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

Maha K. AL-Mishry; Sadiq K.Ali**; Maane N.Al-Shimary* and Nadhim K.Mahdi**

College of Science-Biology Department-Basrah University

*College of Medicine-Al-Qadisiya University, ** College of Medicine – Basrah university

mahaalmishry@yahoo.com

ABSTRACT

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 tests is 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 microscope by the assistant of hematologist. Culture for parasite *Leishmania donovani* was obtained from *Leishmania* center follow to AL-Nahre 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 Purification 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 for 30 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 30 second 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 (χ²) and t tests were used as tests of significance. The differences were recorded as significant whenever probability (p) was less than 0.01 by using SPSS analysis programs version 17.

**Results and Discussion**

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

<table>
<thead>
<tr>
<th>B.M test</th>
<th>NO</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 0.00 \text{, } df = 1, \text{ } p < 0.01 \]

\[ t = 4.359 \text{, } df = 19, \text{ } p < 0.01 \]

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

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

<table>
<thead>
<tr>
<th>PCR test of B.M slides</th>
<th>NO</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 0.800 \text{, } df = 1, \text{ } p < 0.01 \]

\[ t = 5.339 \text{, } df = 19, \text{ } p < 0.01 \]

Fig. (1): positive bone marrow smears show the *L. infantum* bodies (amastigote) inside the macrophage by 100X oil emersion objective.
Fig. (2): A: agarose gel electrophoresis (2%) shows 18S rRNA gene amplification. Lane M = Marker 100 bp. Lane 1 = negative control Lane 2-7 = 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 = \frac{TP}{(TP+FN)} \times 100\% \\
= \frac{12}{(12+0)} \times 100\% = 100\% \\

Specificity = \frac{TN}{(TN+FP)} \times 100\% \\
= \frac{8}{(8+0)} \times 100\% = 100\%

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

Sensitivity = \frac{TP}{(TP+FN)} \times 100\% \\
= \frac{12}{(12+2)} \times 100\% = 85.7\% \\

Specificity = \frac{TN}{(TN+FP)} \times 100\% \\
= \frac{8}{(8+0)} \times 100\% = 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 result of 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-Jawabrehet 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% (Brust-oloniet al., 2007).

In conclusion the sensitivity of molecular techniques is 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


تشخيص اللشمانيا الاشحائية باستخدام تقنية التفاعل السلسلي لإنزيم البلمرة التقليدي للحامل النووي المستخلص من عينات نخاع العظام المصبגה والمثبتة على الشرائح الزجاجية

مها خلف المشري ، صادق خلف علي* ، معاني ناجي الشمري*، وناطم كاظم مهدي**
قسم علوم الحياة-كلية العلوم-جامعة البصرة. كلية الطب-جامعة القادسية. كلية الطب-جامعة البصرة

mahaalmishry@yahoo.com

الخلاصة

اللشمانيا الاشحائية Kala azar هي إصابة الجهاز البطاني الشبكي باللشمانيا الدونوفانية أو لشمانيا الأطفال وكلاهما يعود لتنوع العالم القديم تصنيفيا أو اللشمانيا الجاكازية وهي تتتبع العالم الحديث في التصنيف. خطورة داء اللشمانيا مرتبطة بالإمراض الإحشائية 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 الاشحائية للشرائح الزجاجية المصبجة لنخاع العظم للمصابين المشخصة إصابتهم سريريا ومقارنة نتائج هذا الفحص PCR مع الفحص التقليدي المباشر للعينات التقيتي لعينات نخاع العظم المتثبتة على PCR كالفحص المجهري. إضافة لذلك التشخيص بالشرائح الزجاجية تعتبر طريقة مناسبة لتأكيد الإصابة بهذا المرض الخطير للحالات الصعبة التشخيص بالإضافة لسهولة خزن عينات نخاع العظم المتثبتة 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 المجهري المباشر بينما ثماني سالبة واثني عشر موجبة بتقنية PCR. بيئة هذه التقنية حساسية ونوعية عالية تصل إلى 100% في حين الاختبار المجهري المباشر أعطى نسبة حساسية 85.5% ونوعية 100% إضافة إلى ذلك تقنية PCR لها القدرة في الكشف عن 10% من العينات السالبة بالفحص المجهري.