Diagnosis of visceral leishmaniasis by polymerase chain reaction of DNA extracted from Giemsa's solution-stained slides.

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Abstract

Visceral leishmaniasis (VL) is caused by the protozoan parasite *Leishmania donovani* and is a potentially fatal disease in endemic areas of the world. Nepal is an endemic area in which VL causes major public health problems in the lowland areas of the southeast regions. The aim of the present study was to evaluate the sensitivity of polymerase chain reaction (PCR) amplification for the detection of *Leishmania* DNA from Giemsa's solution-stained bone marrow slides. Bone-marrow samples were aspirated from a total of 115 VL suspected patients and used to prepare smears on glass slides and for the initiation of *in vitro* culture. Bone-marrow slides were used for microscopic observation and for DNA extraction, and subsequent PCR amplification. PCR analysis showed that all the positive samples were of *Leishmania* parasites. The PCR assay also showed a higher sensitivity (69%) than microscopic examination (57%) and culture (21%). In addition, PCR was able to detect VL in 12% of samples which were negative by microscopy. PCR of DNA extracted from Giemsa's solution-stained bone-marrow slides is a suitable tool for confirming diagnosis in patients with VL and may also be useful in the diagnosis of difficult cases. Bone-marrow smears are easily stored, and can be easily sent to research centers where PCR is available. This makes PCR is good option for diagnosis in the field.

Introduction

Visceral Leishmaniasis (VL) is a parasitic disease caused by the protozoan parasite *Leishmania donovani*, and is transmitted by sand fly vectors. VL has been reported from 51 countries around the world and has an annual incidence of 500,000 cases. VL is responsible for about 59,000 deaths per year and 2.4 million disability-adjusted life years are lost worldwide. India, Nepal and Bangladesh account for 300,000 cases annually, and thus account for 60% of the total global burden of this disease. In Nepal, VL causes major public health problems as well as mortality in the lowland areas of the southeast regions which is border Bihar Indian state, a known VL endemic area. Early treatment is a major pillar of the current VL elimination programme, which has been launched by the governments of India, {Aransay, 2000} Nepal and Bangladesh {Sundar, 2008}.{Al-Jawabreh, 2003}

The gold standard method for VL diagnosis is microscopical examination of *Leishmania donovani* bodies (LD bodies) in bone marrow aspirates. Such detection of *Leishmania* parasites in a clinical sample is necessary to confirm a suspected case of VL. The most common method for VL diagnosis is direct detection of parasites, either by microscopical examination or *in vitro* cultivation, for which sensitivity is low {Herwaldt, 1999}. Other methods for diagnosis of VL, such as parasitological or serological tests are difficult even in well-equipped hospitals {Sundar, 2003} and {da Silva, 2005}. As an alternative at the district level, serological tests (direct agglutination test or rK39 immunochromatographic test) were used. The minimum basis for starting treatment for VL is positivity for rK39 immunochromatographic test. The sero-diagnosis is negative in early acute stage of the disease and do not differentiate between active, past or subclinical infection. It remains positive well beyond the time of cure, thus limiting their use for the diagnosis of relapses or reinfection {Chappuis, 2007}. Thus, improved tools for diagnosis of VL are

needed. Molecular methods including polymerase chain reaction (PCR)-based techniques have proven to be highly sensitive and specific as they analyse parasite DNA and may be applied to a variety of clinically obtained samples {Marfurt, 2003}; {Bensoussan, 2006}; {Schallig, 2002}; {Reithinger, 2007}; {Brustoloni, 2007}. For clinical samples, Giemsa's solution-stained bone-marrow smears on glass slides are potentially very valuable sources of DNA for PCR-based diagnosis. Firstly, PCR-diagnosis using DNA extracted from Giemsa's 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. Secondly, historical slides or archived materials may be assessed, thus allowing retrospective studies. Lastly, bone-marrow smears can be easily stored and sent to research centres where PCR-diagnosis can be readily achieved.

A number of PCR-based diagnoses from Giemsa's solution-stained samples on glass slides have previously been reported, including blood smears for *Plasmodium* spp. {Li, 1997}, for cutaneous Leishmaniasis {Motazedian, 2002} and faecal smears containing *Cryptosporidium parvum* {Amar, 2001}. However, PCR-based *Leishmania* diagnosis from bone-marrow smears on glass slides have not been widely applied although we have recently described the use of this strategy to confirm VL infection of a Nepali patient {Pandey, 2009}. Thus, in the present study we have investigated the sensitivity of PCR-diagnosis from bone marrow samples obtained from clinically suspected VL patients in comparison with microscopy and parasite culture**.**

Material and methods

Sample collection

Bone marrow samples were collected from clinically suspected VL patients on the basis of clinical history (continuous fever for 2 week, headache, splenomegaly) in Nepal during 2003-2005. Bone marrow was aspirated by sternal puncture and used for smear preparation and to initiate *Leishmania* culture. Bone marrow smears were fixed with methanol and stained with Giemsa's solution. More than 100 microscopic fields were observed in order to detect the parasite. The slides were kept at room temperature and brought to Japan for further analysis. Informed consent from each of the patients was obtained after an explanation of the uses of a bone marrow aspirates. The research protocol was approved by the Ethical Committee of Nagasaki University.

DNA extraction

After shipping to Japan, all bone marrow smear samples were re-examined microscopically for *Leishmania* parasite infection. Then, Giemsa's solution-stained smears were wiped with fresh paper wipes (Kimwipe wipers S-200; Kimberly-Clark, Dallas, TX) to remove immersion oil, wetted with sterile phosphate buffered saline, and scraped with a sterile scalpel. The DNA was extracted by using DNeasy Blood and tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, yielding a final elution volume of 25 μl. Two different reference strains of *L. donovani*; Dd8 Indian strain (accession no. Y11401) and T4 Nepalese strain (accession no AB458390), were used as positive controls for PCR amplification.

PCR amplification

We selected the conserved region of minicircle kinetoplast DNA because its copy number is more than 10^4 per parasite, maximizing the possibility of detection. {Akkafa, 2008; Smyth, 1992; Salotra, 2001}. PCR analysis consisted of two steps, he details of which were given previously {Aransay, 2000} and {Pandey, 2008}. Briefly, first round PCR amplification was carried out in a total of 10 μl reaction mixture with primers LINR4 (5′–GGGGTTGGTGTAAAATAGGG–3′) and LIN17 (5′–TTTGAACGGGATTTCTG–3′) and 2 μl of DNA solution. The second round semi-nested PCR was carried out in a 20 μl reaction mixture volume as for the first round by adding primer LIN19 (5′–CAGAACGCCCCTACCCG–3′) and 1 μl of first PCR product. Two negative control and two positive controls were set for each experiment. The first negative control was normal mouse bone marrow and the other was DNA negative reaction mixture. Two positive controls contained DNA from cultured *Leishmania donovani* parasites. Ten microliters of the PCR amplified products were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. A 100-bp DNA ladder was used as a marker.

Results and discussion

Microscopical examination, parasite culture, and PCR-diagnosis using bone marrow smears were performed for 115 samples obtained from clinically suspected VL patients. Samples which produced a 720 bp band following PCR amplification and electrophoresis were judged to be positive for VL. Figure 1 shows an example of an agarose gel electrophoresis image. The positive controls produced the expected 720 bp band which was absent from the negative controls. Among 115 samples, *Leishmania* DNA was successfully detected in 79 samples. PCR diagnosis showed the highest sensitivity (79 positive/115 samples, 68.7%) compared to microscopical examination (65/115, 56.5%) and parasite culture (20/115, 21%) (Table 1). All

samples positive by microscopic examination or parasite culture for *Leishmania* infection were positive by PCR-diagnosis and all negative samples by PCR-diagnosis were negative by other methods.

Despite its high sensitivity and specificity, PCR-diagnosis may have limitations in VL diagnosis. PCR-diagnosis alone cannot differentiate between asymptomatic and acute infections of VL,{Deborggraeve, 2008}, thus this method may be used only for the confirmation of suspected cases of acute VL. Currently, asymptotic VL patients in endemic regions are not treated with {Kennedy, 1984}drugs, because the available drugs for VL are highly toxic and their unnecessary use may stimulate the emergence and spread of drug-resistant parasite strains. However, it should be noted that PCR-diagnosis of *Leishmania* DNA in asymptomatic VL patients is beneficial for epidemiological studies.

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Table 1. Comparison of PCR-amplification, microscopical examination, and parasite culture to diagnose visceral leishmaniasis.

	total	microscopy		culture	
		(+)	(-)	(+)	(-)
PCR-diagnosis (+)	79 (68.7%)	65	14	20	59
PCR-diagnosis (-)	36 (31.3%)	0	36	0	36
total	115	65 (56.5%)	50 (43.5%)	20 (21.0%)	95 (79.0%)

Figure legends

Figure 1. PCR amplification of the *Leishmania* DNA obtained from Giemsa's solution-stained smears on glass slides. Lanes 1-7, bone marrow samples; pos1 and -2, DNA from cultured *Leishmania* parasite; neg1, DNA from mouse bone marrow without *Leishmania* infection; neg2, without DNA template. In this experiment, sample numbers 1, 3, 4, 6, and 7 were judged to be positive for *Leishmania* infection.