SHORT REPORT: OCCURRENCE OF *LEISHMANIA DONOVANI* DNA IN DONATED BLOOD FROM SEROREACTIVE BRAZILIAN BLOOD DONORS

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Abstract. Human visceral leishmaniasis (kala-azar) transmitted by blood transfusion has been described in previous reports. Seroprevalence of antibodies to *Leishmania donovani* was shown to be related to prior blood transfusions in multiply transfused hemodialysis patients in Natal, Rio Grande do Norte, Brazil. In this study, a possible correlation between seroreactivity and the presence of *L. donovani* DNA was investigated in asymptomatic healthy blood donors. Sera were tested using the fucose mannose ligand (FML) ELISA, which was shown to have a sensitivity of 100%, a specificity of 96–100%, reliability, and diagnostic and prognostic potential for the detection of human and canine kala-azar, respectively. Leishmanial DNA was assessed by the polymerase chain reaction (PCR) and dot-blot hybridization techniques in blood and bone marrow samples. Among 21 FML-seroreactive asymptomatic blood donors, 5 (24%) were positive by the PCR and 9 (43%) were positive in a dot-blot assay of blood samples, showing a significant correlation ($\chi^2 = 14.24, P < 0.01$). No *Leishmania* DNA was detected in 20 FML non-reactive blood donors. Our results point to the need for control of transmission of kala-azar by blood transfusion in areas endemic for this disease.

Human visceral leishmaniasis (kala-azar) is a severe chronic disease caused by parasites of the *Leishmania donovani* complex, and it may be lethal if not treated soon after the onset of the symptoms. Its incidence is dramatically increasing in India, Sudan, and Brazil. As intracellular parasites of monocytes, the *Leishmania* parasites are expected to be present in the blood for an undefined period between the skin infection following the bites of sand flies and their final localization in target organs. The development of the disease starts with an undetermined asymptomatic subclinical period in which parasites may already be circulating in the peripheral blood, but no clinical or hematologic changes are noted. Kala-azar transmitted by blood transfusion has already been described in individuals from nonendemic areas of the United Kingdom, Belgium, France, and India, either the presence or absence of a history of foreign travel. The finding of congenital transmission of kala-azar has confirmed the blood-borne characteristic of the disease.

The fucose-mannose ligand (FML) is a surface glycoprotein and a species-specific antigen of *L. donovani*. The FML-ELISA has shown a sensitivity of 100% and a specificity of 96% in the diagnosis of kala-azar when tested in a recent outbreak of the disease in Natal, Rio Grande do Norte, in northeastern Brazil, an area endemic for kala-azar. This assay identified patients with overt disease as well as inhabitants who had subclinical infection with the potential for evolution towards fatal disease (22%). Seroreactivity in Natal to the FML was 9% among healthy blood donors ($n = 1,194$), increasing to 25% in inhabitants of a peri-urban focus for kala-azar and to 37% in polytransfused hemodialysis patients. Risk factors included blood transfusion, whose association with the presence of antibodies to *Leishmania* was statistically significant. In contrast, potential exposure to the bites of sand flies showed no correlation. The prevalence decreased significantly to 7% in hemodialysis patients from Rio de Janeiro, an area in which kala-azar is found only occasionally, and was 0% in patients subjected to continuous ambulatory peritoneal dialysis who had not received a blood transfusion. The prospective analysis of 27 FML-seroreactive donors from Natal detected amastigotes of *Leishmania* in the bone marrow of 1 subject, while 4 had clinical complaints, including splenomegaly and hepatosplenomegaly. However, blood donors might already have antibodies to *Leishmania* but no circulating parasites that could contaminate a recipient. The detection of leishmanial DNA in blood units could clarify this point and provide confirmation of potential infectivity.

In this investigation, we used different assays for the analysis of *L. donovani* infection in kala-azar patients: FML-ELISA of sera, a *Leishmania*-specific polymerase chain reaction (PCR) and dot-blot hybridization assays of bone marrow and blood samples, detection of amastigotes by microscopic examination of bone marrow, and monitoring the decrease in hematocrit values. In addition, blood samples from asymptomatic blood donors, either reactive or nonreactive in the FML-ELISA, were analyzed by the PCR and dot-blot hybridization approaches. The FML-ELISA was performed with 125 ng of FML/well. Sera were analyzed at a dilution of 1:100. Antibodies were detected using peroxidase-labeled protein-A (Sigma, St. Louis, MO). The absorbance values at 492 nm were compared with those of negative and positive controls. The cut-off value of the method was determined according to the Youden test, which circumvents the arbitrariness of other methods, and considers possible errors such as negative results for diseased individuals and positive results for healthy individuals. The use of the mean + 2 SD value (absorbance at 492 nm = 0.204) in the FML-ELISA led to a maximal Youden index value of 1, corresponding to the absence of false-positive or false-negative results. The difference between the kala-azar patients and the asymptomatic blood donors was highly significant ($P < 0.001$). The FML-ELISA reactivity decreased during treatment with antimony and became negative after the parasitologic cure. The FML-ELISA showed negative
**L. DONOVANI** DNA IN BLOOD FROM BRAZILIAN BLOOD DONORS

TABLE 1

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>FML-ELISA serum</th>
<th>PCR blood</th>
<th>Dot-blot blood</th>
<th>Microscopy* blood</th>
<th>PCR BM</th>
<th>Dot-blot BM</th>
<th>Leishmanial† BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kala-azar patients</td>
<td>12</td>
<td>12/12</td>
<td>10/12</td>
<td>5/7</td>
<td>12/12</td>
<td>7/7</td>
<td>4/4</td>
</tr>
<tr>
<td>Blood donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seropositive</td>
<td>21</td>
<td>21/21</td>
<td>5/21</td>
<td>(23%)</td>
<td>9/21‡</td>
<td>0/21</td>
<td></td>
</tr>
<tr>
<td>Seronegative</td>
<td>20</td>
<td>0/20</td>
<td>0/20</td>
<td></td>
<td>0/20</td>
<td>0/20</td>
<td></td>
</tr>
</tbody>
</table>

* Hematocrit values <30%.
‡ Presence of *Leishmania* amastigotes by microscopic examination of Giemsa-stained smears of bone marrow punctures.
‡ Includes 5 that were positive by the PCR.

Results in 1 of 116 samples that were seroreactive for Chagas' disease (22 patients with chronic disease and 94 blood donors; Hemocontro-São Paulo, São Paulo, Brazil), 1 of 21 cases of cutaneous and mucocutaneous leishmaniasis, 0 of 1 case of *Paracoccidioides brasiliensis* infection, and 0 of 2 patients with lymphoma.11

For PCR analysis, genomic DNA was extracted from blood using the RapidPrep Kit (Pharmacia Biotech, Uppsala, Sweden). This kit showed a sensitivity of 97% in tissue samples from patients with leishmaniasis.13 The DNA eluted by the chromatographic procedure was precipitated with sodium acetate and ethanol, and resuspended in 10 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). A hot start PCR was performed with a pair of oligonucleotides that anneal to the origin of replication of both strands of the minicircle molecules that are one of the components of the genus *Leishmania* mitochondrial DNA: primer A: 5'-G(G/C)G/(C/G)C(G/C)CC(A/C)CTAT(A/T)TTTACCACCAACCC-3' and primer B: 5'-GGGGAGGGGCGTTCTGCGAA-3'. These oligonucleotides amplify the conserved region of the minicircle molecule. The PCR was performed using 35 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec. The reactions were performed using 200 ng of each nucleotide, 200 µM of a mixture of the 4 dNTPs, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 2.5 units of *Taq* polymerase. The 120-basepair amplification products were analyzed by electrophoresis on 2% agarose gels followed by staining with ethidium bromide and visualization under UV light. For hybridization, 10 µl of PCR products were denatured in 0.4 N NaOH, applied to a nylon membrane using a dot-blot apparatus, and hybridized with a cloned *L. donovani* minicircle as a specific molecular probe for the *L. donovani* complex.14 The probes were radiolabeled with α-³²P-dATP by hexamer random priming.15 Filters were then hybridized using the bovine lacto transfer technique optimizer16 at 60°C, washed in 0.5× SSC (0.15 M NaCl, 0.017 M sodium citrate) at the same temperature, and exposed to x-ray film overnight with an intensifying screen at –70°C.

Informed consent was obtained by the attending physicians from all patients and blood donors participating in this investigation by means of a standard blood bank questionnaire. Blood donors were aware of their participation in a scientific investigation and that their blood units would not be used for transfusions if they tested positive in the FML-ELISA or in either the PCR or dot-blot assay. Confidentiality was ensured. This study was reviewed and approved by the National Foundation of Health of the Brazilian Ministry of Health (FNS-MS), the National Council of Health (resolution no. 01/88, decree no. 93-933, January 14, 1987), the Federal University of Rio de Janeiro, and the Centro de Matologia e Hemoterapia HEMONORTE (Natal, Brazil).

As shown in Table 1, all blood samples obtained from confirmed kala-azar patients were seroreactive to FML and showed decreased hematocrits (<30%). All bone marrow samples from these patients showed positive PCR and dot-blot assays results. However, microscopic examination of bone marrow for parasites and PCR and dot-blot assays on blood samples were less sensitive. A comparative analysis of blood samples in asymptomatic blood donors showed that of 21 FML-seropositive individuals, 5 (23%) were also positive in the PCR assay. Nine of the samples (43%) were positive in the dot-blot hybridization assay; among them were the 5 PCR-positive specimens already mentioned. Among seronegative blood donors, no reactivity was observed in these 2 DNA assays. Despite the differences in sensitivity among the 3 diagnostic methods, positive results in the PCR and dot-blot assays are highly correlated with seroreactivity to FML (χ² = 14.24, degrees of freedom = 3, P < 0.01, by a critical non-parametric test).

Figure 1 shows products obtained after PCR amplification of samples separated by electrophoresis on an agarose gel stained with ethidium bromide. The positive control corresponds to *L. donovani* mitochondrial DNA. Strong labeling appears in bone marrow and blood sample products from untreated patients showing a positive PCR response (patients 1, 2, and 3). However, the sample from patient 4 shows only a weak reaction in bone marrow and no product was obtained from blood. In a group of individuals undergoing treatment, blood PCR products are labeled in patients 5 and 6 and absent in patients 7, 8, 9, and 10. Sample 11, from a cured patient, is representative of all other negative controls. Weak labeling appears in samples from blood donors that give a positive reaction in the FML-ELISA (b 12). Also shown in Figure 1 are the strong reactions of the PCR-amplified products of blood and bone marrow samples from kala-azar patients 1 and 2 indicated by dot-blot hybridization probes (lower right), and the weak but significant labeling seen in blood products from an asymptomatic blood donor seroreactive to FML (b 12). The PCR and dot-blot assay used in this investigation were previously validated in dogs.17 The PCR has shown a sensitivity of 100% in dogs with infection demonstrated either by culture or inoculation of specimens into hamsters.17
Some parasites may be readily transmitted by transfusion, some only occasionally, and for others the risk is only theoretical. Human cases of transfusion-transmitted kala-azar have already been detected in France, Belgium, and India. All of them corresponded to patients that received blood units from donors resident in areas endemic for kala-azar. Since the donors were considered acceptable by blood bank screening, they were subclinical asymptomatic patients by the time of donation. Overt disease was detected in recipients 6–11 months after the blood transfusion, indicating that the specific blood units contained a minimal but significant parasitic load. In previous work, we demonstrated that blood transfusion is significantly associated with the presence of antibodies to *Leishmania* in polytransfused hemodialysis patients from endemic areas. In this investigation, our preliminary findings on the occurrence of leishmanial DNA in blood of FML-seroreactive healthy blood donors, although corresponding to a relatively small number of samples, are statistically significant. Taken together, these findings lead to the conclusion that some form of screening is advisable to decrease the risk of transmitting kala-azar by blood transfusion, at least in endemic areas. Although most blood banks test their blood donors for human immunodeficiency virus and hepatitis virus, as well as for Chagas’ disease and syphilis (in India and Brazil, respectively), there are several infectious agents for which routine screening is not performed, including *L. donovani*, *Toxoplasma gondii*, cytomegalovirus, and herpes simplex virus. The need for control of blood products to prevent transmission of kala-azar is already recognized in the United States. This recommendation is related to demographic changes and to increases in international travel and human migration from endemic areas. The results of the present investigation support the need to define possible routine preventive measures to evaluate blood samples for leishmanial infection in Brazil and in areas where human visceral leishmaniasis is endemic.

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REFERENCES


