Diagnosis of visceral leishmaniasis

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Leishmaniasis, responsible for two million new cases per year, is considered by the World Health Organization (WHO) to be one of the most important parasitic diseases. The visceral form of this disease producing 500 000 new cases per year. Visceral leishmaniasis (VL) is of higher priority than cutaneous leishmaniasis as it is a fatal disease in the absence of treatment. The search for adequate diagnostic tools is a ongoing struggle and one of the priorities for the WHO. This ongoing effort by the medical/scientific community has produced results in the form of some diagnostic field tests such as rK39 strips or DAT, that are now specific and sensitive enough for field use. These are important tools to decrease the mortality and morbidity world wide. In recent years, the HIV co-infection represents a new reality and it changed the epidemiology of traditional VL adding new problems to common diagnosis procedures. This article reviews the current tools for VL diagnosis, focusing on the problem of the co-infection with HIV.

Keywords Visceral leishmaniasis; diagnostic; HIV co-infection.

1. General remarks

Leishmaniasis is considered by the WHO to be a neglected emerging disease and one of the most important parasitic diseases with 10% of the world population at risk, 350 million people, and two million new cases per year. This disease has a near global distribution being endemic in 88 countries, absent only from Australia and Antarctica due to the lack of suitable vectors. Clinically, leishmaniasis can occur in four major forms: (I) visceral leishmaniasis (VL) and post kala-azar dermal leishmaniasis (PKDL); (II) cutaneous leishmaniasis (CL); (III) diffuse cutaneous leishmaniaisis (DCL) and (IV) muco-cutaneous leishmaniasis (MCL). The VL, also known as 'kala azar', is the most severe form of the disease, with an estimated half million cases reported per year. In this form of the disease, the parasites colonize specific internal organs like the spleen, liver, bone marrow and lymph nodes with almost always fatal outcome if not subjected to appropriate treatment. VL is also prone to large-scale epidemics with high fatality rates. The PKDL, a distinct form of leishmaniasis associated with cured VL patients, may have an important role in the maintenance of the antroponotic cycle of transmission of the disease in certain places. The PKDL, although not as deadly as VL, requires a lengthy expensive treatment program. The CL, DC and MCL are responsible for affecting 1.5 million people per year. In CL the parasite is usually present in the skin where it normally causes self healing sores. DCL occurs in individuals with defective cell-mediated responses. It's a type of leishmaniasis resembling leprosy which never heals spontaneously and is very prone to relapses. In MCL the infection spreads to the mucosal membranes, of the mouth and nose, causing extensive damage. These four distinct clinical phenotypes are caused by more than 20 *Leishmania* species with distinct epidemiological patterns. The HIV/*Leishmania* co-infection represents a distinct challenge to the traditional approach to leishmaniasis with particular epidemiological features, diagnosis and treatment [1]. VL due to its characteristics represents the most severe form of the disease putting at risk a universe of 200 million people. Over 90% of the registered cases occur in five countries: India, Bangladesh, Nepal, Sudan and Brazil. The anthropomorphic form of the disease is prone to epidemic outbreaks like the one in Sudan responsible for mortality of 36%. The conventional treatment for leishmaniasis, though not applicable in HIV-

coinfections, is still based upon pentavalent antimonials and requires a 30 day treatment composed of intramuscular or intravenous injections. Incorrect treatment schemes and poor compliance has lead to an increasing unresponsiveness to the pentavalent antimonials. This treatment carries a significant toxicity; although alternatives exist they are more expensive and often not accessible [2].
The challenges posed to the medical/cientifical community by leishmaniasis are related to the search for new tools of diagnosis, drugs and vaccines. As there is still no vaccine available, early detection of the disease and compliance to the treatment schemes, that should cheap and easy to administrate and cheap, are the most important control measures.

2. Diagnosis

Diagnosis and treatment follow-up is a problematic issue in VL. Clinical follow up prevents relapses and resistance development. In developed countries, where this disease is not prevalent, the existence of laboratory facilities enables an adequate and efficient follow up of the disease. However, in developing countries with large numbers of patients in rural areas simple diagnostic tools are necessary for field use. Diagnosis of leishmaniasis can be made using different approaches: (i) direct visualisation of the parasite in tissue samples or (ii) detection of the parasite DNA and (iii) immunodiagnosis, detecting parasite antigens using specific *Leishmania* antibodies or *Leishmania*-specific cell mediated immunity by skin tests.

2.1 Direct visualization or isolation of the parasite

The demonstration of parasites in relevant tissues such as spleen, bone marrow, lymph nodes, liver or the buffy coat of peripheral blood is commonly used in the diagnosis of VL. Normally the diagnosis is based on finding amastigotes in monocytes or macrophages (seldom are they found free in the tissues except in HIV positive patients). The analysis is done using a special logarithmic scale ranging from 0 (no parasites in the microscope field) to +6 (>100 parasites per field) [3]. This technique requires an experienced set of eyes behind the microscope as they must recognise an oval shape of 2 to 3 μm inside a monocyte or a macrophage. In HIV patients this is even more critical as many other organisms might be infecting them leading to false positive results. The two most commonly used tissues are bone marrow and spleen. The sensitivity of this approach using bone marrow is about 60-85% and in spleen is more than 95%, making direct detection of parasites one of the most powerful techniques and the spleen one of the more sensitive tissues to detect *Leishmania*. Although less painful than the bone marrow aspirate, the splenic aspirate is still delicate a procedure and requires very skilled personnel as fatal bleeding is a possibility. This makes a splenic puncture a very delicate procedure not suitable for routine diagnosis. Cytological analysis of the above depicted tissue sources by Giemsa staining is more common, the presence of *Leishmania* per 100 host cell nuclei is registered. Long searches may be required for detecting the parasites. To increase the sensitivity fluorescent dye-conjugated antibodies can be used. Overall, the use of these techniques implies sensitivity values similar to what is described for HIV co-infected patients [4]. Culture of parasites can also be used to improve the overall sensitivity of the detection, nonetheless it's time consuming and seldom used in routine clinical practice. These techniques related to the direct detection of the parasite can be used with similar sensitivity in the case of diagnosis HIV coinfection. Furthermore in HIV coinfection, good results are also obtained with blood. The amastigote forms can be found freely in blood from most coinfected patients so non-invasive sampling is more useful in coinfection patients than in normal VL. Parasites can also be found in atypical locations during coinfection, such as larynx, rectum, spinal fluid and others. Even the detection of a single parasite in the microscopic examination of tissue smears or in culture, it is enough for a positive diagnosis. However, the sensitivity of these techniques is overall unsatisfactory, with the exception of using splenic aspirate as the source of biological material. Furthermore the procedures for tissue sampling are often invasive and not free of risk. The correct identification of the parasite requires trained personnel and skilled observers. The special characteristics of this sort of approach to diagnosis make it unfit for field use as it is time consuming and requires expert personnel.

2.2 PCR based techniques

Since the direct visualization of the parasite had certain limitations, indirect ways of detecting the parasite were implemented, most of them based on the detection of parasite DNA. One of the first approaches consisted in simple DNA hybridization using radioactive or non-radioactive probes [5]. This technique although very sensitive and specific is not simple to perform due to complex the hybridization conditions and DNA extraction procedures that are required. Furthermore, this technique is subjected to the same problems as the direct observation methods, the best biological sources of parasites will be the same spleen and bone marrow. Albeit this approach was the basis of all the PCR based protocols. Primers designed for specific conserved sequences of the parasite were used in many PCR-based diagnostic methods. The most commonly used targets for primer design have been mini-exon-derived RNA genes, genomic repeats, ribosomal RNA genes and kinetoplastid DNA [6]. The latter is still one of the most appealing targets for primer design due to the highly repetitive copies of minicircle DNA present in the kinetoplast. Several PCR-based methods for diagnostic of VL exist in the literature with several specificities and sensitivities [7,6]. The sensitivities of the PCR based approaches are similar or even more sensitive than the direct microscopic observation [7,5,6]. The sensitivity of the PCR-based diagnosis will be dependent on the source of biological material much like what happens in direct observation. So, bone marrow and splenic aspirates had very good sensitivities, near 100% in cases of confirmed VL [7,8,6]. The lymph nodes sensitivity range was from 87% to 100% [7,6]. Nonetheless the ideal source of biological material is peripheral blood, due to its non invasive characteristics. Using blood, the sensitivities range described varies from 70% to 100% [9,7,10,6,11]. Buffy coat preparations allow more sensitivity than whole-blood preparations [12]. This enables the use of blood as a first screening technique, the more invasive methods would only be used in the event of a negative blood test [6]. The use of PCR-diagnosis in the HIV coinfection is very useful in coinfected patients due to the existence of circulating parasites in the peripheral blood [13]. Nonetheless this does not reflect in higher diagnostic capacity because similar values of sensitivity have been found in immunocompetent VL patients [14-16]. A PCR-enzyme linked immunosorbent assay (ELISA) is a PCR based method very sensitive and specific. It was adapted to *Leishmania* detection with great success: the authors claim it was able to detect fentograms of genomic material [17]. This technique was applied in diagnosis of VL with promising results using peripheral blood [18,19]. A similar PCR based technique was applied in the diagnosis of VL in HIV patients with good results, using non-invasive sampling [20]. Another interesting approach is a rapid fluorogenic PCR technique: the authors use a fluorescent DNA probe for a conserved rRNA gene that is amplified using flanking primers. This approach using clinical samples showed great sensitivity and specificity [21]. The real-time PCR has the advantage of being quantitative which could be useful in the follow up of treatment allowing for the assessment of the parasite burden [22,23]. The potential of these PCR based methods have not been limited to diagnosis. They have also been useful in distinguishing relapse episodes from reinfection by detecting subtle differences in the genome using restriction fragment length polymorphism analysis [24]. PCR can also be used to evaluate the success of treatment. The detection of parasites in lymph node tissue after a seemingly successful cure of the disease seems to be related to actual relapse and development of PKDL [25]. Nonetheless the value of PCR is not absolute, a positive PCR can be due to non virulent or dead parasites that haven't been cleared, so its use must always be correlated with the clinical data [26]. This is more critical in endemic areas as continuous contact with the parasite results in the existence of DNA that can be detected until the total clearance of parasitic biological material [27]. This could be overcome by using cDNA based approaches as the RNA is more sensitive to the degradation upon parasite death. The existence of apparently asymptomatic humans in an endemic area as regular blood donors raises the possibility of direct infection by blood transfusion in immunocompromised patients [27]. This is a problem that should be looked upon more carefully by the medical community. The use of PCR-based techniques in diagnosis is also not fit for field use but in hospital facilities is undeniably powerful. It is unsurpassed specially when we consider the Real-Time PCR based techniques that have the capacity of determine parasite load. This information will help improve the effectiveness of diagnosis, treatment and even aid vaccine development.

2.3 Serological techniques

The fact that leishmaniasis, specially the visceral form, is accompanied by high antibody titres led to the widespread use of several serological techniques that are often very sensitive. These high antibody titres were exploited for serodiagnosis using techniques such as ELISA, immunoblot, indirect immunofluorescent test (IFAT), direct agglutination test (DAT), gel diffusion, the complement fixation test and the indirect hemagglutination test among others [28]. Most of them were technically very demanding, and with various degrees of specificity and sensibility that made them unfit for diagnostic use in the field. In 1988, the use of Comassie brilliant blue stained formalin fixed trypsinized whole promastigotes in a Direct Agglutination Test (DAT) was the first real field application of a diagnostic test for VL [29]. Sensitivities between 91 to 100% and specificity ranging from 72% to 100% were described for DAT [30-33]. Albeit the usefulness of DAT, the reproducibility of the results was still unsatisfactory due some problems related to batch variation in the antigen [34]. ELISA is the preferred technique for serodiagnosis. This technique is highly sensitive and specific, but these parameters depend on the antigens used. The *Leishmania* total soluble extract is one of the most commonly used antigens in ELISA, the sensibility ranges from 80 to 100%, but several cross reactions are described [35,36,31]. It is possible to improve the specificity using specific *Leishmania* antigens, but often these implicate the loss of sensitivity [37-39]. The search for a reliable serodiagnosis has led to the testing of several molecules in different clinical environments. The attempt to use other sources of biological material was also done urine being the most commonly used with some degree of success, but most of the literature reports the use of blood as the preferred source of biological material [40]. A screening by Maalej and colleagues gave insights into the potential of several *Leishmania* recombinant proteins in diagnosis with sensitivities varying between 38% and 100% for the different proteins tested [38]. Some successful antigens include the fucose-mannose ligand with a sensitivity of 95 to 100% and a specificity of 95% when used in conjunction with the ELISA technique [41-43]. Another interesting approach was performed by Boarino and colleagues that created a synthetic chimeric peptide with several immunogenic fractions from immunogenic proteins (K39,K9-K26) that had a sensitivity of 82% and specificity of 100% in the detection of VL [44]. ELISA with *Leishmania* membrane antigens are also capable of detecting PKDL patients with 100% sensitivity and 96 % specificity [45]. Among all the different candidates the most extensively tested is the recombinant protein rK39. The rK39 is a 39 amino acid repeat from a kinesin like protein isolated from *Leishmania chagasi* with proven diagnostic value in the diagnose of VL [36,46,31]. Monitoring anti-rK39 during active disease, therapy and subsequent follow-up monitoring showed that anti-rK39 antibody titre correlates with the active disease [47,36]. Other proteins such as a tryparedoxin peroxidase from *Leishmania infantum* also have this characteristic what can make them also attractive targets for diagnosis in active leishmaniasis [48]. Recent improvements in the technique claim to bring more reproducibility to the assay, nonetheless it is a generally accepted technique for diagnosis [49]. In spite being described as having some predictive value in the HIV co-infection [50], several authors report low sensitivities ranging from 22% to 62% [51,52]. Although some ELISA based techniques have proven to be very sensitive and specific, this is a technique that requires some technical expertise rendering it almost useless for direct use in the field. Nonetheless it helped to make rK39 the basis of the first rapid test, with good specificity and sensitivity, for use by paramedic personnel without any specific expertise. The rK39 immunochromatographic strip is composed of antigen immobilized in a piece of nitrocellulose membrane. Above it we find anti-protein A. Half a drop of blood is all the biological material needed. It is placed on the lower part of the strip, after the addition of 5 drops of phosphate buffered saline and the mixture flows along the strip by capillarity dragging the conjugate (protein A colloidal gold) that was pre-dried on the strip. If antibodies for rK39 are present in the blood they will bind to the immobilized rK39 yielding a pink band. The presence of two pink bands is the final outcome of a positive test, for the rK39 and the anti-protein A band control. A large scale a trial in India found the test 100% sensitive and 98% specific [53]. Independent studies from the region reported also near 100% sensitivity [54-58]. The test sensitivity for PKLD was determined as 91% [59]. Beyond the Indian subcontinent different values of sensitivity for the test were achieved. In Sudan the sensitivities varied from 67% to 93%, while the specificities were

under 60% [60,61]. These low specificities in Sudan were somewhat surprising as the Indian subcontinent trials report higher specificities [62-64]. In Southern Europe the sensitivity was described as 71% [65,61]. In Brazil, Carvalho and colleagues report 90% sensitivity and 100% specificity [66] It was proposed that this could be due to distinct antibody responses for different genetic backgrounds [31]. In 2002 a study revealed that 12,5 % of an endemic control group was positive using this test [58]. Some of these putatively false positives were confirmed by PCR indicating that this test is also able to detect asymptomatic subclinical infections [11,58]. Nonetheless the rK39 immunochromatographic strip test has good specificity and sensitivity and it's simple enough to be used by non-trained personnel in the field. Western blotting can also be used in diagnosis using total soluble extract of promastigotes. The proteins are separated by electrophoresis, according to their molecular weight, on a sodium dodecyl sulphate-polyacrylamide gel, transferred to a nitrocellulose membrane and then incubated with patient serum. The proteins recognised can be detected using a anti-human antibody bound to alkaline phosphatase or more commonly to a peroxidase. This is a good method for screening potentially immunogenic antigens but not often used in diagnosis. It is not very user friendly requiring trained personnel; furthermore it is technically burdensome not fit for field use. The place it could have in diagnosis is occupied by other more standardized techniques like ELISA. Western blotting based techniques are used in the basic research and not in diagnosis [67]. As an example PKDL patients can be distinguished from VL patients by the recognition pattern of certain peptides [45]. Nonetheless it is used indirectly in the rk39 strip and all like tests. Immunodiagnosis is not restricted to antibody detection; antigen detection can also be used in diagnosis, some authors have described it as more specific that antibody-based approaches [28]. Sensitivities of 96% and specificities of 100% were reported using a polypeptide fraction found in the urine of VL patients. Furthermore these peptides seem to disappear upon successful treatment so it has also prognostic value [37]. This type of approach seems potentially useful in the HIV co-infection as it is not dependent of antibody production. In the last decade other easy to use diagnostic methods were developed. An improved version of DAT with freeze dried antigen enables a better reproducibility [68]; KAtex a latex agglutination test based on the detection of a heat stable carbohydrate antigen from *Leishmania* in the urine [69,70]. A comparative study in India with 452 subjects demonstrated the suitability of the use of rK-39 strip test and DAT in the field with close to 100% specificities and sensitivities, KAtex with 67% sensitivity was less sensitive [63]. This corroborates previous works that showed no significant difference between the use of the rK39 dipstick or the DAT [62,64]. KAtex tended to perform less well, with sensitivities between 44,7 and 67% [71,63]. The use of these tests in HIV co-infection is not so critical because the follow up of HIV patients is normally done in hospitalary facilities. Nonetheless the sensibility of DAT in a group of co-infected patients in Ethiopia was 90% [72]. The rK39 dipstick had poor performance with scattered reports of weak sensitivity [1]. In a test done in Spain, the KAtex was 100% specific and 96% sensitive in immunocompromised patients [73]. In another study a 87% specificity was obtained for primary VL in co-infected patients [74] and also had some predictive capabilities in the follow up of treatment and in the detection of subclinical infections [74].

2.4 Skin tests

The Montenegro skin test is a test for delayed type hypersensitivity. Injection of 5 x 10^7 phenol killed promastigotes into the forearm leads to the formation of an induration measurable after 48/72 hours. Comparing the size of the induration with a control injection with phenol-saline in the other forearm it is possible to determine either an individual is infected or not. In active untreated VL we find negative DTH due to T-cell anergy characteristic of the acute phase of VL [75,76]. The recovery of positivity is associated with treatment for VL as the T-cell anergy is overcome. A study in Ethiopia demonstrated that 66% of VL patients undergoing treatment were leishmanin positive while none of the non-treated patients were positive [75]. One of the big drawbacks of this approach is the lack of standardized leishmanin [77].

3. Conclusions

Diagnosis of VL in an equipped laboratory is a well established fact where there are various tests with excellent specificities and sensitivities. These tests are not commercially available requiring trained personnel, equipment and some expertise. This is the case of direct parasite diagnosis either by culture, direct observation or PCR. Serological tests are also well established and are the basis of the only field diagnosis tests in existence: rK39 strip test, the DAT and the KAtex. These field tests represent an important step in the efficient management of VL in problematic endemic areas enabling rapid and accurate screening and in timely intervention by the medical authorities. New challenges will come from the diagnosis of HIV co-infection as these appear without the typical clinical signs. The immunosuppression associated with both HIV and *Leishmania* makes specially challenging the serological approach to diagnosis although good results were found for KAtex. But still we do not have a true "gold standard" diagnostic test for visceral leishmaniasis.

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