RESEARCH NOTE

Investigating the dynamics of *Leishmania* antigen in the urine of patients with visceral leishmaniasis: a pilot study [version 1; referees: 2 approved with reservations]

Prakash Ghosh, Israel Cruz, Albert Picado, Thomas Edwards, Md. Anik Ashfaq Khan, Faria Hossain, Rajashree Chowdhury, Emily R. Adams, Rupen Nath, Joseph M. Ndung'u, Dinesh Mondal

1Department of Formulations, Infectious Disease Research Institute, Seattle, WA, 98102, USA
2Nutrition and Clinical Services Division, International Center for Diarrhoeal Diseases Research, Dhaka, 1212, Bangladesh
3Foundation for Innovative New Diagnostics, Geneva, 1202, Swaziland
4Research Centre of Drugs and Diagnostics, Liverpool School of Tropical Medicine, Liverpool, L3 5QA, UK

Abstract

**Background:** Detection of *Leishmania* antigens in the urine provides a non-invasive means of diagnosis and treatment monitoring of cases of visceral leishmaniasis (VL). *Leishmania* antigen load in the urine may vary between different time-points within a day, thus influencing the performance of antigen-detection tests.

**Methods:** We investigated the dynamics of *Leishmania* antigen in urine collected at three different time points (08:00, 12:00 and 16:00 hours). All urine samples collected were tested with the *Leishmania* Antigen ELISA (VL ELISA) kit, produced by Kalon Biological Ltd., UK.

**Results:** The median concentration of *Leishmania* antigen in urine collected at 08:00 (2.7 UAU-urinary antigen units/ml) was higher than at 12:00 (1.7 UAU/ml) and at 16:00 (1.9 UAU/ml). These differences were found to be statistically significant (08:00 vs. 12:00, p=0.011; 08:00 vs. 16:00, p=0.041).

**Conclusion:** This pilot study indicates that the *Leishmania* antigen concentration is higher in urine samples collected in the morning, which has important implications when the VL ELISA kit or other tests to detect *Leishmania* antigen in urine are used for diagnosis of VL and treatment monitoring.

**Keywords**
Visceral Leishmaniasis; *Leishmania* Antigen; ELISA; Urine; Diagnosis; Treatment monitoring
Introduction

Visceral Leishmaniasis (VL), also known as kala-azar, is a potentially fatal vector-borne disease that, in the Indian subcontinent, is caused by *Leishmania donovani* protozoa, which are transmitted by female *Phlebotomus argentipes* sand flies. The number of cases of VL per year worldwide is estimated to be 0.2–0.4 million, with 20,000 to 40,000 associated deaths. Just six countries, in which the disease is transmitted as part of an anthropo- (Bangladesh, India), zoono- (Brazil) or a probable anthropo-zoonotic cycle (Ethiopia, South Sudan, Sudan), account for 90% of VL cases worldwide.

In patients presenting with VL-compatible signs, namely fever for more than two weeks plus splenomegaly and/or weight loss, VL is usually diagnosed by serology, either with a direct agglutination test or rK39 antigen-based rapid diagnostic tests. When parasite confirmation is required, the main approach is tissue aspirate microscopy (from spleen, bone marrow and to a lesser extent lymph node), which has a variable sensitivity and, because of the invasiveness of the procedure (especially spleen aspiration), requires experienced personnel and should be performed in hospitals where blood transfusion and surgical facilities are available. Besides, the accuracy of microscopic examination is influenced by the ability of the laboratory technician and the quality of the reagents and equipment used.

Parasite confirmation by tissue aspirate microscopy is also used for treatment monitoring, test-of-cure (TOC) and diagnosis of relapses, since serology is useless for this purpose, as anti-*Leishmania* antibodies may remain detectable up to several years after cure. Initial cure rates vary between 49% and 94%. Therefore, alternative, less invasive options to invasive tissue aspiration and microscopy are needed to monitor treatment responsiveness, diagnose relapses and assess cure. Although molecular methods, such as PCR, have shown to be effective in VL diagnosis and treatment monitoring using less invasive samples; unfortunately, these require sophisticated laboratory and trained personnel, and there are no standardized protocols that can be used across endemic settings, which hinders their application.

Antigen detection tests, ideally in less invasive samples such as blood, serum/plasma or urine, are an interesting option, as antigen levels should reflect the parasite load in the patient. These tests also present an advantage over antibody detection in immunocompromised patients with low antibody response, as in *Leishmania/HIV* co-infection. In chronic infections, such as VL, the detection of antigens of the pathogen in blood or serum/plasma can be 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 antigens. Nevertheless, Gao *et al.* proved that it was possible to detect *Leishmania* antigen in the sera of VL patients from China with high sensitivity and specificity. However, many of the problems described above may be avoided by searching for antigens in urine. Several studies have demonstrated *Leishmania* antigens in the urine of VL patients using different approaches, such as countercurrent immunoelectrophoresis, Western blot, latex agglutination test and ELISA.

Fluctuations in the quantity of *Leishmania* antigens excreted through urine might influence the sensitivity of these assays. According to the Clinical and Laboratory Standards Institute guidelines, and confirmed by other authors, urine collected in the early morning contains urinary components at the highest concentration and is more reliable for quantification of urine markers. However, there is no evidence concerning the persistence and levels of *Leishmania* antigen in urine collected in the early morning versus other time points. Therefore, given the utility of antigen detection tests in VL diagnosis and treatment monitoring, we set out to study the dynamics of *Leishmania* antigens in urine in order to determine which time point is the most appropriate to detect *Leishmania* antigens in VL patients using the *Leishmania* Antigen ELISA (VL ELISA) kit (Kalon Biological, Ltd., UK). Further, in a recent study we showed that the parasite load in relapse VL is higher than the primary VL cases. Therefore, we hypothesized that the level of *Leishmania* antigens in urine might differ in different states of VL. In our current study we compared the *Leishmania* antigens level in patients with primary VL and relapse VL.

Methods

Study sites and subjects

This study was conducted at the Emerging Infections and Parasitology Laboratory, International Centre for Diarrheal Disease Research, Bangladesh (icddr,b), between 15 March and 30 April 2016. The study population was a convenience sample of 16 patients with VL (seven primary VL, seven relapse VL and two with treatment failure) who were invited to participate in the study while hospitalized at Surya Kanta Kala-azar Research Centre (SKKRC), the only specialized hospital for VL treatment in Bangladesh. Patients were eligible if they had VL. Patients in the study were grouped as type-1 (primary VL) or type-2 (patients presenting with either relapsed disease or treatment failure). The patients were diagnosed according to the national guidelines for VL management in Bangladesh: a patient from a VL-endemic area presenting with fever for more than 2 weeks, splenomegaly and positive by rK39 rapid diagnostic test (here, Kalazar Detect, InBios Intl., USA was used). Information on clinical and demographic characteristics of the patients is provided in Table 1.

Specimen collection

Urine samples were collected at 4-hour intervals compatible with routine activities at SKKRC from each of the 16 enrolled patients before initiation of treatment. A total of 50 ml midstream urine was collected in a tube containing 0.1% NaN₃ at 8:00, 12:00 and 16:00 hours. Immediately after collection, all samples were stored at -20°C in SKKRC facilities and then transported to icddr,b, maintaining the cold chain. A 2-ml aliquot of urine from each of the subjects and time points was used for this study.

*Leishmania* antigen ELISA

The *Leishmania* Antigen ELISA (VL ELISA) (Kalon Biological Ltd., UK) uses a set of polyclonal antibodies against non-proteic *Leishmania* antigens. As the antigens detected in urine with this kit remain largely uncharacterized, the unit Urinary Antigen Unit (UAU) is used to express the amount of *Leishmania* antigens detected. ELISA was performed...
according to the manufacturer’s instructions, described elsewhere\textsuperscript{16}. Briefly, samples were diluted using the assay diluent provided with the kit and a 1:20 dilution was used to determine the antigen concentration. A total of 100 µl diluted urine were tested in triplicate together with duplicates of the antigen calibrators included in the kit using 96-well ELISA plates. After incubation at room temperature optical density (OD) was read at 450 and 620 nm (Biotek, microplate reader). OD at 620 nm was subtracted from OD at 450 nm for further calculations of UAU. A four-parameter logistic standard curve was constructed for each plate using the calibrator provided with the kit. Then \textit{Leishmania} antigen level in each sample was estimated from the standard curve.

**Statistical analysis**

The difference between antigen concentrations at three different time points of all urine samples was investigated. Based on the distribution of data, a non-parametric test (Wilcoxon matched-pairs signed Rank test) was performed to determine significant differences between medians. To find out any correlation between the antigen concentrations at different time points with participants’ age, Spearman’s test was performed. Mann-Whitney U-test was performed to investigate the difference in the antigen concentrations at different time points within sex and the difference between type-1 and type-2 patients. Statistical analyses were performed using the GraphPad Prism software version 7.03 and SPSS version 20.0.

**Ethics approval and consent to participate**

This study was approved by the icddr,b Ethical Review Committee, research protocol number PR-14093. Informed written consent was collected from each participant, or the legal guardian in the case of children.

**Results**

The median concentration of \textit{Leishmania} antigens was 2.7 UAU/ml, 1.7 UAU/ml and 1.9 UAU/ml in urine samples collected at 08:00, 12:00 and 16:00, respectively (Figure 1). Most of the study subjects (9/16, 56.3%) showed highest urinary \textit{Leishmania} antigen concentration at 08:00 (Table 2). The five patients presenting the highest antigen concentration at other time points had either identical or similar levels at 08:00. Only two patients (ID, 7 and 16) showed a marked decrease in antigen concentration at 08:00 compared to the 16:00. The median concentration of \textit{Leishmania} antigens in urine collected at 08:00 was significantly higher than the median concentration of \textit{Leishmania} antigen in urine collected at 12:00 (p=0.011) and at 16:00 (p=0.041) (Figure 1). However, we did not find significant differences in the \textit{Leishmania} antigen levels between urine samples collected at 12:00 and 16:00 (p=0.820). Further, the investigation did not find any correlation between the antigen concentrations at different time points with participants’ age and sex (Table 3). In addition, the concentration of antigen in urine of primary VL cases did not differ with the antigen concentration in patients with VL relapse or treatment failure (Table 3).

**Discussion**

One of the antigen detection tests most widely used in VL diagnosis is the KAtex latex agglutination test (Kalon Biological, Ltd., UK). Although the first studies showed very promising results, further evaluations proved that this test returns variable sensitivity (36–100%) and specificity (64–99\%\textsuperscript{20}), which has limited its wide use for both diagnosis and treatment.

**Table 1. Clinical and demographic characteristic of the VL patients included in the study (N=16).**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, mean ± SD</td>
<td>33 ± 21</td>
</tr>
<tr>
<td>Children (&lt;17 years), n (%)</td>
<td>3/16 (19%)</td>
</tr>
<tr>
<td>Adults (≥17 years), n (%)</td>
<td>13/16 (81%)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>8/16 (50%)</td>
</tr>
<tr>
<td>Splenomegaly, n (%)</td>
<td>16/16 (100%)</td>
</tr>
<tr>
<td>Fever for ≥2 weeks, n (%)</td>
<td>16/16 (100%)</td>
</tr>
<tr>
<td>Duration of fever in days, mean ± SD</td>
<td>20.25±4.20</td>
</tr>
<tr>
<td>ESR, mean ± SD\textsuperscript{*}</td>
<td>88.3 ± 11.92</td>
</tr>
<tr>
<td>Hepatomegaly, n (%)</td>
<td>11/16 (69%)</td>
</tr>
<tr>
<td>Darkening of skin, n (%)</td>
<td>14/16 (87%)</td>
</tr>
<tr>
<td>Pancytopenia, n (%)</td>
<td>7/16 (43%)</td>
</tr>
</tbody>
</table>

\textsuperscript{*}mm/h. ESR, erythrocyte sedimentation rate.
Table 2. Description of participants and antigen concentrations at different time points. Bold figures indicate highest daily concentration.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Patient VL type</th>
<th>Time of antigen concentration assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Relapse</td>
<td>08:00, 0.757</td>
</tr>
<tr>
<td>2</td>
<td>Primary</td>
<td>08:00, 1.016</td>
</tr>
<tr>
<td>3</td>
<td>Relapse</td>
<td>08:00, 12.587</td>
</tr>
<tr>
<td>4</td>
<td>Treatment failure</td>
<td>08:00, 3.103</td>
</tr>
<tr>
<td>5</td>
<td>Primary</td>
<td>08:00, 6.415</td>
</tr>
<tr>
<td>6</td>
<td>Relapse</td>
<td>08:00, 1.31</td>
</tr>
<tr>
<td>7</td>
<td>Primary</td>
<td>08:00, 0.484</td>
</tr>
<tr>
<td>8</td>
<td>Treatment failure</td>
<td>08:00, 37.292</td>
</tr>
<tr>
<td>9</td>
<td>Primary</td>
<td>08:00, 28.784</td>
</tr>
<tr>
<td>10</td>
<td>Primary</td>
<td>08:00, 2.383</td>
</tr>
<tr>
<td>11</td>
<td>Primary</td>
<td>08:00, 6.161</td>
</tr>
<tr>
<td>12</td>
<td>Primary</td>
<td>08:00, 1.915</td>
</tr>
<tr>
<td>13</td>
<td>Relapse</td>
<td>08:00, 52.269</td>
</tr>
<tr>
<td>14</td>
<td>Relapse</td>
<td>08:00, 4.940</td>
</tr>
<tr>
<td>15</td>
<td>Relapse</td>
<td>08:00, 0.678</td>
</tr>
<tr>
<td>16</td>
<td>Primary</td>
<td>08:00, 0.422</td>
</tr>
</tbody>
</table>

Mean [UAUAg] 10.03 | 4.62 | 5.60
SD [UAU] 15.05 | 6.17 | 12.63
Median [UAU] 2.7 | 1.7 | 1.9
IQR 0.8-11.04 | 0.6-6.8 | 0.9-3.45

VL, visceral leishmaniasis; UAU, urinary antigen unit.

Table 3. Correlation between participants’ age, sex and disease type with the antigen concentrations in urine collected at three different time points.

<table>
<thead>
<tr>
<th>Time points</th>
<th>Correlation between participant age and Ag concentration, r (P)</th>
<th>Difference in Ag concentration by sex</th>
<th>P value</th>
<th>Difference in Ag concentrations by Patient type</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male median (IQR)</td>
<td>Female median (IQR)</td>
<td></td>
<td></td>
<td>Type 1, median (IQR)</td>
</tr>
<tr>
<td>8:00</td>
<td>-0.411 (0.114)</td>
<td>4.04 (1.1-24.73)</td>
<td>2.74 (0.53-6.05)</td>
<td>0.505</td>
<td>1.92 (0.48-6.2)</td>
</tr>
<tr>
<td>12:00</td>
<td>-0.441 (0.088)</td>
<td>2.81 (0.60-9.4)</td>
<td>1.6 (0.60-5.24)</td>
<td>0.721</td>
<td>1.7 (0.48-5.92)</td>
</tr>
<tr>
<td>16:00</td>
<td>-0.435 (0.092)</td>
<td>1.84 (0.70-6.2)</td>
<td>2.3 (1.1-3.0)</td>
<td>0.721</td>
<td>1.84 (1.03-3.11)</td>
</tr>
</tbody>
</table>

Type 1, Primary VL; Type 2, VL relapse or treatment failure.

Given the potential applications of Leishmania antigen detection tests, the interest in developing new approaches has been sustained and recent efforts have resulted in the development of two standardised, user-friendly, quantitative and direct ELISA tests that may prove to be useful for VL diagnosis and treatment monitoring; the Leishmania Antigen Detect™ ELISA (InBios International Inc., USA) and the Leishmania Antigen ELISA (VL ELISA) (Kalon Biological, Ltd., UK). Moreover, strategies for VL control in the Indian subcontinent (ISC) seems to be working well, currently the main foci to be considered are South America and Eastern Africa, being VL zoonotic in the first and anthropo-zoonotic in the second. Till date, no study has yet been performed to evaluate this test for canine leishmaniasis where it might have the potential to diagnose canine leishmaniasis thereby improving surveillance of reservoirs, proper treatment, monitoring transmission and assessing the
efficacy of control activities in endemic areas including South America and the Mediterranean basin where canine leishmaniasis is an important veterinary issue.

Although these two ELISAs have the potential to be useful for treatment-monitoring in human VL, they also showed that at the same time point, especially at the day of diagnosis, the parasite load can be very different from patient to patient. This could be due to the fact that patients are not in the same moment of the VL episode when they seek for diagnosis, or because the samples were taken at different times of the day. In this pilot study we have tried to address the second explanation, and have found that the highest level of *Leishmania* antigen in urine is obtained with early-morning urine samples. A recent study explored that urine collected in the early morning improves the sensitivity of urinary lateral flow LAM assay for diagnosis of TB in HIV-infected patients, which is congruent with our study finding.

The Kala-azar Elimination Programme in the ISC has been conducting diverse activities since 2005, with active case detection being one of the key activities to stop transmission of VL. However, to eliminate the disease, proper follow-up of treated VL cases and prompt relapse management is no less important, since in the ISC 1–16% of treated VL patients relapse and 10–20% develop post kala-azar dermal leishmaniasis (PKDL). At present icdcb, in collaboration with the Liverpool School of Tropical Medicine and the Foundation for Innovative New Diagnostics, is evaluating the efficacy of the *Leishmania* Antigen ELISA (VL ELISA) kit for diagnosis of VL, PKDL and asymptomatic infection in Bangladesh. Thus it is critical to ensure that the urine samples are taken at a time and in conditions that increase the chances of detecting *Leishmania* antigens. In this pilot study we have assessed the dynamics of *Leishmania* antigens in urine from VL patients attending the SSKRC hospital in Bangladesh, and we have found that urine collected at 08:00 contains the highest amount of *Leishmania* antigens. These findings can be used as a guide to ensure the best performance of the *Leishmania* Antigen ELISA (VL ELISA) kit when used either for VL diagnosis or treatment monitoring, as well as for implementation of this method in endemic regions in future where this disease is zoonotic. Furthermore, prospective studies are warranted to explore the efficiency of *Leishmania* Antigen ELISA (VL ELISA) kit as a predictor of VL relapse.

**Conclusion**

The *Leishmania* antigen load in the urine of VL patients varies at different times during the day, and is highest in the morning. This should be taken into account in order to increase the sensitivity of the *Leishmania* Antigen ELISA (VL ELISA) kit, and to harmonize sample collection time points during treatment follow-up, so the comparison of the measurements taken on different days can be reliably compared.

**Data availability**

Dataset 1. Details of patient symptoms, demographic information and results of ELISA for *Leishmania* antigens. Leishmania antigen load is not found in this Dataset, but can be found in Table 2. https://doi.org/10.5256/f1000research.16181.d217632
text.

**Grant information**

This work was supported by funds from the Ministry of Foreign Affairs, Government of the Netherlands (Activity Ref. Nr. 22211, Developing Innovative Diagnostics to Address Poverty-related Diseases; www.minbuza.nl).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Acknowledgements**

We are grateful to all the study participants. We are also thankful to icdcb and its core donors: GOB, UKAID, USAID and SIDA.

**References**


The authors have evaluated the leishmanial antigens in urine (as a non-invasive means) in primary and relapse VL as well as in cases of treatment failure, at different time points. Though the pilot study is interesting, it is very preliminary. There are few concerns regarding the manuscript.

1. The sample size is too low.
2. What is the sensitivity and specificity of the assay? Is it better than others reported so far?
3. The authors may assay the PKDL samples as well.
4. The authors mention the advantage of antigen detection in the urine of patients as in *Leishmania* /HIV co-infection. They need to complement their pilot study with few such co-infected cases.
5. The authors also state the importance of antigen detection in urine (that reflects parasite load in the patient) over serum antibody response, as the later are detectable up to several years after cure. The authors need to validate this by including urine from cured patients in their assay.

Minor concerns:
The English language needs to be improved. For instance’ Although molecular methods, such as PCR, have shown to be effective in VL diagnosis.’

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly
**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

---

**Summary**

1. This is a small diagnostic study of a Leishmania antigen ELISA from Bangladesh that explores the variation of antigen load in urine at three different time points over the course of a day.

**General comment:**

1. The overall research question and rationale is of limited scope but sufficiently relevant for publication. However, there are a number of substantial issues that need to be addressed in order to make this research and its reporting scientifically sound.

**Specific comments:**

1. While the authors justify why primary and relapse cases are compared separately, this is not done for age and sex. Unless there are specific reasons to expect clinically relevant differences in outcomes between age and sex for this particular analysis, no formal statistical comparisons with p-values should be done. The authors should either provide a rationale for this subgroup analysis or stick to descriptive comparisons for age and sex.

2. The authors state that “urine collected in the early morning contains urinary components at the highest concentration and is more reliable for quantification of urine markers”. Please refer to the biological rationale behind this phenomenon, and in which other markers this has been observed. If markers in morning urine are considered to be higher than during the day because of urine retention overnight, the factor “time since last urination” becomes important for the 12pm and 4pm measurements in this study. Was this captured? Please add this information if available.

3. In general, the literature search should be updated and more systematic to include the most important relevant recent studies. This is not the case. E.g. four of the five references the authors use to substantiate their statement that “Several studies have demonstrated Leishmania antigens in the urine of VL patients using different approaches, such as countercurrent immunoelectrophoresis, Western blot, latex agglutination test and ELISA” are from 1987, 1995, 2001, and 2002. However, at least two relevant VL antigenuria diagnostic studies have come out in 2018 alone.

4. The sample size of 16 is very small and seems to be a convenience sample. Why was no systematic approach used to ensure representativeness of the sample to the target population? This weakness should be discussed and the study patient characteristics should be compared to the characteristics of the primary/relapse VL patients in the hospital.

5. What was the rationale for including exactly 16 patients? Normally sample size should be prespecified based on estimated parameters. Please justify your sample size decision.

6. HIV status is an important factor associated with antigen expression in urine. Please add this information from your patients if available, and if not please add HIV prevalence estimates from the target population.
7. Time since symptom onset is also very important. Please add this data to Table 1 if available. If this information is not available, please discuss this as a limitation.

8. For relapse patients, how was it established that patients actually had VL? Merely by oral patient history, or through patient treatment charts? If only by asking the patient, please discuss this as a limitation.

9. Table 1 should be improved. Children and adults seem to be mutually exclusive categories, hence one of them can be removed without loss of information. All clinical and lab variables should be clearly defined in the table footnotes. Patients with primary and relapse VL episode should be presented in separate columns.

10. Figure 1 should be improved. A box-whisker plot should be used. It is not clear what the asterixis and horizontal lines refer to.

11. There might be a danger to accidentally de-anonymise your study patients with the data included in Dataset 1. Better remove the following variables: Date of collection, Upazila, District.

12. You refer to Table 1 in the Methods section. However, patient characteristics are part of the results and should be referred to in that section, not earlier.

13. The data of Table 2 should be merged with Dataset 1 to facilitate replication of your analyses.

14. The abstract should include the number of patients included and the time when the study was conducted as well as the place where the study took place.

15. Interquartile ranges should be be added to the median point estimates in the results section.

16. Table 3 should show which statistical test was used for each column. Also show the absolute number of patients in the different strata and columns.

17. The discussion needs substantial reworking:
   - 17.1. The first paragraph of the discussion section should be a succinct summary of the main findings, instead of referring to a test that was not used in this study (KAtex).
   - 17.2. It is not clear why such prominent reference is made to the InBios Antigen Detect ELISA throughout the discussion. It played no role in this particular study. If that test was evaluated in parallel to the presented Kalon ELISA on the same patients, this should be reported together in the same manuscript. Please explain or change.
   - 17.3. Too little reference is made to the existing body of literature. The authors should compare their concrete results to findings from other studies, and discuss how and why their findings might differ. A proper interpretation of the study findings should be presented as well. Keep elaborations about things that are unrelated to your study at a minimum. Instead, the concrete implications of the study findings should be elaborated in more detail.
   - 17.4. Methodological strengths and in particular the weaknesses of the study should be discussed. This part is entirely absent for now.
   - 17.5. Since the sample size was extremely small and probably not representative of the VL patients at that hospital, any conclusions should by default be very cautious. No conclusive findings, can be drawn from this study, and in particular no diagnostic decisions should be taken based on this study. The need for future, properly designed and sufficiently powered studies should be highlighted instead of suggesting that the research question is settled after having conducted this study. Currently the conclusions are too firm and too broad, given the limitations of this research.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly
Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Research methodology, epidemiological and clinical. Infectious diseases outbreak research

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

---

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com