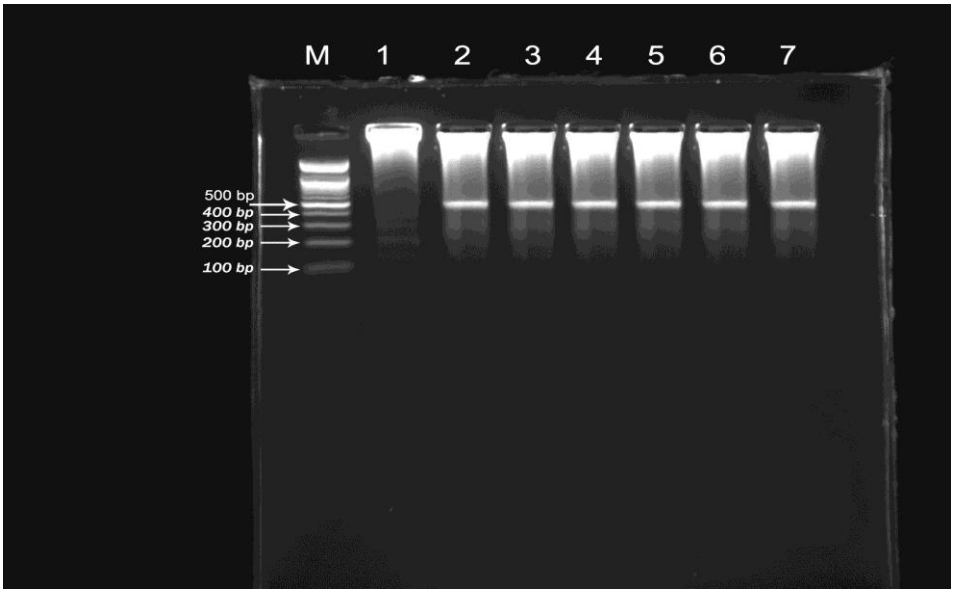
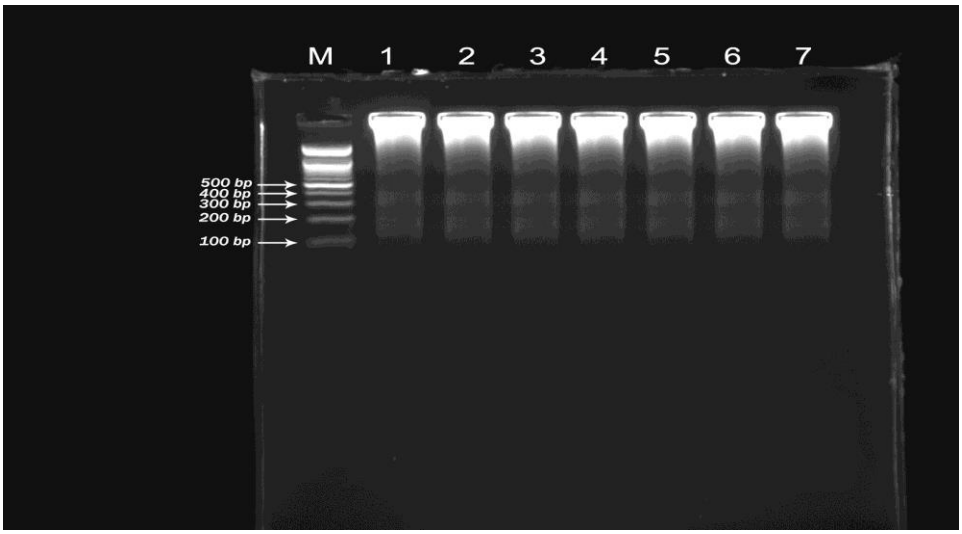


Fig.(1):positive bone marrow smears show the *L.infantum*bodies (amastigote) inside the macrophage by 100X oil emersion objective.



A



B.

Fig.(2): A:agarose gel electrophoresis (2%) shows 18S rRNA gene amplification. Lane M= Marker 100 bp. Lane1= negative control Lane2-7 = positive Patients sample.**B:**agarose gel electrophoresis (2%) shows negative result of microscopically Negative bone marrow slide smear samples

Measurements of Sensitivity and Specificity of PCR Test in B.M .of Giemsa stained slides were as fellow:

Sensitivity =TP/ (TP+FN) X 100%

= 12/ (12+0) x100%=100%

Specificity = TN/ (TN+FP) X100%

= 8/ (8+0) x100% = 100%

And Measurements of Sensitivity and Specificity in B.M .of Giemsa stained slides according to PCR test were:

Sensitivity =TP/ (TP+FN) X 100%

= 12/ (12+2) x100 =85.7%

Specificity = TN/ (TN+FP) X100%

= 8/ (8+0) x100 = 100%

One of the difficulties in defining the sensitivity and specificity of any test is the need for a gold standard that can correlate with the direct detection of the parasite (Dye *et al*., 1993). The aims of this study were to compare the result of parasitological microscopic examination with the resultsof molecular examination test.

Parasitological diagnosis remains the gold standard in leishmaniasis diagnosis because of its high specificity (Herwaldt, 1999). This is typically undertaken by microscopical examination of Giemsa-stained lesion biopsy smears of lymph node, bone marrow, and spleen aspirates .There are more disadvantages of this method, The sensitivity of microscopical examination tends to be low and can be highly variable (Reithinger and Dujardin, 2007) depending on the

number and dispersion of parasites in biopsy samples, the sampling procedure, and most of all the technical skills of the personnel. At present, definitive diagnosis of VL relies mainly on demonstration of parasites in bone marrow or splenic aspirates, or serological tests. However, the scarcity of parasites in bone marrow aspirates from many patients complicates parasitological confirmation and the sensitivity with microscopy is very low (Al-Jawabreh*et al*., 2006). As a result, bone marrow aspirate is the most commonly utilized material for the diagnosis of pediatrics VL (Kafetzis and Maltezou,2002).

In this study (20) Bone marrow slides were used for microscopic observation which was 10 positive and 10 negative of VL infection, DNA extraction and subsequent PCR amplification. PCR analysis showed that all positive samples of VL parasites and 2 of negative samples were positive of VL .The PCR assay also showed a higher sensitivity (100%) than microscopic examination (85.7%) and there was no statistically different significant between the two methods, this indicates near in efficiency between two test . In addition, PCR was able to detect VL in (10%) of samples which were negative by microscopy.The PCR assay for Giemsa stained slides which showed by Pandey*et al.*, (2010) with a higher sensitivity (69%) than microscopic examination (57%) and culture (21%). Among the 91 children slides with a diagnosis of VL, PCR was positive in 84, yielding a sensitivity of 92.3% PCR specificity was 97.5% (Brustoloni*et al*.,2007).

In conclution the sensitivity of molecular techniqueis high than direct bone marrow examination. Molecular biology based assays for detecting parasite DNA have

been high specificity and sensitivity it is developed but need more works

References

Al-Jawabreh, A.; Schoenian, G.; Hamarsheh, O. and Presber, W. (2006). Clinical diagnosis of cutaneous leishmaniasis: a comparison study between standardized graded direct microscopy and ITS1-PCR of Giemsastained smears. Acta. Trop., 99: 55-61.

Brustoloni ,Y.; Lima, R.; da Cunha, R.; Dorval, M.E.; Oshiro, E.T.; de Oliveira, A. and Pirmez, C. (2007). Sensitivity and specificity of polymerase chain reaction in slides for diagnosis of visceral leishmaniasis in children MemInstOswaldo Cruz, Rio de Janeiro, 102(4): 497-500

da Silva, E.S.; Gontijo, C.M.; Pacheco Rda, S. and Brazil, R.P. (2004). Diagnosis of human visceral leishmaniasis by PCR using blood samples spotted on filter paper. Genet. Mol. Res., 3(2): 251-257.

Dye, C.; Vidor, E. and Dereure, J. (1993). Serological diagnosis of leishmaniasis: on detecting infection as well as disease. Epidemiol.Infect., 110: 647-656.

Herwaldt, B.L. **(**1999). Leishmaniasis. Lancet, 354: 1191–1199.

Kafetzis, D.A. and Maltezou, H.C. (2002).Visceral leishmaniasis in pediatrics.Curr.Opin. Infect. Dis., 15: 289-294.

Lachaud, L.; Chabbert, E.; Dubessay, p.; Reynes, J.;Lamothe, L. and Bastein, P. (2001).Comparison of various sample preparation methods of PCR diagnosis of visceral leishmaniasis using peripheral blood.J. Clin. Microbiol., 38: 613-617.

Laurence, L.; Marchergui, H.S.; Chabbert, E.1.; Jacques, D.; Dedet,J.P .and Bastien, P. (2002).Comparison of Six PCR Methods Using Peripheral Blood for Detection of Canine Visceral Leishmaniasis. J. Clin. Microbiol., 40: 210-215.

Pandey, K.; Pandey, B.D.; Mallik, A.K.; Kaneko, O.; Uemura, H.; Kanbara, H.; Yanagi, T. andHirayama, K. (2010). Diagnosis of visceral leishmaniasis by polymerase chain reaction of DNA extracted from Giemsa's solution stained slides.Parasitol. Res., 107(3): 727-730.

Qiagen (2010). QIAamp DNA Mini and Blood Handbook, sample and Assay Technologies. Third Edition 04/2010.

Reithinger, R. and Dujardin, J.G. (2007). Molecular Diagnosis of Leishmaniasis: Current Status and Future Applications. J. Clin. Microbiol., 25(1): 21-25.

Schalling, H.; Canto-Cavalherio, M. and Silva, E.S.D. (2002).Evalution of the direct Agglut-ination Test and the rK39 Dipstick test for the seroDiagnosis of visceral leishmaniasis.Memorias do institutoOswalado Cruz, 97(7): 1015-1018

Wijeyaratne, P. M., L. K. Jones Arseault, and C. J. Murphy.(1994). Endemicdiseases and development: The leishmaniases. Acta Trop. 56:349–364.

تشخيص اللشمانيا الأحشائية باستخدام تقنية التفاعل السلسلي لانزيم البلمرةالتقليدي للحامض النووي المستخلص من عينات نخاع العظم المصبغة والمثبتة على الشرائح الزجاجية

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الخلاصة

اللشمانيا الاحشائية Kala azar هي إصابة الجهاز البطاني الشبكي باللشمانيا الدونوفانية أو لشمانيا الأطفال وكلاهما يعود لانواع العالم القديم تصنيفيا او اللشمانيا الجاكازية وهي تتبع العالم الحديث في التصنيف . خطورة داء اللشمانيا مرتبطة بالإمراض الاحشائية الناتجة عن الإصابة بالطفيلي ولذا كان الهدف من هذه الدراسة هو تقدير حساسية ونوعية اختبار التفاعل السلسلي لأنزيم البلمرة في الكشف عن الطفيلي في العينات الأرشيفية للشرائح الزجاجية المصبغة لنخاع العظم للمصابين والمشخصة إصابتهم سريريا ومقارنة نتائج هذا الفحص PCR مع الفحص التقليدي المباشر للعينات كالفحص ألمجهري . إضافة لذلك التشخيص بال PCR التقليدي لعينات نخاع العظم المثبتة على الشرائح الزجاجية تعتبر طريقة مناسبة لتأكيد الإصابة بهذا المرض الخطير للحالات الصعبة التشخيص بالإضافة لسهولة خزن عينات نخاع العظم المثبتة على الشرائح الزجاجية وشحنها إلى المراكز البحثية حيث سهولة العمل بهذه التقنية.

استخدمت 20 عينة نخاع عظم مثبتة على الشرائح الزجاجية ، عشرة منها سالبة وعشرة موجبة بالفحص ألمجهري المباشر بينما ثمانية سالبة واثني عشر موجبة بتقنية ال PCR. بينت هذه التقنية حساسية ونوعية عالية تصل إلى 100% في حين ألاختبار ألمجهري المباشر أعطى نسبة حساسية85.5% ونوعية 100% إضافةإلى ذلك تقنية أل PCR لها القدرة في الكشف عن 10% من العينات السالبة بالفحص ألمجهري.