Evaluation of the use of real-time PCR for human T cell lymphotropic virus 1 and 2 as a confirmatory test in screening for blood donors

Análise do uso da PCR em tempo real para HTLV-1 e 2 como teste confirmatório na triagem de doadores de sangue

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ABSTRACT

Introduction: HTLV-1/2 screening among blood donors commonly utilizes an enzyme-linked immunosorbent assay (EIA), followed by a confirmatory method such as Western blot (WB) if the EIA is positive. However, this algorithm yields a high rate of inconclusive results, and is expensive. Methods: Two qualitative real-time PCR assays were developed to detect HTLV-1 and 2, and a total of 318 samples were tested (152 blood donors, 108 asymptomatic carriers, 26 HAM/TSP patients and 30 seronegative individuals). Results: The sensitivity and specificity of PCR in comparison with WB results were 99.4% and 98.5%, respectively. PCR tests were more efficient for identifying the virus type, detecting HTLV-2 infection and defining inconclusive cases. Conclusions: Because real-time PCR is sensitive and practical and costs much less than WB, this technique can be used as a confirmatory test for HTLV in blood banks, as a replacement for WB.

Key-words: HTLV. Blood donors. Real-time PCR. Western blot.

RESUMO

Introdução: A triagem para HTLV-1/2 em doadores de sangue geralmente utiliza imunoensaio enzimático, seguido de um método confirmatório como Western blot quando o EIA é positivo, mas este algoritmo mostra alta taxa de resultados inconclusivos, e elevado custo. Métodos: Dois ensaios qualitativos de PCR em tempo real foram desenvolvidos para detectar HTLV-1 e 2 e um total de 318 amostras foram testadas por PCR (152 doadores de sangue, 108 de portadores assintomáticos, 26 pacientes HAM/TSP e 30 indivíduos soronegativos). Resultados: A sensibilidade e especificidade das PCR em relação aos resultados de WB foram de 99,4% e 98,5%, respectivamente. As PCR foram mais eficientes em identificar o tipo viral, a infecção pelo HTLV-2 e úteis para definir casos inconclusivos. Conclusões: Por serem sensíveis, práticas e de custo muito inferior ao do WB, as técnicas de PCR em tempo real podem ser usadas como teste confirmatório do HTLV em bancos de sangue, em substituição ao WB.

Palavras-chaves: HTLV. Doadores de sangue. PCR em tempo real. Western blot.

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in Indian tribes, although it is also found in urban populations, particularly among intravenous drug users. Blood donor screening for HTLV was introduced in Brazil in November 1993.

According to the Brazilian standard, screening for HTLV-1/2 among blood donors must be done by a highly sensitive test such as an enzyme-linked immunosorbent assay (EIA) or a chemical luminescence test. Use of a second test to confirm the reactivity of a sample is not mandatory. The majority of blood banks use EIA as screening test for HTLV-1/2. If the test result is positive or indeterminate, the sample is tested again using EIA in duplicate.

If it remains positive or indeterminate, the associated blood products are discarded. The donor is asked to provide a second sample, and if it is EIA-positive or indeterminate, a Western blot (WB) test is used to confirm the serological status. This algorithm has a high cost, making the use of WB unviable for many Brazilian blood centers. In addition, it generates a relatively high rate of inconclusive results, due especially to the indeterminate nature of WB results.

Detection of the viral genome of samples using PCR is a gold standard with high sensitivity and specificity. Its use, which was initially restricted to research studies, is becoming more and more applicable to routine services due to reductions in the costs of molecular biology reagents and the introduction of real-time PCR methods. Several studies have described different real-time PCR protocols for HTLV-1/2 with the aim of quantifying the proviral load, which is considered to be a risk marker for the development of virus-associated diseases.

Differently from the previous studies, we evaluated the performance of two qualitative real-time PCR protocols for specific detection of HTLV-1 and HTLV-2 that might present high sensitivity and low cost, in order to increase the viability of their use as routine confirmatory tests in blood banks.

**METHODS**

**Samples**

We tested a total of 318 blood samples collected in Vacutainer® tubes containing EDTA. One hundred and fifty-two (47.7%) samples were obtained from blood donors whose initial test results had been EIA-positive or indeterminate for HTLV-1/2, and who returned to the Hemominas Foundation to have a second sample collected and tested by EIA and WB. The remaining 166 (52.3%) samples were from GIPH (Interdisciplinary HTLV Research Group) cohort participants. This cohort is following up HTLV-positive/indeterminate blood donors at the Blood Center of Belo Horizonte (part of the Hemominas Foundation), and also includes seronegative blood donors and HAM/TSP patients from the Sarah Network of Rehabilitation Hospitals (Belo Horizonte, State of Minas Gerais). The samples from the cohort included asymptomatic carriers (n = 108), HAM/TSP patients (n = 26), and seronegative individuals (n = 30), along with two individuals whose serological tests were inconclusive (EIA positive/WB indeterminate).

**Serological tests for HTLV-1/2**

During the study period (2007 to 2008), two HTLV-1/2 EIA kits were used for screening tests: Murex HTLV-1/2+ (Murex Biotech Ltd, UK) and Ortho HTLV-1/HTLV-2 Ab-Capture ELISA Test System (Ortho Clinical Diagnostics Inc., USA). These kits use recombinant antigens and synthetic peptides, respectively. The tests were conducted in accordance with the manufacturer’s instructions. Serum samples with OD/CO (optical density sample/cutoff) lower than 0.8 were considered negative. Results between 0.8 and 1.2 were deemed indeterminate, and results greater than or equal to 1.2 were considered positive for HTLV-1/2. A WB kit (WB HTLV-2, Genelabs Diagnostics, USA) was used as a confirmatory test and the results were interpreted in accordance with the manufacturer’s criteria, as follows: (1) HTLV-1 positive: GAG reactivity (p19 with or without p24) and two ENV bands (GD21 and gp46-I); (2) HTLV-2 positive: GAG reactivity (p24 with or without p19) and two ENV bands (GD21 and gp46-II); (3) HTLV-positive without virus type: GAG reactivity (p19 and p24) and ENV (GD21); (4) indeterminate: detection of specific bands without fulfilling the criteria of positivity for HTLV-1/2; or (5) negative: without reactivity for any specific HTLV bands.

**Real-time PCR for HTLV-1 and 2**

Peripheral blood DNA was extracted and purified in columns (QIAamp DNA Blood kit, QIAGEN GmbH, Hilden, Germany), in accordance with the manufacturer’s instructions. We performed two qualitative molecular tests for specific detection of HTLV-1 and HTLV-2, using real-time PCR with TaqMan chemistry. One reaction was designed to detect both HTLV-1 and the human albumin gene. Because this was a qualitative assay, human gene amplification was used as a control for amplification of the DNA sample and not for estimating the input of the cells present in each sample. The primers and probe for the albumin gene amplification were applied at a lower concentration than that used to amplify the viral gene, in order to avoid competition for reagents during the reaction. HTLV-2 detection was performed in a separate reaction vessel, but using the same HTLV-1 PCR temperature conditions, which enabled testing for both virus types simultaneously.

The primers and probes were as previously described. DNA (5μl) was used in the HTLV-1 reaction, presenting a final volume of 25μl consisting of 12.5μl of TaqMan PCR Master Mix (Applied Biosystems, CA, USA), 250nM of HTLV-1F (5' -GAACGCCCTTAATGGCACTTTAAGAAC–3'), 250nM of HTLV-1R (5' -GTGGTTGATTGTCCATAGGGCTAT–3') and 200nM of HTLV-1P (5'-FAM-ACAAACCCGCTACCC-MGB-3') for HTLV-1 pol region amplification; and 150nM of ALB-F (5' -GCTCACTCCCTATGTTCTAACC–3'), 150nM of ALB-R (5' -GGGCAAGCAGGTTTTGTCAAA–3') and 100nM of ALB-P (5'-VIC-TGTGCTGCTGAAATCAT-MGB-3') for human albumin gene amplification.

The reaction to detect HTLV-2 (pol region) was performed using 5μl of DNA in a volume of 25μl containing 12.5μl of TaqMan PCR Master Mix (Applied Biosystems, CA, USA), 250nM of HTLV-2F (5’-CAACCCCACACGTCAAGG–3'), 250nM of HTLV-2R (5' -GGGAAAGGTTAGGACGTCTAGTAGATA–3') and 250nM of HTLV-2P (5’-FAM-TGAGAGAACATGGTATAAT-MGB-3').

Both reactions were performed in the ABI 7,300 apparatus (Applied Biosystems, CA, USA), under the following cycle conditions: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15s at 95°C and 1 min at 60°C. DNA from the MT2 cell line, which has a complete proviral copy integrated in the genome of the cell and other defective proviral sequences, was used as a positive control for the HTLV-1 assay. For HTLV-2, a plasmid containing an HTLV-2 DNA sequence was utilized as a positive control. DNA samples from two seronegative individuals were used as negative controls, in addition to the “mix” control, without the addition of DNA.
Ethics committee approval
This study was approved by the Ethics Committee for Research Involving Human Beings of the Hemominas Foundation.

RESULTS

The real-time PCR results for HTLV-1 and HTLV-2 from the 318 samples tested are shown in Table 1, according to the serological results. Taking into account the samples that were WB-positive (n = 174) or negative (n = 39), and the ones that were EIA-negative (n = 96) but were not tested using WB, the sensitivity and specificity of the PCR were 99.4% and 98.5%, respectively.

<table>
<thead>
<tr>
<th>Real-time PCR</th>
<th>EIA neg</th>
<th>EIA ind</th>
<th>EIA pos</th>
<th>EIA pos</th>
<th>EIA pos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WB neg</td>
<td>WB neg</td>
<td>WB pos</td>
<td>WB ind</td>
<td></td>
</tr>
<tr>
<td>HTLV-1 pos</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td>HTLV-2 pos</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>HTLV-1 and 2 neg</td>
<td>66</td>
<td>18</td>
<td>19</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>GIPH cohort</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTLV-1 pos</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>130</td>
<td>1</td>
</tr>
<tr>
<td>HTLV-2 pos</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>HTLV-1 and 2 neg</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>18</td>
<td>21</td>
<td>174</td>
<td>9</td>
</tr>
</tbody>
</table>


We found only one false-negative PCR result. This was from a sample from a GIPH cohort participant, an asymptomatic 38-year-old male whose EIA and WB tests done in 2004 and 2008 were positive for HTLV-1. The samples collected in 2004, 2007 and 2008 were tested by means of nested PCR for HTLV-1 at the time of collection, but they were negative. The last sample collected from this individual (in 2008) was tested using real-time PCR, but it was also negative.

Two other results were discordant between the molecular and WB tests. In these cases, the samples were EIA-positive (OD/CO of 25.1 and 2.5), but with a negative result from WB. We tested these samples twice using PCR and they were both negative for HTLV-1 and positive for HTLV-2, thus suggesting that WB failed to detect the type 2 virus.

We also analyzed nine samples that were EIA-positive and WB-indeterminate. Seven were negative for HTLV-1 and 2 using PCR, while two were positive for HTLV-1. One of these samples was from a GIPH cohort participant who had been followed up. As shown in Table 2, he had been tested three times using EIA and WB over a five-month period, maintaining the indeterminate pattern on WB. Nevertheless, PCR confirmed the presence of virus infection in his first sample.

Out of the 40 blood donor samples that were both EIA and WB-positive (excluding the two individuals who were WB-negative and PCR-positive for HTLV-2), two (5%) did not present reactivity on WB for either of the recombinant proteins (rgp46-I and rgp46-II) that define whether the infection consists of HTLV type 1 or type 2, respectively. This discrimination was possible through using PCR, such that both samples yielded positive results for HTLV-1.

TABLE 2 – Serological and molecular results for HTLV-1 and 2 on an individual in the GIPH cohort with HTLV-indeterminate WB results, Belo Horizonte, Brazil.

<table>
<thead>
<tr>
<th>Date</th>
<th>EIA (OD/CO)*</th>
<th>WB</th>
<th>HTLV-1</th>
<th>HTLV-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov 23, 2007</td>
<td>positive (6.1)</td>
<td>GD21 + rgp46-I not done not done</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feb 11, 2008</td>
<td>positive (8.5)</td>
<td>GD21 + rgp46-I positive negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apr 17, 2008</td>
<td>positive (6.9)</td>
<td>GD21 + rgp46-I positive positive</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*OD/CO: optical density/sample cutoff, EIA: enzyme-linked immunosorbent assay, WB: Western blot.

Among the asymptomatic carriers and HAM/TSP patients from the GIPH cohort, only three (1.7%) were positive for HTLV-2 on PCR; all the rest (98.3%) being identified as infected by HTLV-1. In the 42 blood donors who were PCR positive, we identified three cases (7.1%) of HTLV-2 infection (two of which were not detected on WB), and all the remaining cases (92.9%) showed evidence of infection by type 1 virus (HTLV-1).

DISCUSSION

In order to diagnose HTLV infections, both the ability to detect them and the capability to differentiate between types 1 and 2 are required. The strategies currently used in Brazilian blood banks for such detection are based on serological tests (EIA followed by WB). The development of new EIA kits for HTLV that use recombinant proteins and synthetic peptides has increased the sensitivity and specificity of such kits, in comparison with kits that use viral lysate. In contrast, the WB test for HTLV-1/2 has shown little development over recent years, and is unsuitable for blood banks because of its high costs and high proportion of indeterminate results. This situation suggests that there is a need to confirm virus infection by means of molecular tests.

In this study, we evaluated two qualitative analyses for HTLV proviral DNA, using real-time PCR. The assays proved to be highly sensitive and specific for HTLV-1 and 2 in the great majority of samples. The results indicated that WB failed to detect infection by HTLV type 2, and this was also observed in two other cases that were not included in this study. Previous studies have shown that the confirmatory sensitivity of WB for HTLV-2 is worse than for HTLV-1 and presents a greater frequency of indeterminate or false negative results.

Two (5%) out of the 40 WB-positive samples from blood donors did not present reactivity in the WB test for either of the recombinant proteins (rgp46-I or rgp46-II). This distinction was only possible through using the PCR, thus showing one more advantage of the PCR test in comparison with WB. In addition to confirmation of the presence of the virus, it is very important to differentiate between HTLV-1 and HTLV-2, because the virus types present associations with different outcome diseases.

Controversy surrounds the interpretation of indeterminate WB patterns because of the inability to correctly define positive and negative cases from such patterns. Indeterminate WB profiles are a problem for blood banks, because they lead to discarding of blood components and rejection of donors as unsuitable for future blood donations. Such results are disturbing for the donors, as they are left without any definition regarding their true status (infected or uninfected). This situation generates great difficulty.
in donor counseling. In general, indeterminate WB findings do not represent current HTLV infection\(^5\), but in high-risk populations or in endemic areas, an indeterminate WB result may represent a case at the seroconversion stage\(^6\). By using real-time PCR in the present study, we found nine samples that were EIA-positive and WB-indeterminate, and two of them (22.2%) were positive for HTLV-1. The data showed that PCR was able to define cases with inconclusive serological tests, thereby identifying the current viral infection status, because proviral DNA can be detected before an immune response intense enough to be detected in the WB serological test has developed. The proportion of true positive individuals among the indeterminate cases emphasizes the need for caution when considering the indeterminate cases in our population as false-reactive ones.

Countries such as France, Germany and the USA have introduced NAT (nucleic acid testing) for identifying the HIV and HCV genomes during blood donor screening, with the objective of diminishing the immunological window and increasing the safety of transfusions. Unlike the use of NAT for HIV and HCV, we proposed the use of PCR for HTLV testing in routine blood donor screening, only for confirmatory tests on samples that are EIA-positive or indeterminate. Replacing WB with PCR would lead to improvement of the sensitivity and reduction of the costs of the screening. Thus, our approach was to develop a qualitative assay, differently from many real-time PCR tests, which were designed to quantify the proviral load of HTLV-1/2. Because of the sensitivity, simplicity, rapidity in obtaining results and lower cost (approximately one tenth of the cost of WB) of real-time PCR, qualitative PCR assays for HTLV-1 and 2 were shown to be viable as confirmatory tests in blood banks.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

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24. Kamihira S, Dateki N, Sugahara K, Hayashi T, Harasawa H, Minami S, et al. HTLV-1 proviral load in peripheral blood mononuclear cells quantified in 100 countries such as France, Germany and the USA have introduced NAT (nucleic acid testing) for identifying the HIV and HCV genomes during blood donor screening, with the objective of diminishing the immunological window and increasing the safety of transfusions. Unlike the use of NAT for HIV and HCV, we proposed the use of PCR for HTLV testing in routine blood donor screening, only for confirmatory tests on samples that are EIA-positive or indeterminate. Replacing WB with PCR would lead to improvement of the sensitivity and reduction of the costs of the screening. Thus, our approach was to develop a qualitative assay, differently from many real-time PCR tests, which were designed to quantify the proviral load of HTLV-1/2. Because of the sensitivity, simplicity, rapidity in obtaining results and lower cost (approximately one tenth of the cost of WB) of real-time PCR, qualitative PCR assays for HTLV-1 and 2 were shown to be viable as confirmatory tests in blood banks.

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