A Study of rK39 Antigen Strip Test in Bone Marrow Aspiration Test Confirmed Kala-azar Patients And Control Group

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ABSTRACT
INTRODUCTION
Demonstration and isolation of parasites is the most reliable and conventional method for diagnosis of visceral leishmaniasis (kala-azar). The parasites are commonly demonstrated in the splenic or bone marrow aspirates. However facilities for parasitological diagnosis are often difficult in rural areas. The rK39 strip test is a new immuno-chromatographic strip test using the recombinant Leishmania chagasi K39 antigen and suitable for use under field condition.

OBJECTIVE
The aim of this prospective study is to determine the usefulness of rK39 strip test, as a non-invasive method of diagnosing visceral leishmaniasis under field conditions by testing serum antibody to leishmania antigen rK39.

METHODOLOGY
It is an descriptive study. The material used is Kala-azar detect test(rK39 strip test) which is an immuno-chromatographic test designed for the qualitative detection of antibodies against Leishmania chagasi rK39 antigen in serum of patients with visceral leishmaniasis.

The study enrolled 30 patients with bone marrow confirmed visceral leishmaniasis and 30 healthy controls from same endemic zone. Out of 30 patients 20 patients were from Sukraraj Tropical and Infectious Disease Hospital Teku which is a tertiary care center for infectious and tropical diseases in Nepal and the remaining 10 patients were from Rangeli District Hospital Morang which is situated in visceral leishmaniasis endemic zone of Nepal. For healthy controls attending relatives or friends of the patients, visiting Rangeli District Hospital, Sukraraj Tropical and Infectious Disease Hospital Teku and NAMS Bir Hospital which is a tertiary referral center of Nepal, were included. rK39 strip test was performed with sera of these patients and healthy controls.

MAIN OUTCOMES AND MEASURES
Sensitivity, specificity, positive predictive value and negative predictive values of rK39 strip test were calculated taking bone marrow aspiration test positive case as a standard.

rK39 strip test was positive with sera of all patients and it was negative with sera of all healthy controls in this present study sensitivity of rK39 strip test is 100% and specificity 100%

Positive predictive value 100%, negative predictive value 100% and P Value is 0.00001

CONCLUSION
The study shows that rK39 test is a rapid, accurate and simple test which can be performed in peripheral health centers of endemic countries like Nepal where equipped laboratory facilities and expertise to perform invasive methods such as bone marrow and splenic aspiration for the diagnosis of visceral leishmaniasis cases is lacking. The study shows that rK39 strip test is as equally effective as bone marrow aspiration test in diagnosing active visceral leishmaniasis patients, who had no past history of visceral leishmaniasis.

Key words: Bone marrow, kala-azar, rK39 strip test, visceral leishmaniasis,

INTRODUCTION
The term Leishmaniasis refers collectively to various clinical syndromes caused by obligate intracellular protozoa of the genus Leishmania.

Although leishmaniasis is a worldwide disease, affecting 88 countries; 90% of cases of VL occur, in poor rural areas of Bangladesh, India, Nepal, Sudan and Brazil. In Nepal 5.5 million population are estimated to be at risk of kala-azar. The main kala-azar affected districts in...
Nepal are Bara, Parsa, Rautahat, Siraha, Dhanusha, Sarlahi, Saptari and Morang.  

In India as well as Nepal the visceral leishmaniasis is caused by *Leishmania donovani* and man appears to be chief host. In other areas it is endemic affecting mostly children and visitors. 1,4,5,6 The *Leishmania* species responsible for Visceral Leishmaniasis (VL) are *L. donovani L infantum* and *L chagasi*. 1,4,5,6

The leishmaniasis is an intracellular parasite. It infects and divides within macro phases. 7,8

Visceral leishmaniasis (VL) typically present as a chronic systemic illness with fever, weight loss, splenomegaly, hepatomegaly, hypergammaglobulinemia and bone marrow suppression with pancytopenia. 7,8,9,10

Demonstration and isolation of parasites is the most reliable and conventional method for diagnosis of Visceral leishmaniasis (kala-azar). The parasites are commonly demonstrated in the splenic or bone marrow aspirate and buffy coat of peripheral blood (in HIV co-infection). 7,9,10,11,12

The parasite also can be demonstrated in lymph nodes or liver biopsy and aspirate specimens. 8,9,12

Other methods of diagnosis are immunodiagnosis and detection of parasites DNA in tissue sample using PCR. 1,7

In the study of Zijlstra EE, Ali MS, el-Hassan AM et al, splenic aspiration appeared to be the most sensitive method with sensitivity more than 96%, 13 while the sensitivity of bone marrow aspiration test was reported to be 70%. 13 In the same study the sensitivity of lymph node aspiration test was up to 58%. 13

Culture on NNN or Schneider’s medium has also given successful results. 14

Immunodiagnosis of VL consists of detection of immunoglobulins, non specific or specific anti-leishmaniasis antibodies and detection of parasite antigen. 12 Serological diagnosis is based on the presence of specific humoral antibodies in cases of visceral leishmaniasis. There is a range of serological methods available for the diagnosis of visceral leishmaniasis varying accuracy and specificity. With ongoing research newer better methods are continually becoming available. 14,15,16

Conventional methods for antibody detection includes gel diffusion, compliment fixation test, indirect haemagglutination test, immuno- enzymatic techniques, counter current immuno-electrophoresis, Immunofluorescent Antibodies test 7,10,12,14,15. But all the above test are expensive, sophisticated need a equipped laboratories facilities and difficult to use in peripheral laboratories and field condition.

There are a few tests which are relatively easy to perform in field condition: Direct agglutination test, latex particle agglutination test, dot Elisa and fast Elisa and rK39strip test 7,14,15.

Direct agglutination test: it is highly specific and sensitive test. The method uses whole stained promastigotes, either as a suspension or in freeze-dried forms. The test performed in Nepal and other countries were found to be 91-100% sensitive and 72-100% specific. 13,17,18,19,20 The main limitation of the DAT is its relatively sophisticated procedure and cost which impairs its wide application peripheral health structure. 17,18

Enzyme linked immuno sorbent assay (ELISA) has been used as a potential sero-diagnostic tool in almost all infectious disease including leishmaniasis. The test have excellent sensitivity and varying specificity depending upon antigen used. 12,14,15,16 Several antigens have been studied but the most promising one has been the recombinant antigen rK39. 21 The study done by Kumar K, Pai K, Pathak K et al reported that the high level of anti rK39 antibodies in VL cases suggest the application of rK39 for sensitive and specific sero-diagnosis and rK39 ELISA also valuable in sero-diagnosis in monitoring drug therapy and detecting relapse of the disease. However these require expensive equipment, continues power supply and skilled manpower and thus not appropriate in peripheral laboratories and in field setting. 21

Burns JM, Shreffler Jr WG, Benson DR et al reported a study about a cloning of *Leishmania chagasi* antigen gene and an evaluation of Leishmaniasis patient antibody responses to the recombinant protein rK39. rK39 contains a 39 amino acid repeat that is part of a 230 –KDa protein predominant in *Leishmania chagasi* tissue amastigotes. Sequence analysis showed this protein, LcKin, to be related to the kinesin superfamly of motor protein s. Southern blot analyses demonstrated LcKin –related sequences.
in seven species of Leishmania, with conservation of the repeat between L. chagasi and Leishmania donovani. Serological evaluation revealed that 98% of Brazilian and 100% of Sudanese visceral leishmaniasis patients have high antibody levels to the rK39 repeat. Detectable anti–rK39 antibody was virtually absent in cutaneous and mucosal leishmaniasis patients.22

The study done by Badaro RD, Eulalion MC, Freire M et al, showed that rK39 antigen was very specific with no cross reaction with any of the 100 sera from patients with other diseases or the 100 healthy individual.23

Sundar S, Steven S, Reed G et al., reported that a recently developed nitrocellulose strip test that detects antibody to the recombinant amastigote antigen rK39 is highly sensitive and specific for the diagnosis of active VL in a clinical setting.24

The invasive method and sophisticated methods for visceral leishmaniasis are not appropriate in rural endemic areas due to the lack of trained medical personnel and equipped laboratory facilities. For rural endemic regions, a simple, cheap and reliable test with good sensitivity and specificity is required. rK39 strip test serves the purpose.

OBJECTIVE

The aim of this prospective study is to determine the usefulness of rK39 strip test, as a non-invasive method of diagnosing visceral leishmaniasis under field conditions by testing serum antibody to leishmania antigen rK39.

METHODOLOGY

It is a descriptive study, was carried out in Sukraraj Tropical and Infectious Disease Hospital, Teku Kathmandu which is a tertiary referral center of Nepal, for tropical and infectious diseases, Rangeli District Hospital Morang, situated in VL endemic zone of Nepal and National Academy for Medical Sciences Bir Hospital a tertiary referral center of Nepal. Duration of study was from March 2003 to July 2005.

The study enrolled 30 patients with bone marrow confirmed visceral leishmaniasis (kala-azar) and 30 healthy controls from the same endemic zones. As far as possible case and control were matched with age sex and occupation. Out of 30 patients 20 patients were admitted in Sukraraj Tropical and Infectious Disease Hospital Kathmandu. The patients were from different visceral leishmaniasis endemic districts of Nepal, such as Sarlahi, Saptari, Bara, Rautahat etc. The remaining 10 patients were admitted in Rangeli District Hospital in Morang.

The healthy controls were from Morang, Sarlahi, Bara, Saptari, Dhanusa, etc. For healthy controls, attending relatives or friends of the patients visiting Rangeli District Hospital, Sukraraj Tropical and Infectious Disease Hospital Teku and NAMS Bir Hospital Kathmandu, who had come from endemic zones, having no fever, without splenomegaly and had no past history of VL were included.

1. Inclusion criteria:

Patients of all ages and both sex, patients having fever for fourteen days or more with splenomegaly, bone marrow aspiration test for leishmania donovani (LD) bodies positive were included in the study.

2. Exclusion criteria

Patients having past history of visceral leishmaniasis and bone marrow aspiration test negative for LD bodies were excluded from the study.

Materials

Kala-azar detect test (rK39 strip test) is an immuno-chromatographic test designed for the qualitative detection of antibodies against Leishmania (L) chagasi rK39 antigen in serum during active infection.

Test Procedure

20μl of room temperature sera is added to the test strip in the area beneath the arrow.

The test strip is placed into a test tube so that the end of the strip is facing downward as indicated by the arrows on the strip.

2-3 (150μl) drops of the Chase Buffer solution is added, which is provided with the test kit.

The result is read in 10 minutes.

A Negative Result:

The test is negative when only the control line appears. No test line is present.

An Invalid Result:

The test is invalid when no lines appear.

A positive test:

The test is considered positive when two red or pink lines appear.
TOOLS AND TECHNIQUE FOR DATA COLLECTION

Each patient was explained about the study and a written consent was taken from the patient or his/her guardians. For each patient a detailed medical history was obtained and complete physical examination was done. Bone marrow aspiration test was done in the same hospital where patients were admitted. If the bone marrow was positive for LD bodies, the patient’s was included in the study. On the same patient, rK39 strip test was done.

rK39 strip test was done in the same hospital laboratory for the patients of Rangeli District Hospital. For patients who were admitted in Sukraraj Tropical and Infectious Disease Hospital, the dipstick test was done in the same hospital laboratory or in the central Public Health laboratory or in the laboratory of Bir Hospital NAMS. Bone marrow aspiration slides from Sukraraj Tropical and Infectious disease Hospital Teku were read either in same hospital laboratory or in Central Public Health Laboratory Kathmandu. Bone marrow slides from Rangeli District Hospital were read in the same Hospital and the slides were later also seen in the Pathology Department of Bir Hospital NAMS. The same result was found in both laboratories. All Bone Marrow aspiration test positive cases were considered true positive and included in the study.

For each patient blood was taken and investigated for:

- Hemoglobin, total cell count and differential cell count, malaria parasites.

For each patient’s formol gel test (Aldehyde Test) was also performed.

For healthy controls, a detailed medical history was taken, complete physical examination was done and only rK39 strip test was performed. Bone marrow aspiration test was not done. From each individual blood was collected and tested for rK39 strip test in the laboratory of NAMS Bir Hospital.

Diagnostic Criteria:

- Leucopenia was defined as a white blood cell count less than 4x10^9/liter.

- Anemia was defined as a hemoglobin level less than 12 g/dl for women and less than 13g/dl for men.

Statistical Method

The data were processed with SPSS for windows version 10.0.5 (SPSS Inc Chicago IL).

A patient with a positive bone marrow aspiration test was considered true positive (a kala azar case).

A healthy control with negative rK39 strip test is considered true negative.

The sensitivity, specificity, positive predictive value, negative predictive value is calculated by using standard formula

CHI -SQUARE TEST is used for testing the significance of difference between two methods.

P value less than 0.05 was considered statistically significant.

The continuous variables were reported as Mean ±SD.

Age distribution among patients and control group.

The mean age of the patients included in this study is 25.5 ± 14.33 SD. In this study the most frequent age group is 26-30 (20%), followed by age group 0-10 (17%), age group 16-20 and 21-25 are of 13% of each. Number of patients older than 40 years of age is less in this study.

The age distribution is shown in the following bar diagram.

![Figure 1](image-url)

The mean age of the individuals in the control group is 27.13 ± 16.12 SD. In this study the most frequent age group is 21-25 (30%) followed by 26-30 (20%) and 16-20 (17%). The age distribution of the control group is shown in the following bar diagram.
T-test was done assuming two samples with equal variance. The calculated P-value is equal to 0.6799, which is statistically not significant.

Sex distribution among patient and control group:

In this patient group, there were 53% males and 47% females. The male to female ratio is 1.14:1.

In the control group there were 57% of male and 43% of female. The male to female ratio is 1.3:1.

Occupation among the patients group and control group:

Most of the patients under study were farmer (33%) and housewife (33.3%) followed by student (10%) the result is shown in bar diagram.

Similarly in the control group most of the individuals were housewife (30%), farmers (26%) and student (20%) The result is shown in the bar diagram.

Sign and symptoms observed in patients:

All patients had fever. The mean temperature was 103.26°F ± 0.69 SD with duration of 12.69±8.25 weeks.

Anemia was seen in all patients.

Weight loss was seen in all patients. The mean weight loss was 7.56±3.75 SD.

All patients had splenomegaly. The mean spleen size was 8.48±3.46 SD.

Liver was palpated in 90% of the patients. The mean liver size was 2.8±1.6 SD.

Sign and symptoms are shown in the table below:

<table>
<thead>
<tr>
<th>Sign and symptoms</th>
<th>frequency</th>
<th>Percent</th>
<th>Remark(mean value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatomegaly</td>
<td>27</td>
<td>90</td>
<td>2.8±1.6</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>30</td>
<td>100</td>
<td>8.48±3.46</td>
</tr>
<tr>
<td>Fever</td>
<td>30</td>
<td>100</td>
<td>103.26±0.69</td>
</tr>
<tr>
<td>Weight loss</td>
<td>30</td>
<td>100</td>
<td>7.56±3.57</td>
</tr>
<tr>
<td>Anaemia</td>
<td>30</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Laboratory Investigation:

Leucopenia was seen in 33% of the patient. The mean level of TC was 4900/cm³± 1580 SD. The result is shown in table:

Anemia was found in all patients (100%). The mean hemoglobin level was 6.82±1.1 SD.

Frequency of Leucopenia in Patients
rK39 strip test was performed with serum of each patient.
All 30 patients gave positive result with rK39 strip test.

Chi-Square Test

Chi-Square Test is used to test the significance of difference between two tests; bone marrow aspiration and rK39 test. P value is 0.00001. It is highly significant.

DISCUSSION

Definitive diagnosis of VL required demonstration of Leishmania amastigotes in aspiration of bone marrow or spleen. However, facilities for parasitological diagnosis are often difficult to access for patients living in villages. We can use serological test as alternative test in field condition and in the remote rural areas. The direct agglutination test (DAT) is a highly sensitive serologic test for VL, and has been widely used in developing countries. However, DAT antigen produced commercially is expensive, and maintenance of local DAT antigen production is challenging.

The rK39 strip is a new immuno-chromatographic strip test using the recombinant Leishmania K39 antigen. The test is suitable for use under field condition across the world. The rK39 strip test has the ideal format for use under field conditions. It is quick and no special equipment is needed. It is simple to carry out and the result can be read easily. It just need small amount about 1-2 ml of blood. It has high sensitivity and specificity, the result of this present study shows that rK39 strip test is a highly sensitive and reliable indicator of active kala-azar with high specificity.

Our study has shown sensitivity 100% and specificity 100% for rK39 strip test.

Sensitivity expresses the ability of a test to identify correctly all those who have the disease that is true positive. A 90% sensitivity means that 90% of the disease people screened by the test will give a “true positive” result and the remaining 10% a false negative result.

Specificity expresses the ability of the test to identify correctly those who do not have the diseases that is true negative. Similarly Predictive value reflects the diagnostic power of the test. The predictive accuracy depends upon sensitivity, specificity and disease prevalence. The predictive value of a positive test indicates the probability that a patients with positive test result has in fact the disease in question.

As the present study is done with patients of endemic area having high prevalence of kala-azar the accuracy of the test is high. Our study has positive Predictive value 100%.
Similar to our study other studies done in Nepal, by Chappuis F, Real S, Singh R et al, at BPKIHS Dharan the sensitivity of rK39 strip test was 97%\(^2\) and in a study carried out by Gyawali K, Thapa S, Devkota B et al, the sensitivity was 96%.\(^6\) However the sensitivity of rK39 strip test was 87% only in the study carried out by Marleen Boelaert et al in BPKIHS Dharan, Nepal.\(^2\) Similar to our study other studies carried out in India also showed the 100% sensitivity of rK39 strip test in confirmed VL cases.\(^{12,28}\)

In a study carried out in Brazil, the sensitivity of rK39 strip test was 90% in parasitologically proven Brazilian visceral leishmaniasis.\(^{29}\) In comparison to India, Nepal and Brazil, the sensitivity of rK39 strip test was found low in Sudan and other countries.

In a study carried out by Henk-Schalling et al., the rK39 has a sensitivity of 85.7%.\(^{19}\) In a study carried out, in Sudan, the sensitive of rK39 strip test was only 67% in parasitologically proven VL patients,\(^{30}\) while in Kuwait it was found to be 80%.\(^{31}\)

According to them the reason for low sensitivity of rK39 strip test in their study is unclear; but differences in the antibody responses elicited, ethnic backgrounds and times since infection may be relevant variables and need to be considered.\(^{30,31}\) According to Silvio F, Carvalho G, Lemos EM et al, there are several hypothesis that could explain this regional variation of the result of the rK39 antigen strip test. First there may be difference in the test accuracy between sub-species of the Leishmania donovani complex, similarly within these sub-species, there may be regional differences as a result of variations in the rK39 antigen. Another possible explanation involves genetic differences in individual patients or in racial sub-groups, similar to differences in host response in patients exposed to Leishmania chagasi in which only 95% of the patients develop progressive disease. There may be non-identified genetic factors mediating the degree of host immune response. In other factors affecting the level of antibody response also may explains the regional difference observed.\(^3\) In contrast to variations in sensitivities for region to region, the rK39 antigen strip test has uniformly high specificity in most of the studies.

The present study showed specificity of 100% and similar result were observed by other studies done in Nepal. In the study of Bern C, Jha S N, Joshi A B et al, the specificity of the test was 100%,\(^18\) and in the study of Boelaert M, Rijal S, Regmi S et al carried out in BPKIHS, Dharan, the estimated specificity was 93%.\(^{29}\) Similar to our study in other studies carried out in India, the specificity of rK39 strip test was found high.

In the study of Goswami RP, Bairagi B, Kundu PK et al, the specificity of the rK39 strip test was 98%.\(^{27}\) In the study of Sundar S, Steven S, Reed G, et al it was 98%.\(^{24}\) and in the study of Singh S, Kumari V, Singh N it was 100%.\(^{32}\) In the study of Silvio F, Carvalho G, Lemos EM et al; in Brazil the specificity was 98%\(^9\) in other study of Iqbal J, Hira P R, Saroj G et al, the specificity was 100%.\(^3\) All these studies have showed that rK39 strip test is a very reliable and rapid test to use in field conditions, where it is difficult to carry out invasive procedures to diagnose visceral leishmaniasis. Besides this in comparative to other test like DAT and ELISA, the strip is very cheap and be used in resource limited setting.

Testing sera from patients other than those with leishmaniasis was not included in present work but other studies have found no cross reaction in sera from patients with other parasitic diseases or from non-endemic control using either rK39 Elisa or rK39 strip test.\(^{24,28,29}\) One of the draw back of this test is that it remains positive long after cure.

The test becomes rarely positive in healthy control.\(^{20,32}\)

In a study carried out by Goswami RP, Bairagi B, Kundu PK, out of 20 healthy controls of endemic area only two were weakly positive. Thirty subjects with past history of VL showed positive results at time points 1, 3, 6, 24, 36 months after complete successful therapy.\(^{28}\)

The study of Singh S, Kumari V, and Singh N showed that IgG anti rK39 antibodies reached a titer of up to $10^6$ within 6 months of infection, started declining thereafter, and completely disappeared in two to three years in successfully treated cases.\(^{32}\)

Although previous studies showed rK39 strip test positive in few endemic healthy control.\(^{19,28}\) Our studies didn’t have a single case of positive healthy control, although we included few healthy controls that had family history of kala-azar.

This shows the high specificity of rK39 strip test in our study.

The strength of our study was that it was carried out in patient’s representatives of the population in whom
this new test would be applied in normal clinical practice. The test was done, on only bone marrow confirmed kala-azar (VL patients). All cases were truly positive. All patients received chemotherapy for kala-azar. In our study patients who had a past history of kala-azar were excluded.

The cause of high sensitivity of rK39 strip test in our study may be because all our patients had typical sign and symptoms of kala-azar. All patients had splenomegaly, all had high grade fever above 102ºF for more than 2 weeks of duration, in most of the patients liver was palpated and all patients gave history of weight loss.

One of the limitations of our study is that comparison of rK39 strip test was done with only bone marrow confirmed VL cases, which has sensitivity of about 70%. 13

If the test were compared with splenic aspiration test confirmed VL cases, it would have been more confirmative, because the sensitivity of splenic aspiration test is more than 96%. 13

In its present form, the rK39 strip test has the ideal format for use in the field, but also has important limitation and should be used with caution. In general positive test results in patients who presents with the classical clinical features of VL, chronic fever, hepatosplenomegaly, wasting, strongly will support the diagnosis of VL.

However it should be noted that a positive test results may be the result of previous sub-clinical infection and therefore not relevant to current illness.

CONCLUSION

Visceral Leishmaniasis is a tropical disease causing a lot of mortality and morbidity mainly in developing countries. Nepal is also Visceral Leishmaniasis endemic country. It is more common in rural areas of Nepal. Confirmed diagnosis of visceral leishmaniasis (kala-azar) needs invasive procedures such as bone marrow aspiration test and splenic aspiration test. Splenic aspiration test is considered as a gold standard but it is rarely performed except in referral centers because of fatal complications and due to lack of trained medical personnel and good laboratory facilities. Bone marrow aspiration test is also not performed in most of the health centers of Nepal. Treatment of visceral leishmaniasis is expensive and most of the drugs are toxic, so confirmed diagnosis before starting treatment is vital to the patients. Because of this, a simple and reliable test which can be performed in peripheral health centers as well as in field settings is required, rK39 strip test is meet all the criteria for the diagnosis of visceral leishmaniasis.

The result of this present study shows that, the sensitivity of rK39 test is 100% and specificity of the test is 100%. The test was carried out with individuals from endemic areas who had suffered from active visceral leishmaniasis and confirmed by bone marrow aspiration test and with endemic healthy controls.

The study shows that rK39 strip test is as equally effective as bone marrow test in diagnosing active Visceral leishmaniasis patients, who had no past history of visceral leishmaniasis, The study shows that in visceral leishmaniasis endemic areas of Nepal where there is lack of trained medical personnel and well equipped laboratories, the rK39 strip test would be a useful confirmatory test for visceral leishmaniasis patients with clinically consistent diseases. rK39 strip test is easy to perform, can be read by paramedical personnel, can be done in any rural areas and is reliable test, so it is very suitable for visceral leishmaniasis endemic countries like Nepal.

There is no false positive response of rK39 test in our study but still studies involving large number of healthy control from endemic areas are recommended to confirm the specificity of rK39 test. As it is a non-invasive procedure and has no complication the patients will also accept it easily and it will be a method of choice equally in tertiary care centers also.

The limitation of this test is that it may remain positive long after cure. Therefore aspiration of bone marrow or spleen is needed for relapsed or re-infected cases of visceral leishmaniasis.

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