Detection of human T-cell lymphotropic virus type 1 in plasma samples

Fabio Cabral\textsuperscript{a,1}, Liá Barbara Arruda\textsuperscript{c,1}, Marilia Ladeira de Araújo\textsuperscript{a}, Patricia Montanheiro\textsuperscript{a}, Jerusa Smid\textsuperscript{b}, Augusto César Penalva de Oliveira\textsuperscript{b}, Alberto J.S. Duarte\textsuperscript{a}, Jorge Casseb\textsuperscript{a,b,c,}\textsuperscript{*}

\textsuperscript{a} Laboratory of Immunodeficiencies and Dermatology, Sao Paulo University Medical School, Sao Paulo, Brazil
\textsuperscript{b} HTLV Outpatient Clinic, Institute of Infectious Diseases “Emilio Ribas”, SP, Brazil
\textsuperscript{c} Tropical Medicine Institute of Sao Paulo, University of Sao Paulo, Brazil

\textbf{Abstract}

Human T-cell lymphotropic virus type 1 (HTLV-1) is an RNA virus responsible for diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia/lymphoma (ATL). Cell-to-cell contact and Tax-induced clonal expansion of infected cells are the main modes of virus replication, making virus detection during the viremic stage difficult. Consequently, the proviral load is the current virologic marker for disease monitoring, but the mechanisms of progression have not been established yet. Thus, this study investigated the presence of virus in plasma from asymptomatic HTLV-1 carriers and from HAM/TSP patients. Real-time PCR was performed on DNA from 150 plasma samples; 12 (8%) had detectable DNA amplification, including 6 (4%) asymptomatic HTLV-1 carriers and 14 (26%) HAM/TSP patients ($p < 0.005$). Of the 33 samples submitted for nested PCR, six (18%, $p = 0.02$) were positive for HTLV-1 RNA in the plasma. Additionally, 26 plasma samples were treated with DNase enzyme to eliminate any DNA contamination before RNA extraction. Two of them (8%) showed amplification for HTLV-1 ($p = 0.5$). Therefore, this study described for the first time the detection of free HTLV-1 RNA in plasma from HTLV-1-infected subjects, regardless of their clinical status. Thus, HTLV-1 viral replication does occur in plasma, and other transmission pathways for HTLV-1 should be investigated further.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is the etiological agent of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). Currently, 10–20 million people worldwide are persistently infected with HTLV-1 and at risk of developing HAM/TSP and adult T-cell leukemia/lymphoma (ATL). Although most HTLV-1-infected carriers remain healthy, 2.5% may eventually develop HAM/TSP and other inflammatory disorders that have been correlated with HTLV-1 such as myositis, arthritis, dermatitis, uveitis and alveolitis (Hall et al., 1996; Kaplan et al., 1990). HAM/TSP is a chronic debilitating inflammatory disease of the central nervous system characterized by axonal damage and demyelination, which is most pronounced in the mid-thoracic spinal cord (Iwasaki, 1993).

For many viral infections, viral load is used to indicate the degree of viral replication and to determine the probability of host damage during disease progression. One well-known example of viral replication being associated with disease progression is in HIV-1 infection, where plasma viral load correlates with disease progression and is an important surrogate marker in predicting acquired immunodeficiency syndrome (AIDS) development (Mellors et al., 1997).

HTLV-1 replication occurs when a virion comes into contact with immune system cells. GLUT-1, a glucose transporter, is the membrane receptor that allows virus entry into the cell through attachment with the virus surface protein (Manel et al., 2004). More recently, other cell surface molecules have been reported to be important to HTLV-1 attachment and entry, such as NRP-1 and HSPG (Ghez et al., 2010; Pais-Correia et al., 2010). It has been shown that HTLV-1 can be transmitted by cell-to-cell interaction via the induction of cell polarization and transmission of viral genetic material from an infected cell to an uninfected one (Bangham, 2003). Because of this, few HTLV-1 virions are found in human plasma, which does not facilitate virus detection and infection control.

Unlike the large amount of information correlating HIV and its viral load to AIDS progression, few studies have addressed the relationship between the HTLV-1 viral load and the clinical...
infection outcome. The main problem is that HTLV-1 infection has a long incubation time, and less than 5% of carriers will actually progress to an associated disease. It has been previously demonstrated that HTLV-1 replication is higher in HAM/TSP patients than in asymptomatic HTLV-1 carriers (Montanheiro et al., 2005) and that replication occurs in the blood (Best et al., 2006; Kira et al., 1991) and cerebrospinal fluid (Lezin et al., 2005); however, this finding is not yet conclusive. Additionally, the mechanisms behind HAM/TSP development are still unclear. Additionally, HTLV-1 models present two main mechanisms for replication, cell-to-cell and/or Tax induced clonal expansion, thus making the detection of virions in plasma samples very difficult. In this report, we investigated the presence of HTLV-1 in plasma from asymptomatic carriers and HAM/TSP individuals.

2. Materials and methods

2.1. Study population

A total of 216 adult patients (161 asymptomatic and 55 with HAM/TSP) under clinical monitoring in the HTLV out-clinic of the Emilio Ribas Institute of Infectious Diseases, Sao Paulo, Brazil, were enrolled in this study from July 1997 to December 2010. The written informed consent for collecting blood samples and the protocol for this study were approved by Ethical Committee Board of the Emilio Ribas Institute.

After signing the informed consent, patients had 10 mL of venous blood collected in tubes containing EDTA. Plasma samples were separated in two aliquots of 1 mL and stored at –70 °C. DNA was extracted from plasma using the GFX Genomic Blood DNA Purification Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer’s instructions.

2.2. Real-time PCR for DNA HTLV-1 viral load detection and quantification

This step was performed in 150 plasma samples from 123 HTLV-1 asymptomatic carriers and 27 HAM/TSP patients (Group 1) using specific probes (TaqMan system) and primers SK110 and SK111, as previously described (Montanheiro et al., 2005). Probes carried a 5′ FAM reporter dye (6-carboxy fluorescein) and a 3′ TAMRA quencher dye (6-carboxy tetramethyl rhodamine). The pHTLV-ALB plasmid (kindly supplied by Marcelo de Carvalho from Institut Pasteur Fort-de-France, Martinique), whose concentration was determined by UV spectrometry at 260 nm, was used as a quantitative control for each reaction. Albumin DNA detection was performed as an endogenous reference (housekeeping gene) to normalize variations due to differences in the HTLV-1 viral load and/or DNA extraction. Quantitative values were calculated by taking the mean HTLV-1 copy number divided by the mean albumin copy number (Dehee et al., 2002; Montanheiro et al., 2005).

The 50-µL PCR mixture for HTLV-1 or albumin DNA amplification consisted of 10 µL DNA, 200 nM of each primer (SK110 and SK111 or Alb-S and Alb-AS, respectively), 100 nM of HTLV-1 or albumin TaqMan probe, 400 nM dUTP, 200 nM of the remaining dNTPs, 5 mM MgCl2, 0.5 U uracil DNA glycosylase, 1.25 U Taq platinum polymerase and 1 × PCR buffer. The cycle conditions for HTLV-1 and albumin DNA amplifications were as follows: one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, and 45 cycles of 15 s at 95 °C and 1 min at 65 °C. Amplification and data acquisition were carried out using the I-cycler Sequence Detector System (BioRad, Hercules, CA, USA).

2.3. Nested PCR assay

This step was carried out to confirm the source of the amplified material for real-time PCR. A subset of 40 plasma samples (20 asymptomatic carriers and 20 with HAM/TSP) was extracted without previous treatment with DNase enzyme (Group 2) while another subset of 26 plasma samples (18 asymptomatic and 8 with HAM/TSP) was extracted after DNase enzyme treatment to eliminate DNA contamination (Group 3) in accordance with the manufacturers’ instructions (RNase-Free DNase Set, Mainz, Germany). RNA extraction from plasma was performed using the QiAamp RNA Blood Mini Kit (QiAGEN, Hilden, Germany) according to the manufacturer’s instructions. Therefore, nested PCR was performed in a total of 66 samples.

RNA samples were submitted to cDNA synthesis and nested PCR using primers to the HTLV-1 tax/rex region, as previously described (Tuke et al., 1992). cDNA was synthesized from RNA samples using Sensiscript Reverse Transcription (QiAGEN, Hilden, Germany) according to the manufacturer’s instructions and then stored at –20 °C.

Amplification was performed using a conventional thermal cycler according to the following steps: outer PCR was done by an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The conditions for a 50-µL final volume PCR were 2 × PCR buffer (10×), 2 mM MgCl2, 2 µM of each primer, 0.2 µM of each dNTP, 2 U DNA Taq polymerase and 5 µL cDNA. Inner PCR was performed by an initial denaturation step at 94 °C for 5 min, followed by 25 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The conditions for a 50-µL final volume PCR were 2 × of PCR buffer (10×), 2 mM MgCl2, 2 µM of each primer, 0.2 µM of each dNTP, 2 U DNA Taq polymerase and 2.5 µL of outer PCR product.

PCR products were submitted to electrophoresis in a 1.5% agarose gel at 100 V for 60 min. Visualization was performed by ethidium bromide staining under UV light. Fragment length determination was performed using a 100 bp molecular weight ladder reference. PCR products of approximately 128 bp were considered positive.

2.4. Statistical analysis

Differences in patient characteristics and laboratory values were evaluated using a one-way Mann–Whitney’s test or a χ² test with a Yates’ correction test. In both cases, p values <0.05 were considered statistically significant.

3. Results

From the 216 subjects included in this study (mean age 48 years, 36% males), 55 had HAM/TSP and 161 were asymptomatic HTLV-1 carriers. Real-time PCR was performed on DNA from 150 plasma samples; 12 (8%) subjects showed amplification, including 5 (4%) asymptomatic HTLV-1 carriers and 17 (26%) HAM/TSP patients (p < 0.005) (Group 1).

Due to the possibility of the presence of cellular debris and, consequently, proviral DNA in the plasma, an additional subset of 66 patients was assessed for proviral DNA contamination in plasma. In the first step, 40 samples (Group 2) had their RNA extracted and then amplified by nested PCR without retro-transcription to cDNA. Seven samples were proviral DNA positive and were next assessed by nested PCR to amplify the beta-actin gene, and all were positive, suggesting that these samples could have been contaminated by cellular debris or genetic material from the host cells. Thus, the 33 remaining samples were retro-transcribed to cDNA and amplified
by nested PCR using our standard protocol for the molecular diagnosis of HTLV-1 infection (Novoa et al., 2007; Tuke et al., 1992).

Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 150)</th>
<th>Group 2 (n = 33)</th>
<th>Group 3 (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean, years)</td>
<td>48</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Clinical status</td>
<td>Asymptomatic (n = 123)</td>
<td>HAM/TSP (n = 27)</td>
<td>Asymptomatic (n = 19)</td>
</tr>
<tr>
<td>Gender F/M</td>
<td>84/39</td>
<td>13/14</td>
<td>11/8</td>
</tr>
<tr>
<td>Detectable HTLV-1 DNA (%)</td>
<td>5 (4%)</td>
<td>7 (26%)</td>
<td>NA</td>
</tr>
<tr>
<td>HTLV-1 RNA (%)</td>
<td>NA</td>
<td>NA</td>
<td>2 (11%)</td>
</tr>
<tr>
<td><strong>p = 0.02 (IC 95% 2–34)</strong>, Seven of 40 samples had DNA HTLV-1 positive (cellular debris) and were excluded from the analysis.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p = 0.5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable.

**Table 1**

Presence of HTLV-1 RNA or DNA in plasma of asymptomatic and HAM/TSP cases.

4. Discussion

The HTLV-1 proviral DNA load is currently used as a reference for disease progression and has a noted association with HAM/TSP (Matsuzaki et al., 2001; Nagai et al., 1998). Additionally, there is no previous publication reporting HTLV-1 viremia (free virions in the plasma); therefore, little is known about plasma viral load and its impact on HAM/TSP development (Olliondo et al., 2003; Takenouchi et al., 2003).

DNA amplification from the plasma samples of asymptomatic HTLV-1-infected patients and HAM/TSP patients was analyzed in 150 individuals by real-time PCR, and 8% of samples had detectable HTLV-1 amplification in the plasma. There was also a significant difference in amplification between asymptomatic patients and HAM/TSP patients.

The amplification of HTLV-1 DNA in the plasma probably reflects the presence of cellular debris. Thus, further studies with more standardized plasma separation should be performed to assess this hypothesis (Table 1).

Because the presence of HTLV-1 virions in the plasma is not often detected, a more sensitive and specific method was performed to verify HTLV-1 RNA copies in the plasma and to address the possibility of DNA contamination in extracted RNA samples. Nested PCR was carried out in RNA samples with and without DNase pre-treatment because this approach possesses high sensitivity for HTLV-1 detection and is capable of amplifying one copy of HTLV-1 in 10^5 PBMCs (Tossillii et al., 1998).

A subset of 40 HTLV-1 RNA samples was amplified by nested PCR prior to cDNA synthesis. Seven samples were positive for HTLV-1 DNA, indicating the possibility of cellular debris or host genetic fragments in the plasma. The 33 remaining samples were retro-transcribed to cDNA and amplified by nested PCR, yielding six (18%) positive samples for plasma HTLV-1 RNA. This time, however, all the amplified samples belonged to HTLV-1 asymptomatic individuals and no significant difference in the statistical analysis was found. From the 26 plasma samples treated with DNase, the amplification of HTLV-1 RNA was positive for two samples, both from asymptomatic HTLV-1 patients.

There were differences between amplification methods as well as in the size of the groups analyzed. Nonetheless, these findings indicate a possible presence of HTLV-1 in the plasma, suggesting that HTLV-1 could be transmitted by biological fluids in addition to cell-to-cell transmission and clonal cell expansion.

The high sensitivity of this assay allows its use in the clinical setting and provides confidence in the results presented in this study, where HTLV-1 viremia is described for the first time to our knowledge.

Because it was not possible to specifically evaluate HTLV-1 messenger RNA as the source of the HTLV-1 RNA detected, we cannot be completely certain of the viral origin (in terms of a cell free viral particle), although this is the most likely source. The findings of this study indicated that free RNA could be detected in plasma samples among HTLV-1-infected subjects regardless of their clinical status. However, further studies should be carried out to assess whether the HTLV-1 RNA found in the present study could be HTLV-1 mRNA from cellular debris.

Acknowledgements

We would like to acknowledge all the patients who participated in this study. We thank Dana Gallo for the review and discussion. The financial support for this work was provided by FAPESP 99/11188-1 and 2008/56427-4.1.

References


