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Original Article

Detection of LD body in Peripheral Blood Buffy-coat from Suspected Kala-azar Cases of Bangladesh

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Abstract

The present study has been carried out in an attempt to observe the usefulness of peripheral blood buffy-coat microscopic examination for detection of LD-body from clinically suspected Kala-azar cases. Total 127 individuals are included in this study as cases and controls. Among them 67 are clinically suspected Kala-azar cases included from one of the Kala-azar endemic areas of Bangladesh and 60 are taken as endemic and non-endemic healthy controls. Peripheral venous blood and bone marrow aspirate collected and tested for LD bodies from clinically suspected Kala-azar cases in Rajshahi Medical College Hospital, during the period of July 2006 to June 2007. Serum from all cases and controls were tested by rK39 ICT for serological identification of visceral leishmaniasis. Laboratory examinations were performed in the Department of Microbiology and Immunology, BSMMU, Dhaka. Out of 67 study cases bone marrow smear microscopy for LD bodies positive in 44 (66.67%) and buffy-coat smear microscopy positive for 21 (31.34%); diagnostic sensitivity and specificity of buffy-coat smear microscopy was 47.72% and 100% in comparison with bone marrow examinations. Buffy-coat smear made form the 23 splenomegalic patients, microscopy revealed significant number 17 (73.91%) of the cases were positive for LD-body and all of the four hepato-splenomegalic patients were positive (p-value >0.05, reached from ^{2} test). Results of rK39 ICT for detection of visceral leishmaniasis cases show 100% sensitivity and 90% specificity. Peripheral blood buffy coat examination for LD body in splenomegalic cases of Kala-azar may replace bone marrow examinations.

Key words: Buffy-coat, LD-body, rK39 ICT.

Introduction:

The group of diseases known as the leishmaniases is caused by obligate intracellular protozoa of the genus Leishmania. The geographical distribution of leishmaniasis is limited to the areas of natural distribution of the sandfly, the vector for the disease. Leishmaniasis prevalence is estimated to be 12 million people, and, in developing countries, approximately 350 million people, mainly poor, are at risk of contracting the disease[1].

Visceral leishmaniasis (VL), commonly known as Kala-azar is a chronic febrile disease caused by the protozoan parasite, Leishmania donovani and its sub-species[2]. The visceral form, also known as black sickness or kala-azar in Asia, is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia and is complicated by serious infections. It is the most severe form of the disease and, left untreated, is usually fatal[3].

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During the last few years, increasing number of visceral leishmaniasis cases has been reported from various parts of Bangladesh [4, 5, 6]. At present, the disease is a re-emerging major public health problem in our country. Reports from the district health authorities indicate that >15000 new cases per year would not be an overestimate[10]. Current prevalence is estimated as 40,000 cases per year and more than 20 million population of Bangladesh is at risk of developing the disease[7].

Detection of Leishmania parasite in patients by classical methods at the early stage of infection is limited. Demonstration of parasite in tissues of relevance by light microscopic examination of the stained specimen, in vitro culture, or animal inoculation. Detection of parasite DNA in tissue samples now days became a popular diagnostic method. Immunodiagnosis by detection of parasite antigen in tissue, blood, or urine samples, or by detection of nonspecific or specific antileishmanial antibodies (immunoglobulin) are commonly using by the diagnostic laboratories[1, 2].

The commonly used method for diagnosing VL has been the demonstration of parasites in splenic or bone marrow aspirate. The presence of the parasite in lymph nodes, liver biopsy, or aspirate specimens or the buffy coat of peripheral blood can also be demonstrated. The sensitivity of bone marrow aspirate smear was estimated at 70% or lower [8]. Splenic aspirate, though associated with risk of fatal hemorrhage, is one of the most valuable methods for diagnosis of Kala-azar, with a sensitivity exceeding 90% [9]. The parasites can also be demonstrated by staining buffy coat smear of peripheral blood and revealed 67-99% sensitivity in Indian VL patients and around 50% in African patients[10].

Several serological tests have been developed for diagnosis of VL. Conventional methods for anti-leishmania antibody detection include gel diffusion, complement fixation test (CFT), direct agglutination test (DAT), indirect haemagglutination test (IHT), indirect fluorescent antibody test (IFAT) and countercurrent immunoelectrophoresis. However, aside from practical difficulties at peripheral laboratories, the sensitivities and specificities of most of the above tests have been the limiting factors. Except for the IFAT, which is used on a limited scale, these tests are rarely used at present for routine diagnosis of VL [11].

A recombinant antigen, rK39, has been shown to be specific for antibodies in patients with VL caused by members of the L. donovani complex [12]. Another important facet of anti-rK39 antibody is that the titer correlates directly with the disease activity, indicating its potential for use in predicting response to chemotherapy [13].

Considering the threat of kala-azar and the possibilities of the risk of acquiring the infection, early laboratory diagnosis is essential. Definitive diagnosis of kala-azar requires demonstration of parasites by smear or culture of tissues (spleen, bone marrow, or lymph node), and entails at least one invasive procedure. The major problem with conventional smear is low sensitivity particularly in detecting the occult and subclinical infections.

Efforts to avoid these invasive methods of diagnosis have focused on testing more accessible samples, serum or whole blood for detection of active VL cases. In the present study, staining and microscopy of peripheral blood buffy-coat smear done for detection of LD body for early laboratory diagnosis of VL. Though preparation of buffy–coat needs experience, this diagnostic procedure is comparatively more safe than collection of bone marrow and splenic tissue. Further, the study is aimed to evaluate the diagnostic potential of rK39 Immunochromatographic test (ICT) among the Kala-azar patients in one of the endemic areas of Bangladesh.

Aims and Objectives:

The main objective of the study is to observe the diagnostic role of peripheral blood buffy-coat examination to detect VL cases. To observe the role of rK39 dipstick test for VL case detection in endemic area of Bangladesh

Materials ans Methods:

The protocol of this study was approved by the 'Ethical Review Committee' of Bangabandhu Sheikh Mujib Medical University (Ref: ERC/BSMMU/2007/01). The study was carried out from July 2006 to June 2007. Clinically suspected Kala-azar cases and endemic healthy controls were included from Godagari Upazila of Rajshahi district. Laboratory works were performed at the department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Shahbagh, Dhaka.

Clinically suspected 67 Kala-azar patients of different age and sex from kala-azar endemic area (Thakurjoubana,

Babudayng and Deopara villages of Godagari upazila, Rajshahi) were included in this study as cases. Age and sex matched 30 healthy endemic controls were selected from Thakurjoubana and Babudayng villages in Mohanpur union of Godagari upazila of Rajshahi district. Thirty non-endemic healthy controls were selected from different areas of Dhaka city.

Cases were selected according to the following clinical features as mentioned by Bern et. al., 2000. Fever for more than 3 weeks with at least one of the conditions (Prolonged low grade fever, Discomfort in the hypochondrium, Non-tender splenomegaly with or without hepatomegaly, Loss of body weight, Darkening of skin) with more of the followings and showed no clinical evidence of malaria or tuberculosis were considered as kala-azar suspects and included as cases. Bone-marrow aspiration for the detection of LD-body in the smear was done from all the suspected cases. The number of such patients in this group was 67.

Healthy individuals of comparable age and sex living in Kala-azar endemic area without having past history of prolonged fever, splenomegaly or other clinical features of visceral leishmaniasis mentioned for cases were included as healthy endemic controls. Number of control in this group was 30. Individuals of comparable age and sex living in kala-azar non-endemic area and having no disease at the time of taking blood were included as non-endemic healthy controls. Number of control in this group was 30.

Peripheral venous blood samples were collected from both study cases and controls. Bone-marrow aspirations were done only from study cases. Informed written consent was taken from each patient and control subject or from the legal guardian (in case of minor) before performing invasive procedures like bone-marrow aspiration and venipuncture for blood collection. Following proper precaution and standard techniques, bone-marrow aspiration was performed by attending physician of the concerned word of Rajshahi medical college hospital. Just after aspiration at least three smears were prepared on clean glass slide, air dried and collected for staining and microscopy. Serum was separated after clot retraction from 1.0 ml blood kept in the test tube without anticoagulant. 20µl of separated serum from each sample was used for rK39 Immunochromatographic test (ICT) for Kala-azar on the same day of collection. Buffy-coat was prepared in the department of microbiology and immunology of BSMMU. About 30 minutes after collection of blood, the test tube containing anticoagulant mixed blood was centrifuged at 3000 rpm for 30 minutes. Using Pasteur pipette, 200µl of buffy-coat was collected from top of the centrifuged RBC deposit and at least three smears were made on clean glass slides. Slides were allowed to air dry and preserved for staining and microscopy.

The Kalazar Detect^{TM} Rapid Test (InBios International, Inc., Seattle, WA 98104. USA. P/N 900003.8) for VL is a rapid membrane based immunochomatographic strip assay for the qualitative detection of antibodies to members of L. donovani in human serum. The membrane is pre-coated with a novel recombinant VL antigen on the test line region and chicken anti-protein A on the control line region. During testing, the serum sample reacts with the dye conjugate (protein A-colloidal gold conjugate) which has been pre-coated in the test device. The mixture then migrates upward on the membrane chromatographically by capillary action to react with recombinant VL antigen on the membrane and generates a red line. Presence of this line indicates a positive result, while its absence indicates a negative result. Regardless of the presence of antibody to VL antigen, as the mixture continues to migrate across the membrane to the immobilized chicken anti-protein A region, a red line at the control line region will always appear. The presence of this red line serves as verification for sufficient sample volume and proper flow and as a control for the region. Buffy-coat and bone-marrow smears were stained with Leishman stain and examined under oil-immersion lens of light microscope for the detection of amastigotes of L. donovani (LD-body).

Pre-designed questionnaire and laboratory result forms were used for collection of data and recording of relevant informations for study population. Obtained data from the study was analyzed by using SPSS (Statistical Package for Social Sciences), a computerized software package for biostatical data analysis.

Results:

Bone-marrow and buffy coat smear microscopic examination for LD-body among the all 67 clinically suspected cases of Kala-azar, buffy-coat smear microscopy revealed 21 (31.34%) LD-body positive and bone-marrow smear microscopy revealed 44 (65.67%) LD-body positive. Buffy-coat microscopy among the 60 controls from endemic and non-endemic area all are negative for LD-body.

Table : 1. Findings of Bone-marrow and Buffy coat smear microscopic examination for LD-body.

LD-body in smear microscopy		Study population, N=127		
		Study cases, n=67	Endemic controls, controls, n=30	Non-endemic n=30
Buffy-coat	LD-body found	Φ 21 (31.34)	00	00
	LD-body not found	46 (68.66)	30 (100)	30 (100)
Bone-marrow	LD-body found	Φ 44 (65.67)	00	00
	LD-body not found	23 (34.33)	30 (100)	Not done

Figures within parenthesis indicate percentage
LD-body: Leishmania Donovan-body
Φ (p-value <0.05, reached from χ^2 test)

The result of rK-39 ICT among the study population is shows in Table: 2.

Among the study cases 57 (85.07%) were positive by ICT and 10 (14.93%) were ICT negative. Out of 30 healthy controls from endemic area, 3 (10%) were positive by IC and rest 27 (90%) were negative. While all healthy controls from Kala-azar non-endemic area were found negative by ICT. The diagnostic sensitivity of ICT was 100%; buffy-coat smear microscopy sensitivity was 47.72%. Specificity of all of the laboratory methods except ICT was 100%. Diagnostic specificity of ICT was 90%.

Table: 2. Result of rK-39 ICT among the study population, N=127.

rK-39 ICT	Study cases, n=67	Study population (N=127) Controls, n=60	
		Endemic, n=30	Non-endemic, n=30
ICT Positive	57 (85.07)	03 (10)	00(00)
ICT Negative	10 (14.93)	27 (90)	30 (100)

Figures within parenthesis indicate percentage
ICT : Immunochromatographic test.

Table: 3. Among the 23 only splenomegalic patients 100% showed bone-marrow smear microscopy positive and 17 (73.91%) were positive by buffy-coat smear microscopy. All four (100%) hepato-splenomegalic patients were found positive both bone-marrow and buffy-coat smear microscopy for LD-body.

Table : 3. Detection of LD-body in relation with Hepato-splenomegaly.

Organomegaly		LD-body microscopy positive	
		Bone-marrow, n=44	Buffy-coat, n=21
Splenomegaly	Positive, n=23	23 (100)*	17 (73.91)*
	Negative, n=44	21 (47.72)	00 (00)
Hepato-splenomegaly	Positive, n=04	04 (100)	04 (100)
	Negative, n=63	40 (63.49)	00 (00)

Figures within parenthesis indicate percentage
*(p-value >0.05, reached from χ^2 test)

Among the study cases, 11-20 years age group constituted the highest number 20 (29.85%) followed by up to 10 years (26.86%) and 21-30 years (23.88%) age groups. Highest numbers of controls from endemic and non-endemic area were found in 11-20 year age group, 33.3% and 26.6%.
Out of 67 study cases, 26 (38.8%) were male and 41 (61.2%) were female; male to female ratio was 1:1.57. Out of 30 endemic control 18 (60%) were male and 12 (40%) were female with male to female ratio 1.5:1, while among the non-endemic controls, equal 15 (50%) number were male and female; male to female ratio was 1:1.

Discussion:

The main objective of the study was determined the diagnostic sensitivity and specificity of the peripheral blood buffy-coat microscopy for LD-body in clinically suspected kala-azar cases. Buffy-coat smear microscopy results compared favorably in the diagnosis of VL with the parasitologic examination of bone marrow combined with rK39 dipstick test.

Validation studies of VL diagnostics are usually based on parasitologic examination as a reference test. This should not be too problematic if spleen aspirates can be used for case ascertainment in all subjects, but this may associated with fatal haemorrhage and are advocated only in tertiary level hospitals. Bone-marrow examination was done in this study for parasitologic evaluation. Furthermore it is not possible to obtain spleen aspirate for all patient with a negative bone-marrow because of contraindications or patient refusal. When a reference test with sub-optimal sensitivity for case ascertainment is used, true VL cases are missed and therefore included in the group of controls. They will generate a positive result in any new test (assuming this new test is 100% sensitive). For those cases, the new test is actually right

while the reference test is wrong, and the specificity of the new test will thus be systematically underestimated.

In the present study, out of 67 clinically suspected cases of kala-azar, bone-marrow smear microscopy detected LD-body in 44 (65.67%) cases (Table: 1). This result is comparable with microscopic detection of LD-bodies in bone-marrow smear by other investigator[8]. Although the sensitivity of parasite detection in the bone-marrow smear is reportedly high next to splenic aspirate smear in comparison with other tissues, but due to its inherent limitations of smear examination like low parasite concentration or inadequate smear preparation probably might have missed some cases. Leishmania culture has not been performed in this study, but it is assumed that the parasite detection rate could have been better if culture could be done.

Diagnostic sensitivity and specificity of buffy-coat microscopy was 47.72% and 100% respectively. The diagnostic sensitivity of buffy-coat microscopy by other investigator was much higher than this study [10]. Diagnostic sensitivity of buffy-coat microscopy is higher in the diagnosis of Indian VL cases (69-99%) than in African VL cases (50%). Reasons may be due to preparation of buffy-coat was not satisfactory for the smear preparation and microscopy. Sometimes buffy-coat preparation was not done soon after the collection of blood specimens; it may cause haemolysis and poor quality buffy-coat smear.

In this study we have performed rK39 Immunochromatographic (ICT) dipstick test for the detection of anti-leishmanial antibodies in the serum of all study population. There were 57 (85.07%) cases detected positive by ICT out 67 clinically suspected VL patients. ICT was found positive in three (10%) endemic healthy controls out of 30 in this group and was negative in all 30 non-endemic controls (Table: 2). Diagnostic sensitivity and specificity of rK39 ICT were 100% and 90% in this endemic area of kala-azar. However, it is important to point out the strip test for anti-rK39 antibody may not be equally sensitive and specific among patients of different regions. In the first extensive field trial in 323 patients, other investigators found the strip test to be 100% sensitive and 98% specific[14]. In the Sudan study, all the parasitologically confirmed VL patients who tested negative by the rK39 strip test showed IgG against rK39 by micro-ELISA (though at lower titers)[130]. High levels of specificity (97 to 100%) have been reported uniformly for this test; however, with a later version of the rK39-treated strips, some (12.5%) healthy endemic control subjects also tested positive. While such reactions might be considered to be false positive, these probably represent subclinical infections: Anti-rK39 IgG may be present in serum for an extended period after successful treatment for VL; thus, patients with suspected relapse of VL with a past history of infection would not be candidates for diagnosis by strip testing. Another drawback of this format is that an individual with a positive rK39 strip test result may suffer from other diseases (malaria, typhoid fever, or tuberculosis) with clinical features similar to those of VL yet be misdiagnosed as suffering from VL. Not withstanding these limitations, the rK39 immunochomatographic 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 and simple and can be performed even by paramedics in prevailing difficult field conditions[14].

Among the 23 splenomegalic cases 100% revealed LD-body in bone-marrow smear microscopy and 17 (73.91%) in buffy-coat smear. All four cases of hepato-splenomegalic cases were positive for LD-body both in bone-marrow and buffy-coat (Table: 3). Findings of organomegaly and the good correlation of buffy-coat smear microscopy positivity was observed in this study. Unfortunately no study report was found available to compare the result of LD-body detection rate in organomegalic patients of VL.

The study population was distributed into different age groups. Among the age groups 11-20 years constituted the highest number 20 (29.85%) cases followed by up to 10 years 18 (26.86%). Further majority of cases 16 (23.88%) were in 21-30 years age group (Table: 4.1). It has been found in different studies that kala-azar is a disease predominantly affecting the young people [8,9,14] and our findings in relation to the age of the patients are in good agreement with that of others. The reasons for the higher prevalence of kala-azar among comparatively younger age groups remain to be explored. But it is speculated that these people are more in their active life and as a consequence there is also more chance to be exposed to the environment favorable for kala-azar transmission.

Both males and females constituted our study population. There was female preponderance in the study cases with

female to male ratio of 1.57:1 (Table: 4.2). Kala-azar affects usually more in male than females has been revealed by other investigators [8,78,80,82]. In our social form, it is expected that males are more exposed to the environment suitable for the transmission of kala-azar because of their nature of work. In this study, cases were selected from the endemic area of Rajshahi and they are tribal group of people (Saotal). In this particular community females are the main working group, usually farmer, so they are more exposed to the environment may be suitable for the transmission of kala-azar.

Conclusion and Recommendations:

Laboratory test always has definite place for confirmation of clinical diagnosis of diseases more so for kala-azar because of its variable clinical manifestations and sharing of host by other diseases. Definitive diagnosis of kala-azar requires demonstration of parasites by smear or culture of tissues (spleen, bone-marrow or lymph node), and thus entails at least one invasive procedure. These techniques are also cumbersome, time consuming, and not suitable for field. Bone-marrow and splenic aspirations requires admission of the patient in hospital and need close supervision after aspiration to manage complications associated with the procedures. LD-body can be demonstrated in peripheral blood buffy-coat, and collection of blood specimens does not require hospital admission of the patient. Considering the results of peripheral blood buffy-coat smear microscopy from the present study, we would like to recommend the laboratory method for the suspected kala-azar case detection especially the patients those have splenomegaly or hepatomegaly. Results of rK39 ICT obtained from the present study recommend this highly sensitive, rapid, easy to perform, and minimally invasive diagnostic test to be used anonymously in the field for screening population at risk. Diagnostic specificity of the rK39 ICT in endemic area should be evaluated with large number of cases and controls in the perspective of Bangladesh.

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