Specific Noninvasive Detection of *Leishmania donovani* in Desquamated Buccal Cell Swab Samples from Human Visceral Leishmaniasis-HIV Coinfected Patients

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Diagnosis of human immunodeficiency virus (HIV) infection with visceral leishmaniasis (VL) coinfection is challenging. Specific diagnosis of VL in HIV-coinfected patients was evaluated by molecular methods in desquamated buccal swab samples, demonstrating 86.3% sensitivity and 98.3% specificity in controls. This test holds significant potential for development as a noninvasive diagnostic tool for VL in HIV-coinfected patients.

Visceral leishmaniasis (VL) and human immunodeficiency virus (HIV) coinfection has emerged as a serious disease prototype and is on the rise in India and Central and South America, posing a difficult challenge to VL subjugation efforts (1, 2). Since its first report in 1985, 35 countries have reported cases of this coinfection (3). In India, the prevalence of HIV-VL coinfection had been reported to be 2% to 5% in a limited number of studies (4). HIV infection increases the risk of developing VL by 100 to 2,320 times in areas of endemicity (5, 6) and greatly increases the probability of relapse cases. Interestingly, VL was also found to promote the clinical progression of HIV disease and AIDS-defining conditions (3). Parasitological diagnosis remains the gold standard in VL diagnosis due to its high specificity and sensitivity; molecular diagnosis is mainly based on PCR assays (7). In contrast, diagnosis of HIV-VL coinfection is very challenging as the clinical manifestations typical of VL are not always present and/or the patients might demonstrate several nonspecific clinical signs. As tissue aspiration for demonstration of the amastigote stage is potentially associated with dangerous risks, diagnosis in field settings is often based on signs, symptoms, and serology. Specifically, splenomegaly is less frequent in coinfected patients (8, 9), and patients have other associated opportunistic infections with analogous symptoms, further complicating the clinical diagnosis of VL (10). Presentation of amastigotes in peripheral blood smears of HIV-VL coinfected patients has a positivity rate of only 50% to 53% (11, 12). Moreover, as HIV-VL patients present a low level of immune response, the validity and usefulness of a rapid immunochromatographic test (ICT) using recombinant antigen k-39 (rk39) would be doubtful even in cases of HIV coinfection (32). Commonly used laboratory diagnostic procedures for VL involve analyses of the cellular and chemical constituents of blood. However, researchers argue that easily collectable sputum samples or buccal swabs would be more practical for VL diagnosis, especially under field conditions (13, 14). Interestingly, several studies have reported the presence of amastigotes in extraordinary locations, such as the spinal fluid, lungs, tonsils, larynx, digestive tract, rectum, etc., in HIV-VL patients (3, 15, 16, 17). Therefore, in the current study, we investigated the potential of the buccal swab as the sample for noninvasive, safe molecular detection of VL in HIV-VL patients on the basis of the presence of leishmanial genomic DNA (gDNA).

The study patient population consisted of (i) 22 diagnostically confirmed HIV patients coinfected with VL, (ii) 31 patients with HIV infection only, (iii) 20 patients with HIV-tuberculosis (TB) coinfection, (iv) 36 confirmed VL patients with negative anti-HIV antibody status, and (v) 28 patients with other diseases such as malaria, viral fever, TB, etc. All patients were randomly recruited from the Rajendra Memorial Research Institute of Medical Sciences, Patna, India. Among the 41 VL-negative healthy individuals (with no history of leishmaniasis), 18 were from a zone of nonendemicity and 23 were from zones of endemicity in Bihar. Fever, pancytopenia, hepatosplenomegaly and hypergammaglobulinemia, and gastrointestinal (GI) symptoms are common indications observed in HIV-VL coinfected patients (18). All individuals were enrolled in the study with informed consent per standard guidelines. This study was approved by the Institutional Ethics Committee of the Rajendra Memorial Research Institute of Medical Sciences (RMRIMS-ICMR), Patna, India. Desquamated buccal epithelial cells of study individuals were collected by Hi-Media sterile transport viscose swab on polypropylene sticks and dipped in transport buffer as described earlier (14). Spiked samples were prepared by mixing counted parasites in decreasing concentrations into the desquamated buccal samples from healthy individuals from areas of nonendemicity as described earlier (14). In addition, a rK39 immunochromatographic test (rK39-ICT) (Kalazar Detect; InBios International) was performed with the excess buccal fluid from the swab stick immediately after sample collection and before suspension of the tube in transport buffer. All samples were subjected to total DNA preparation by the use of a QIAamp DNA Minikit (Qiagen) per the manufacturer’s proto-
col. *Leishmania donovani* (MHOM/IN/83/AG83) parasite gDNA was used as a positive control in all tests.

A quantitative reverse transcription-PCR (RT-qPCR) with an analytical sensitivity of 0.001 parasite DNA equivalents/ml was reported using kinetoplast DNA as a target in VL diagnosis (19). For this study, the 803-bp *L. donovani* (MHOM/IN/80/DD8) kinetoplast minicircle sequence (GenBank accession no. AJ010086) was analyzed using IDT software, and primers were identified as described earlier (20). The two primers used were 5'-TCCTGTTGCCCATTTGTGTGA-3' and 5'-CATTTTCCGTTTTCGGAGA-3', respectively, together designated the kinetoplast minicircle DNA (kDNA) primers, yielding a product of a 231-bp ampiclon size. The gDNA (100 ng) from buccal swab samples was taken for PCR amplification using a recombinant *Taq* DNA polymerase TaKaRa Taq system. A total of 25 μl of a reaction volume was made with rTaq polymerase (1 U) and deoxynucleoside triphosphate (dNTP) mixture stock (2.5 mM each), and kDNA primers (forward and reverse) of 20 pmol/μl (stock) were used to amplify the template gDNA (100 ng) in 10× PCR buffer. This reaction volume was subjected to 35 cycles preceded by 1 cycle of initial denaturation at 94°C for 5 min. Each cycle consisted of denaturation at 94°C for 45 s followed by annealing at 50°C for 30 s and extension at 72°C for 1 min and, finally, extension at 72°C for 10 min. Multiple samples without template were used as negative-control samples to negate the chances of any contamination during the process. Products were analyzed by electrophoresis using a 2% agarose gel containing ethidium bromide (0.5 mg/ml) in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) and were photographed under UV illumination in a gel documentation system (Bio-Rad). Negative controls containing water in place of gDNA as well as negative controls containing gDNA extracted from VL-negative individuals were run concurrently. Later, the product of PCR amplification (231 bp) was subjected to sequencing and, later, to digestion with 3 U of HaeIII (New England BioLabs) at 37°C for 2 h with the compatible buffer. Fragments were separated on 2% agarose, visualized with ethidium bromide, and mapped. The restriction pattern of the kDNA PCR product was analyzed for the purpose of detecting strain differences in the parasites of VL and HIV-VL patients (Fig. 1B and C).

Molecular diagnostics tools for *Leishmania* infections have become popular because of increased sensitivity and accuracy through the use of PCR and quantitation techniques such as real-time PCR (21, 22, 23). We report for the first time, to the best of our knowledge, the presence of *L. donovani* DNA in desquamated buccal swab samples of HIV patients with VL coinfection (Fig. 1A). Nineteen of 22 microscopically confirmed VL cases with HIV coinfection (86.3%) were found to be positive for this test (Table 1). The test was 98.3% specific in the control groups as described region in HIV-VL patients to be 100% homologous to that found in VL patients (Fig. 1C). However, the specificity might be enhanced by newer techniques such as Touchdown (TD) PCR (26). Furthermore, restriction digestion results revealed that the parasite in the HIV-VL buccal swab was similar to that found in VL patients (Fig. 1C). However, in vitro culture of desquamated buccal cells did not render *L. donovani* promastigotes; additionally, amastigotes were also undetectable in microscopy of these buccal cells of HIV-VL patients. It can be concluded that DNA fragments of *L. donovani* parasite were present in the buccal samples. Interestingly, these primers were highly specific for *L. donovani* as it had low homology with common oral micro-

![FIG 1](A) Kinetoplast minicircle DNA (kDNA) PCR of DNA extracted from desquamated buccal swab samples of the study population. Lane 1, molecular DNA ladder; lanes 2 and 3, HIV patients microscopically confirmed for VL; lanes 4 and 5, confirmed VL cases; lane 6, HIV-only infection; lane 7, HIV with TB coinfection; lane 8, patient with malaria; lane 9, healthy subject from zone of endemicity; lane 10, healthy subject from zone of nonendemicity; lane 11, positive control; lanes 12 and 13, negative controls. The arrow denotes the PCR product of 231 bp. (B) Determination of sensitivity of the kDNA PCR for DNA extracted from desquamated buccal swab samples from healthy individuals with *L. donovani* DNA added (spiked samples). The PCR product was visible with as little as 0.1 fg of template DNA. A negative-control (NC) amplification without addition of DNA resulted in no bands. M, molecular DNA ladder. (C) Restriction profile with map showing resultant fragments after digestion of the kDNA PCR product with HaeIII. Lane 1, molecular DNA ladder; lanes 2 and 3, HIV patients microscopically confirmed for VL; lanes 4 and 5, confirmed VL cases.
organisms like Gemella, Treponema, Streptococcus, Veillonella, etc. (27).

Buccal swabs have a distinctive advantage as biological specimens as they do not require special equipment for sampling, conservation, and specialized transport (28). These oral fluids have already been validated for detection of Helicobacter pylori DNA in the saliva of infected individuals (29,30). Other noninvasive approaches for VL diagnosis in coinfections include KAtex (a latex agglutination test; Kalon Biological, United Kingdom), designed to detect Leishmania antigens in urine, which demonstrated 85.7% to 100% specificity and 96% sensitivity in Spain (30,31).

TABLE 1 Test results

<table>
<thead>
<tr>
<th>Study group</th>
<th>No. in the group</th>
<th>PCR</th>
<th>Other test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Confirmed VL patients with HIV coinfection</td>
<td>22</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Patients with HIV infection only</td>
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<td>31</td>
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<tr>
<td>Patients with HIV-TB coinfection</td>
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<td>0</td>
<td>20</td>
</tr>
<tr>
<td>VL confirmed cases/HIV negative</td>
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<td>34</td>
<td>2</td>
</tr>
<tr>
<td>Patients with other diseases</td>
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<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Healthy subjects from zone of endemicity</td>
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<td>2</td>
<td>21</td>
</tr>
<tr>
<td>Healthy subjects from zone of nonendemicity</td>
<td>18</td>
<td>0</td>
<td>18</td>
</tr>
</tbody>
</table>

Notes:
- TB, tuberculosis; NA, not applicable.
- Malaria, viral fever, TB, etc.

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REFERENCES


