

Review

## Utility of PCR for Molecular Diagnosis of Visceral Leishmaniasis

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### Abstract

The diagnosis of visceral leishmaniasis (VL) can be performed by demonstrating the presence of parasites by direct microscopic examination, using invasive techniques (such as aspiration from the bone marrow, spleen, liver or lymph nodes), which present multiple disadvantages, for this reason, various serological techniques have been used, such as the ELISA test, which routinely have employed whole parasites or semi-purified parasite extracts, which are complex mixtures of molecules, which show false positive results against the sera from patients suffering with other diseases (specificity problems) and false negative results against some sera of patients with VL (sensitivity problems). Advancements of recombinant DNA technology have allowed us to develop better antigens, but its limit of sensitivity does not allow us to detect recent infections or follow up after administering chemotherapeutic treatment. Thus, PCR of peripheral blood has emerged as an excellent alternative in such cases where, other diagnostic tests do not show conclusive results. This review is an endeavor to present recent advantages and disadvantages of using PCR as a molecular diagnostic technique for visceral leishmaniasis.

### Introduction

VL or kala-azar is considered one of the most neglected diseases, proven its strong association with poverty and the limited resources and required to utilize new tools for diagnosis, treatment and control. For these reasons, it constitutes a serious threat to public health of great medical and veterinary importance.

VL can be transmitted by infected insects bite such as *Phlebotomus* and *Lutzomyia* most commonly found in the New and Old World, respectively [1]. It is caused by parasites of the *Leishmania donovani* complex that includes *L. donovani* and *L. infantum* [2]. VL is endemic in 98 countries on 5 continents with an estimated global burden of 300 million people, originates an estimated 200.000 to 400.000 new cases and of 20.000 to 40.000 deaths per year worldwide [3], and the risk of spreading of the disease to new regions increases along with climate change [4].

In 2015, more than 90% of new cases has been reported by WHO that occurred in these countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan [5]. The diagnosis of VL is complex because its clinical characteristics are shared by other diseases, such as malaria, typhoid fever and tuberculosis, in addition, there may be coinfection of these diseases

with VL and the sequestration of the parasite in certain organs further complicates this problem. Moreover, about 95% of patients with VL die without appropriate treatment, because this parasitic infection produces serious damages in the spleen, liver and bone marrow. It is characterized by irregular bouts of fever, weight loss, enlargement of the spleen and liver, and anaemia [6].

VL affects the most vulnerable populations in areas of Africa, Asia, South America and the Mediterranean Basin. It is highly endemic in the Indian subcontinent and in East Africa. The kala-azar elimination programmes in South-East Asia are making sustained progress towards elimination, and cases are declining in the three major endemic countries: Bangladesh, India and Nepal.

### Nature of VL Infections

VL is an infection of great relevance due to all the problems that originates, for this reason, several diagnostic methods for detect this disease have been used over many years. The diagnosis conventional of VL is based on methods parasitological and serological tests. Parasitological examination can directly demonstrate the presence of *L. infantum* in aspirates from the liver, spleen, lymph nodes and bone marrow [7], through microscopic examination, but this technique presents many limitations, requires invasive procedures, such as spleen and bone marrow aspiration what is associated with the risk of severe complications when performed by inexperienced health care workers, low sensitivity of the tissue examination, besides that the identification of amastigotes requires considerable expertise, training and is subject to the ability of the observer.

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The serological tests include the Immunofluorescence Assay (IFA) [8] and enzyme-linked immunosorbent assay (ELISA) [9]. However, these techniques may produce cross-reactions with other diseases (false positive results) [10, 11], also has been observed reduced sensitivity in patients with immunosuppression, do not allow to discriminate between recent or past infections and cannot be used to monitor efficacy of chemotherapeutic treatments.

### PCR as a solution for VL diagnosis

To improve case finding, more sensitive diagnostic tests are required and one such option is the application of molecular tests. In recent decades, molecular techniques such as polymerase chain reaction (PCR) have been introduced for the diagnosis of VL, exhibiting high sensitivity and specificity [12]. PCR allows detection of parasite's genetic material as confirmatory methods in acute and asymptomatic infections, which show negative results by serology, and in most cases, do not show seroconversion and having a low parasite load [13].

### Principal of PCR in VL Diagnosis

PCR is a technique based on the principle of complementary bases pairing of the DNA molecule, allowing amplification and detection of a particular region of the target genome using a pair of specific oligonucleotide primers. The reaction can produce tens of billions of DNA fragments from a single molecule, and has high sensitivity, what allows that small amounts of samples to be used. This type of PCR, hereafter referred as "conventional PCR" (cPCR) employ electrophoresis in agarose or polyacrylamide gels along with dyes such as ethidium bromide, SYBR Green or silver nitrate to view the amplified product.

PCR is usually qualitative, with analysis of the presence or absence of bands, or semi-quantitative, when densitometry of bands is used in comparison with known standards. PCR enable rapid diagnosis of disease and can help to identify the parasite species, through the amplification of species-specific genomic regions [14]. Sensitivity and specificity of PCR-based detection depend on both the samples and primers to amplify the region of target genome. The target sequences most frequently used are tandem repeating regions or polymorphic regions, in order to increase the probabilities of amplifying the parasites' DNA among a great amount of the host's DNA in the sample [15]. The selection of target region in the parasite genome is fundamental because the greater the number of copies the greater the sensitivity of the test for detection the parasite's DNA. The highly conserved and repetitive regions are the most used, such as the kinetoplast DNA (kDNA) with about 10.000 copies distributed among 10 different classes of sequences, small subunit ribosomal RNA gene (SSU-rRNA) that has 40-200 copies per cell, internal transcribed spacers (ITS) in the ribosomal operons, among other. kDNA possess thousands of copies of minicircle DNA and dozens maxicircles concatenated in a giant network [16]. The high number of minicircles (10,000-26,000 copies/parasite) of

kDNA [17] makes them an attractive target for *Leishmania* detection with high sensitivity, which allows detecting less than 1 parasite/ml in the blood [18]. The kDNA PCR seems to be a useful tool for diagnosing VL, and it may be a good marker for predicting VL relapses after treatment in co-infected VL/HIV patients with clinical symptoms of the disease [19].

### Idealism of PCR Based Diagnosis of VL

Many PCR-based methods for diagnosis of VL have been described with different specificities and sensitivities; PCR assay sensitivity depends on the sample used, this parameter may vary because some studies have been observed sensitivities close to near 100% in spleen or bone marrow [20] samples. PCR provides a prompt and sensitive method for the detection of *L. donovani* complex using peripheral blood (70%-100%) [22, 21], the collection of which is less invasive [23]. In a study conducted by Fraga (2010), the DNA of the parasite was detected in 95.5% of the samples evaluated, which shows the high sensitivity of kDNA-PCR [24]. As it has also been observed by other authors, with sensitivity of 79-100% [25]. When compared the sensitivity of PCR in peripheral blood and PCR in bone marrow aspirates it can observed that both were almost identical (91,1%). Similar results have been obtained by other authors [26, 15]. This technique has the advantage of replicating the genome of the parasite from the minimum quantity of circulating DNA in different biological samples such as human and canine blood [21], buffy coat [27], canine conjunctival scrapings [12], and urine [28]. Lymph nodes [29], liver, spleen and bone marrow [30] aspirates or biopsies being the most common [31, 33]. Molecular detection of *Leishmania* parasites' DNA in blood might lead to less invasive diagnosis of VL than conventional parasite detection in bone marrow or spleen aspirates [34]. It's also ideal for diagnosis in children, who suffer much pain, discomfort and can present fatal accidents during organ aspiration procedures (very invasive and dangerous techniques) [24]. The experience gained over the years in researchs in molecular biology has allowed the development of several PCR protocols for the detection of parasite's DNA and VL diagnosis. The different PCR methods may vary in different aspects such as: fluorophores, probes, target regions and tissues used for detection of target DNA making it difficult to do a comparative analysis between the different protocols. It is known that the sensitivity and specificity of PCR for detection of *Leishmania* sp. depends on many factors such as the physico-chemical conditions of the reaction, the concentration and nature of the sample DNA, the probes, and oligonucleotide primers selected for the target region [25, 26].

The protocols standardization based on changes in previous parameters is the key step to increased sensitivity, specificity and reproducibility of the tests. The diagnosis of VL by means of PCR using peripheral blood as a source of DNA template is advantageous because it avoids sampling through painful, dangerous and invasive procedures, as is the case of bone marrow aspirates.

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## Conclusions

PCR is a useful tool for the diagnosis of VL because it allows detecting *Leishmania* DNA in very small quantities. The use of this technique in peripheral blood allows to diagnose VL in children, which avoids the use of sample extraction techniques painful, uncomfortable and dangerous. As well can provide an important approach to the diagnosis of VL reactivation in co-infected VL/HIV patients. The molecular diagnosis also allows the therapeutic follow up of patients for long periods of time and the detection of parasites in asymptomatic patients. PCR based methods for detecting parasites are highly sensitive, specific and have the added advantage that they may be performed on dry specimens without the need for cold-storage.

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