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**RESEARCH ARTICLE**

# SUPERIORITY OF ANTIGEN DETECTION OVER ANTIBODY DETECTION IN THE DIAGNOSIS OF *VISCERAL LEISHMANIASIS*

**Kumar Rajesh and *Kumar Sanjay**

Faculty of Advance Studies in the Life Sciences, Chhatrapati Shahuji Maharaj University, Kanpur, 208024, UP, India

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**ABSTRACT**

*Visceral Leishmaniasis* (VL) also known as Kala-azar (Hindi: kala means black, azar means sickness, also known as Assam fever, Dumdum fever, Sikari disease, Burdwan fever, Shahib's disease and tropical splenomegaly). This disease is endemic and widely spread in 88 countries, including India. Because of its importance, was selected by WHO out of six most precarious diseases. Due to diversity of epidemiological situations, no single diagnosis treatment, or control will be suitable for all. Serological tests which have been used and evaluated are aldehyde test, Napier Test, Complement Fixation Test Direct agglutination test, Immuno-fluorescent test, Enzyme linked immunosorbent assay Immuno-chromatographic dipstick test for VL diagnosis. They all are based on detection of antibody titre in patients serum, but antigen detection is most superior diagnostic test over antibody, as in this case HIV-Co infected can be easily detected because there antibody titre is very low and can't be detected upto a certain limit.



## INTRODUCTION

Visceral Leishmaniasis (VL) also known as Kala-azar was first described in 1824 in India, in Jessore District of Bengal, which is now Bangladesh. Visceral Leishmaniasis (Leash'ma NIGH a sis) is a vector- borne anthrozoonotic disease caused by obligate intracellular protozoan parasites of macrophages and dendritic cells i.e *Leishmania donovani*. This disease is endemic, geographically and ecologically widespread parasitic threatens common in warmer parts of the world, and covers 88 countries (16 developed and 72 developing). About 350 million people all over the world are suffering from this disease and their global incidence is estimated to be almost 5, 00, 000 new cases in a year. Neighboring countries like Nepal (Terai region) and Bangladesh also report a significant number of cases with VL. In India, kala-azar (Visceral leishmaniasis; VL) affects millions of people, and the state of Bihar accounts for nearly 90% cases, followed by West Bengal and Eastern Uttar Pradesh. In Uttar Pradesh recently few active cases have been seen from eastern districts e.g. Varanasi, Gorakhpur, Gonda, Faizabad etc. where there is under reporting of kala-azar and post kala-azar dermal leishmaniasis in women and children 0-9 years of age. Untreated cases of kala-azar are associated with up to 90% mortality.

### Diagnosis

Diagnosis of the disease is still a problem which limits the data on prevalence and makes us unable to limit the disease which is easier for India compared to other countries because no animal reservoir exists. Following diagnostic techniques have been used for the diagnosis of *Visceral Leishmaniasis.*

1. Parasitological diagnosis
2. Serological diagnosis
3. Molecular diagnosis

### Parasitological Diagnosis

It includes direct demonstration of LD bodies in smear of aspirates taken from spleen, bone marrow and lymphnode. Thin smear of above aspirates stained with Giemsa stain. Parasitological diagnosis remains the gold standard in Leishmaniasis diagnosis, because of its high specificity. LD bodies density present in smear interpret that severity of the patient suffering from VL disease.

| Grade | Average parasite density |
|---|---|
| 6+ | >100 parasites/fields |
| 5+ | 10-100 parasites fields |
| 4+ | 1-10 parasites fields |
| 3+ | 1-10 parasites/ 10 fields |
| 2+ | 1-10 parasites/ 100 fields |
| 1+ | 1-10 parasites 1000 fields |
| 0 | 0 parasites 1000 fields |

These procedures are invasive, troublesome and are not conveniently applicable under field conditions (Osman, 1998), and there is no possibility to distinguish between leishmania amastigotes belonging to the different species. The accuracy

***Corresponding author:*** skumarbt88@gmail.com

of microscopic examination is influenced by the ability of the laboratory technician and the quality of the reagents used for slide preparation.

**Serological Diagnosis**

Serological diagnosis is very crucial diagnostic techniques used for VL. They were based on antigen antibody reactions.

**Antibody Detection in Patients serum against specific antigen of *Leishmania donovani***

Various serological tests are being used for the diagnosis of the disease. Few of them are very specific and sensitivity for VL but one of the major drawback of these tests they cross react with other chronic diseases (Chatterjee, 1957; Evans, 1973). and can't be differentiate present and past infection.

**Napier Test**

Also known as Aldehyde test, involves the mixing of formalin (40%) with patient serum. In this Test**,** About 1-2 ml of VL patients serum is taken in a small glass tube and 2-3 drop of 40% formalin added into it. The aldehyde test procedure is simple. Five cubic millimeters venous blood is drawn from the patient and left for clotting. Serum is separated by removing supernatant after centrifugation. To one ml of serum, 2-3 drops of commercial formalin (40% formaldehyde) is added. Opaque jellification like white of a boiled egg indicates a positive reaction. Jellification time indicates intensify of infection as indicated in the table below.

| Jellification time | Intensity of reaction |
|---|---|
| 2 minutes | ++++ |
| 20 minutes | +++ |
| 120 minutes | ++ |
| 24 hours | + |

Positive result will be indicated by jellification of milky white opacity like the white of a hard boiled egg. The concentration of gamaglobulins in blood increases considerably following infection with *L. donovani.* Formaldehyde has a tendency to blind these serum immunoglobulins. A positive reaction may also be seen in diseases like tuberculosis, cirrhosis of liver, malaria, etc. Further, in kala-azar, the test becomes positive only when infection is at least three months old and may remain so even after six months of cure.

**Antimony Test**

Antimony test is also based on detection of antibody in VL patients. This also depends upon a rise of serum gamma globulin. For this test VL patient serum mixed with 4 % of Urea Stibamine in small tube. A positive test will be indicated by the formation of a profuse flocculent precipitate. A negative result is indicated when the two fluids mix without precipitation. As a diagnostic aid antimony test is less reliable than the aldehyde test. Similarly this test is also non specific and shows positive response with other infectious disease as aldehyde test.

**Complement Fixation Test with W.K.K. Antigen**

This test depends upon the presence of certain immune bodies in blood sera of kala-azar patients, the antigen used in the test is prepared from either human tubercle bacilli (Witebsky, Kligenstein & Kuhn: WKK antigen) or from Kedrowsky's acid-fast bacillus. The test is essentially an antigen-antibody reaction in which the antibodies present in the serum are bound to an antigen. The test is more sensitive as compared to Aldehyde test as later becomes positive only after three months. However, cross reactions are observed in cases of pulmonary tuberculosis, leprosy and Mycobacterium infections.

**Direct Agglutination Test**

The Direct agglutination test (DAT) is a highly specific and sensitive test. It is inexpensive and simple to perform making it ideal for both field and laboratory use. The test is based on antigen-antibody reaction. The method uses whole, stained promastigotes either as a suspension or in a freeze-dried form. The freeze-dried form is heat stable and facilitates the use of DAT in the field. However, the major disadvantage of DAT is the relative long incubation time of 18 h and the need for serial dilutions of serum. Also, DAT has no prognostic value. The test may remain positive for several years after cure.

**Indirect Immuno flourescent Assay Test (IFAT)**

This is a commonly used technique for Kala azar. This is a specific and sensitive test requiring fewer amounts of antigens and detects the low titer of antibodies present in patient serum, which are demonstrated in the very early stage of infection and are undetectable six to ninth months after cure. If the antibodies persist in low titers, it is good indication of a probable relapse. The sensitivity and specificity of this test is 96 % and 98 % respectively.

**Enzyme linked Immunosorbant Assay (ELISA)**

ELISA has been used as a potential serodiagnostic tool for almost all infectious disease. Enzyme linked immunosorbent assay (ELISA) is also equally sensitive and specific but specificity depends upon the antigen used, requiring less amount of antigen. This is rapid inexpensive and simple to perform and can be used for diagnosis of Kala-azar for epidemiological work. This test cant be used in field condition and required well established laboratory.

**Rapid Immunochromatographic assay (dipstick or strip test)**

A promising ready-to-use immunochromatographic strip test based on rK39 antigen has been developed as a rapid test for use in difficult field conditions. For this test, recombinant antigen is immobilized on a small rectangular piece of nitrocellulose membrane in a band form, and goat anti-protein A is attached to the membrane above the antigen band. Finger of patient suffering with VL is pricked and half of a drop of blood is smeared at the tip of the strip, and the lower end of the strip is allowed to soak in 4 to 5 drops of phosphate-buffered saline (PBS), placed on a clean glass slide or tube. If the antibody is present in the patients blood, it will react with the conjugate (protein A colloidal gold) that is pre dried on the assay strip. The mixture moves along the strip by capillary action and reacts with rK39 antigen on the strip, yielding a pink band. In the strip of patients who are infected, two pinkish lines appear in the middle of the nitrocellulose

membrane (the upper pinkish band serves as a procedural control). Several studies from the Indian subcontinent reported the test to be 100% sensitive). Drawback of this format is that an individual with a positive rK39 strip test result may suffer from an illness (malaria, typhoid fever, or tuberculosis) with clinical features similar to those of VL yet be misdiagnosed as suffering from VL. Notwithstanding these limitations, the rK39 immunochromatographic strip test has proved to be versatile in predicting acute infection, and it is the only available format for diagnosis of VL with acceptable sensitivity and specificity levels which is also inexpensive (~1 to 1.5 U.S. dollars) and simple and can be performed even by paramedics in prevailing difficult field conditions.

**Western Blot**

A new method, the Western blot for detection of anti-leishmania antibodies in the sera is developed which allows specific serodiagnosis of *Visceral Leishmaniasis* in patients living in non-endemic areas (Salotra P, 1999). In non-endemic areas.This process is time consuming, technically cumbersome, and expensive.

**Antigen Detection in Patients serum against specific antibody of *Leishmania donovani***

**Attar *et al., 2001*** worked in the field of antigen detection in leishmaniasis Two urinary antigens of 72-75 and 123 kDa have been reported to be very useful in diagnosis and prognosis of Kala-azar with sensitivity of 96% and specificity of 100%. A capture ELISA system was developed for diagnosis of visceral leishmaniasis (VL) using a monoclonal antibody raised against an antigen previously detected in the urine of VL patients. The results obtained with the capture-ELISA were compared to those obtained with KAtex, a previously described latex agglutination test, showed that the KAtex and the new ELISA are comparable in terms of specificity (100%) but a better sensitivity (94.1%) was found for the capture-ELISA. Moreover, the capture-ELISA adds a useful quantitative dimension to antigen detection. In addition, the boiling of urine samples, which is necessary for KAtex, was not required in the capture-ELISA. These results suggest that the antigen detection in urine by the new capture ELISA system provides a useful method for diagnosis of VL and fulfils the requirements of a non-invasive method for diagnosis of VL.

***Cruz et al. 2006*** demonstrated that the detection of antigens in serum is complicated by the presence of high levels of antibodies, circulating immune complex, serum amyloid, rheumatoid factors, and autoantibodies, all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of antibodies to free antigen. Many of these problems may be avoided by searching for antigens in the urine.

***According to François Chappuis 2007,*** A latex agglutination test based on heat stable low molecular weight carbohydrate protein detection in urine sample of VL patients. It showed good specificity but only low to moderate (48–87%) sensitivity. Apart from its low sensitivity, there are two practical limitations: the urine must be boiled to avoid false-positive reactions and it is difficult to distinguish weakly positive from negative result, which affects the distinguish weakly positive from negative results, which affects the reproducibility of the test.

***Shyam Sunder 2011demonstrated*** that two polypeptide fractions of 72-75 kDa and 123 kDa were detected in the urine of kala-azar patients. The sensitivity of the 72-75 kDa fractions were 96% and the specificity was100%. Another urinary leishmanial antigen, a low-molecular-weight, heat-stable carbohydrate was detected in the urine of VL patients. This antigen showed 79.1-94.1% specificity and sensitivity of 60.4-71.6% in India. However, the sensitivity of this test was low in clinically suspected patients.

## RESULT AND DISCUSSION

All above parasitological as well as serological techniques which are used to diagnose VL in India have some limitations and not is useful in field conditions. As we know that parasitological diagnosis is gold standard due to its specificity. Detection of LD bodies in smear of Spleen, bone marrow are invasive Painful, cumbersome and are not conveniently applicable under field conditions (Osman, 1998), but according to Neva & Sacks, 1990; Weiss, 1995, there is no possibility to distinguish between leishmania amastigotes belonging to the different species. *François Chappuis et al, 2007 demonstrated that,* The accuracy of microscopic examination is influenced by the ability of the laboratory technician and the quality of the reagents used. In the case of gammaglobulin detection (serological diagnosis) in serum of the patients, the serum globulins also increase in a variety of infections and thus this test is considered to be rather non-specific. A positive reaction may also be seen in diseases like tuberculosis, cirrhosis of liver, malaria, etc. Further, in kala-azar, the test becomes positive only when infection is at least three months old and may remain so even after six months of cure. (Napier & Antimony) are non-specific, and show cross reaction with other pathological conditions, hence are unsuitable for diagnosis. Compliment fixation tests by W.K.K. antigen gives better results However, cross reactions are observed in cases of pulmonary tuberculosis, leprosy and Mycobacterium infections.

Most serological tests are non-specific, permitting detection of antigen shared by related organisms. They do not distinguish antigens confined to a single species as group reactions mask specific ones. The major disadvantage of DAT is the relative long incubation time of 18 h and the need for serial dilutions of serum. Also, DAT has no prognostic value. DAT remains positive for a longtime after the disease is cured, and thus cannot be used as a test of cure or for diagnosis of relapses. Immunochromatographic dip stick test rK 39 has a drawback that an individual with a positive rK39 strip test result may suffer from an illness (malaria, typhoid fever, or tuberculosis) with clinical features similar to those of VL yet be misdiagnosed as suffering from VL. Using western blotting, one can find even minor antigenic differences among various organisms and thus detect cross reactive antigens. This process is time consuming, technically cumbersome, and expensive. Two urinary antigens of 72-75 and 123 kDa have been reported to be very useful in diagnosis and prognosis of Kala-azar with sensitivity of 96% and specificity of 100%. This method is also useful in the diagnosis of disease in cases

where there is deficient antibody production (as in AIDS patients) (Riera *et al*., 2004). Because of the conditions prevailing in endemic area, any sophisticated method cannot be employed on a wider scale. There is a need for a simple rapid and accurate test with good sensitivity and specificity, which can be used without any specific expertise. A promising ready to use Latex test (KAtex) based on detection of antigen in urine has been developed as a rapid test for use in difficult field conditions (Attar *et al*., 2001). These antigens were not detectable within three weeks of successful antileishmanial treatment, suggesting that the test has a very good prognostic value

**Conclusion**

Many of the histological, serological diagnostic methods are reported with due course of time. Every diagnostic method have few limitations or demerits. In the case of parasitological diagnostic methods Firstly, demonstration of LD bodies in smear requires well trained microbiologist behind the microscope & secondly this techniques are time consuming most cumbersome and painful in operation. In the case serology, antibody detection in the patient serum has been done with the help of many techniques. But all the antibody detection methods have demerits like gammaglobulins level is also increase in other disease and show the cross reactivity with VL. In the case of HIV Co-infected individuals immunoglobulins titre is too low, in that case antibody detection against visceral leishmaniasis cant be done. These tests cant distinguish present and past infections. Review of several diagnostic approaches revealed that antigen detection is most sensitive and better technique to diagnose VL patients including immunocomprised ones. This technique doesn't require any painful procedures. Even can be diagnosed with urine of the person.This review article will provide definitely the insight to the readers about the various methods and be helpful for the development of new specific antigens for the diagnosis of VL

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