Usefulness of a Nested-Polymerase Chain Reaction for Molecular Diagnosis of Human T-cell Lymphotropic Virus Type I/II

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This study aimed at implementing a Nested-polymerase chain reaction (Nested-PCR) for the molecular diagnosis of human T-cell lymphotropic virus type I/II (HTLV-I and HTLV-II) infections in peripheral blood mononuclear cells of infected subjects in Argentina. The sensitivity and specificity of the assay for the detection of regional strains were assessed by comparing them with the molecular assay of reference PCR-hybridization. The Nested-PCR detected 1 MT-2 cell (≥ 8 proviral copies)/1x10^6 non-infected cells showing high sensitivity for provirus detection. While both molecular assays showed high specificity (100%) for HTLV-I and HTLV-II detection, the sensitivity values differed: 100% for Nested-PCR and 67% for PCR-hybridization assay. Moreover, this technique showed less sensitivity for the detection of DNA sequences of HTLV-II (33%) than for the detection of DNA sequences of HTLV-I (75%).

The high sensitivity and specificity of the Nested-PCR for regional strains and its low costs indicate that this assay could replace the PCR-hybridization assay for the molecular diagnosis of HTLV-I/II infections. It will be interesting to assess the usefulness of this assay as a tool for the molecular diagnosis of HTLV-I/II infections in other developing countries. Other studies that include a greater number of samples should be conducted.

Key words: human T-cell lymphotropic virus type I/II - nested-polymerase chain reaction - Argentina

The human T-cell lymphotropic virus type I (HTLV-I) is the etiologic agent of adult T-cell leukaemia and tropical spastic paraparesis (TSP) (Uchyama et al. 1977, Osame et al. 1986, Höllsberg & Hafter 1993). Other diseases such as chronic inflammatory arthropathy, polymyositis, uveitis, alveolitis, and infective dermatitis have been described in patients infected with HTLV-I (Jacobson 1996).

The HTLV-II has been isolated from patients with hairy T-cell leukaemia (Kalyanaraman et al. 1982); however, its etiologic role as an oncogenic agent has not been confirmed yet.

The diagnosis of these retroviruses infection is based on the initial specific antibody detection by particle agglutination assay (PA) or enzyme immunoassays (EIA) and subsequent confirmation by Western blot (Wb) or indirect immunofluorescence assay (IFA). Due to the HTLV-I and HTLV-II (Cann & Chen 1990) high amino acid sequence homology, there may be cross-reactivity when using serological assays. Therefore, molecular assays are a useful means to differentiate between the two viruses. Furthermore, the retroviral infection can not be discarded nor confirmed in a high percentage of subjects showing reactive screening and indeterminate results by Wb (Lipka et al. 1991, Zaaijer et al. 1994, Gastaldello et al. 2001, Thorstensson et al. 2002). Molecular assays are then useful to the diagnosis of infection in these subjects with persistently indeterminate patterns.

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The first "gold standard" molecular assays used for the detection and typing of HTLV-I/II infections were the polymerase chain reaction (PCR) that involved the hybridization of the amplification products with DNA probes marked with radioactive P^{32} (Barun & Srinivasan 1989). It has been reported that not all specimens are found positive when examined by a single PCR due to the low level of infected lymphocytes in HTLV-I-infected individuals. Therefore, it is necessary to perform a second amplification (Nested-PCR) in order to improve the sensitivity of the assay (Matsumoto et al. 1990, Tuke et al. 1992, Vallejo & García-Sáiz 1995, Vandamme et al. 1997, Silva et al. 2002).

The purpose of this study was to implement and adapt a simple Nested-PCR protocol (Tuke et al. 1992) as well as to assess its sensitivity for the detection of HTLV-I/II strains circulating in Argentina in comparison with liquid PCR-hybridization as a reference assay.

MATERIALS AND METHODS

Nested-PCR assay

Source and isolation of DNA - The following cell lines have been used as a source of DNA: (i) MT-2, human T-lymphotropic virus type-1 (HTLV-1)-infected cell line (Miyoshi et al. 1981) was supplied by Dr Horacio Salomón from the National Reference Center for AIDS Argentina. Each cell carries 8 copies of the HTLV-I provirus; (ii) HT, uninfected cell line was used as a negative control and was kindly provided by Dr Renu B Lal (CDC, Atlanta, US).

To validate the sensitivity of the assay different proviral DNA concentrations were used and serial dilutions of the MT-2 cells were carried out from 2 x 10^6, 1 x 10^5, 1 x 10^4, 1 x 10^3, 1 x 10^2, 10 up to 1 cell. In each tube, DNA carrier was balanced with T- cells up to a concentration of 1x 10^6 .

DNA was obtained digesting with proteinase K. The pellets were suspended in 100 µl of lysis buffer composed

of 5 mM TRIS ClH, 0.5% Tween 20, 0.5% Triton y 80 µg/ml of proteinase K (Fungal 100 mg, Gibco). The cells were digested with proteinase K for 1 h at 60ºC, and then, the enzyme was inactivated for 10 min at 95ºC. The lysates were stored at –20ºC until they were used.

A β-actin PCR was done to control the quality of the extracted DNA in all the samples (Peng et al. 1993).

Nested-PCR procedure - We adopted the protocol previously described by (Tuke et al. 1992) and made some modifications. PCR was performed in a two-step reaction, first, with a pair of outer primers N1 (5'-CGGATACCC AGTCTACGTGT-3') N2 (5'-GAGCCGATAACGCGT CCATC-3') and then with a pair of inner primers N3 (5'-GTGTTTGGCGATTGTGTACA-3') N4 (5'-CCATCGA TGGGGTCCCA-3'). All four primers used hybridize with a sequence of the tax/rex gene preserved and shared by HTLV-I and HTLV-II.

The reaction mixtures basically contained 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 unit of Taq DNA polymerase (Gibco), Buffer Taq 1x (Gibco). Round 1 of the PCR was performed in a total volume of 50 µl with 15 ng of each outer primer and 25 µl of the cell lysate. After an initial 7 min denaturation at 95ºC, 35 cycles of 95ºC 1 min, 55ºC 1 min, 72ºC 1 min were performed in a Thermal Cycler (Uno II Biometra). Round 2 of the PCR was carried out in a total volume of 49 µl of the same reaction mixture with 62.5 ng of each inner primer and 1 µl of the first-amplification product. The reaction was carried out using the same temperature and duration as for the first round but, in this case, 25 cycles were performed.

A volume of 20 µl of the Nested-PCR amplification product was analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. The samples showing the band of the predicted size (128 bp) were considered positive for HTLV-I/II tax/rex sequence.

Afterwards, an analysis with the restriction enzymes *Sau* 3A and *Taq* I was carried out in order to differentiate HTLV-I from HTLV-II. 5 µl of the Nested-PCR product were digested in a 20 µl reaction mixture composed of 10 units of the restriction enzyme and 1x enzyme buffer. The reaction with enzyme *Sau* 3A was carried out at 37ºC for 90 min and the reaction with enzyme *Taq* I was carried out at 65ºC for 90 min.

A 10 µl aliquot of the digestion product was analyzed by electrophoresis on a 4% agarose gel stained with ethidium bromide. As a result of the digestion of the 128 bp tax/rex gene sequence with enzyme *Taq* I, there were obtained fragments of 69 bp, 53 bp, and 6 bp for HTLV-II and fragments of 122 bp and 6 bp for HTLV-I. As a result of the digestion of the 128 bp tax/rex gene sequence with enzyme *Sau* 3A, there were obtained fragments of 104 bp and 24 bp for HTLV-I, while the enzyme did not cut the sequence of 128 bp for HTLV-II.

Liquid PCR-hybridization assay - The PCR reaction was performed in a total volume of 50 µl in a reaction mixture composed of 2.5 mM $MgCl_2$, 0.4 mM of each dNTP (Promega), 1 unit of Taq DNA polymerase (Promega), 0.2 µM of each primer SK110 (5'-CCCTACAATCCC ACCAGCTCAG-3') and SK111 (5'-GTGGTGGATTTGCC ATCGGGTTTT-3') (Kowok et al. 1988), buffer Taq 1x and 20 µl of the cell lysate. The reaction consisted in an initial denaturation at 94ºC for 10 s, 30 cycles of 94ºC for 30 s, 53ºC for 1 min, and 72ºC for 1 min, followed by a final extension at 72ºC for 10 min in a Thermal Cycler (Perkin Elmer Cetus).

The PCR allows the amplification of a 185 bp fragment of a *pol* gene region conserved and shared by HTLV-I and HTLV-II.

Amplified DNA fragments were analyzed by liquid hybridization with ^{32}P end-labeled probes SK 112 (5'-GTACTTTACTGACAAACCCGACCTAC-3') for HTLV-I and SK 188 (5'-TCATGAACCCCAGTGGTAA-3') for HTLV-II (Kowok et al. 1988). Briefly, we hybridized 20 µl of the PCR product with a respective probe (100,000 cpm/reaction) in a total volume of 40 µl in 150 mM ClNa for 5 min at 96ºC and for 15 min at 56ºC. The products were electrophoresed on 5% polyacrylamide gels at 250 V for 1 h. Gels were autoradiographed (Kodak AR5-X-OMAT) at –85ºC for 24 h.

Sensitivity of the Nested-PCR assay for viral strains circulating in Argentina - Blood samples were obtained from blood donors from the Blood Bank of Hospital Garrahan, Buenos Aires, Argentina and from the Blood Bank of the Universidad Nacional de Córdoba, Argentina: (i) 12 HTLV-I infected subjects, (ii) 3 HTLV-II infected subjects, (iii) 9 subjects with reactive serology for HTLV-I/II and indeterminate Wb, and (iv) 12 subjects with negative screening for HTLV-I/II.

The diagnosis of infection in the blood samples was corroborated by the detection of specific antibodies for HTLV-I/II through PA (Serodia, Fujirebio, Tokyo, Japan) and its subsequent confirmation by Wb (Genelabs Diagnostics 2.4) and IFA (Gallego et al. 1997).

Twenty ml of blood with EDTA K_3 were obtained from each subject. The blood was diluted in 20 ml of phosphate-buffered saline (PBS) and centrifuged in a Ficoll-Hypaque gradient (Pharmacia) in order to obtain the peripheral blood mononuclear cells (PBMCs). After three washes in phosphate-buffered saline, the buffer was removed and the cells were conserved as dry pellets in aliquots of 2 x 10^6 at –20ºC. Afterwards, DNA was obtained by lysis with proteinase K, as described previously.

In all samples the amplification of the 128 bp fragment of HTLV-I/II tax/rex gene was performed by the Nested-PCR assay previously described. By contrast, the amplification of a 185 bp fragment of the *pol* gene was performed through the liquid PCR-hybridization assay (taken as a reference assay).

RESULTS

The Nested-PCR assay implemented detected 1 infected cell/1x10^6 total non-infected cells. Since the MT-2 cells carry 8 copies of the HTLV-I provirus, the sensitivity of this PCR would be ≥ 8 copies of viral DNA/1x10^6 cells.

Thirty six subjects were studied, 24 of whom were serologically reactive for HTLV-I/II. Of these 24 subjects only 15 were Wb confirmed, while the other 9 showed an indeterminate pattern.

Both molecular assays used for the diagnosis of HTLV-I and HTLV-II infection allow the amplification of a viral genome sequence that is shared by both retroviruses. In the case of the liquid hybridization PCR used as reference

technique, the amplified sequence corresponds to a fragment of the *pol* gene; while in the Nested-PCR the amplified sequence corresponds to a fragment of the *tax/rex* gene shared by both retroviruses. HTLV-I and HTLV-II are discriminated by the liquid PCR-hybridization by means of specific P^{32} labeled-probes for HTLV-I and HTLV-II. The products were electrophoresed on polyacrylamide gels and later autoradiographed. In the case of the Nested-PCR, the distinction between both retroviruses is made using different restriction enzymes by observing the bands in agarose gels under ultraviolet light.

The samples of the 15 Wb-confirmed subjects were positive by Nested-PCR, 12 of them corresponding to HTLV-I infection and the other 3 corresponding to HTLV-II infection. These results show that the Nested-PCR has 100% sensitivity for both retroviruses. The sensitivity of the PCR-hibridization was lower (67%) since 10/15 Wb-confirmed subjects were detected by the assay. Moreover, this technique had less sensitivity for the detection of HTLV-II DNA sequences (1/3; 33%) than for the detection of HTLV-I DNA sequences (9/12; 75%).

The samples corresponding to the 12 serologically negative cases for HTLV-I/II were negative by both PCR assays, both molecular assays thus showing 100% specificity.

Regarding the 9 Wb-indeterminate subjects, both PCR coincided at detecting only one case of HTLV-II infection, the other 8 being negative.

DISCUSSION

The Nested-PCR showed 100% sensitivity and specificity for the diagnosis of HTLV-I and HTLV-II infections in Argentina. In contrast, although the liquid PCR-hybridization assay showed 100% specificity, it showed 67% sensitivity. It has been reported that in 16% of HTLV-I infected subjects, the number of PBMCs with the provirus circulating in peripheral blood can be low, ranging from 2 to 68 proviral copies in $1.5x10^5$ cells (Matsumoto et al. 1990). On this account, the sensitivity of the technique implemented for the diagnosis of infection by these retroviruses is particularly significant. The Nested-PCR assay we have used proved successful in the detection of HTLV-I with only 8 copies of the provirus in 10^6 cells. The high sensitivity of the Nested-PCR for the detection of HTLV-I/II genomes by amplifying a fragment of the *tax/rex* gene and for the viral typing in different parts of the world, have already been described (Matsumoto et al. 1990, Tuke et al. 1992). All this indicates that HTLV-I/II genomes would be highly conserved.

In areas of high endemicity for HTLV-I/II it is very frequent to find subjects serologically reactive by screening techniques but indeterminate by Wb. While in the majority of cases HTLV-I/II sequences are not detected by PCR, there are some cases in which it is possible to amplify by PCR sequences of different HTLV-I/II genes (Saito et al. 1989, Delaporte et al. 1991, Miyata et al. 1995), since some of them become seropositive over time. The data reported suggests the existence of HTLV-I/II infected subjects who are serologically negative. This fact should be taken into account as these retroviruses can be transmitted by blood products and by organ transplants. In relation to this, in this study we found a HTLV-II infection case confirmed by the two PCR assays used, but which presented an indeterminate profile for specific antibodies by Wb. The differences frequently found in the results obtained by different serological assays, emphasizes the difficulty encountered in the serological diagnosis of HTLV-I/II infection and corroborates the need to use a high sensitive and specific molecular assay as a complementary technique in the confirmatory diagnosis of the viral infection. This is in agreement with the report of other authors (Vandamme et al. 1997, Liu et al. 1999, Thorstensson et al. 2002).

We believe that the Nested-PCR protocol described here has some advantages over the reference technique liquid PCR-hybridization in terms of safety, cost and simplicity. Moreover, as a result of the high sensitivity of this technique, we could use simple agarose gel electrophoresis and ethidium bromide staining, thus eliminating the need for radioactives.

In summary, the high sensitivity and specificity of the Nested-PCR showed in this study and its low cost of implementation indicate that this assay could replace the PCR-hybridization assay for the molecular diagnosis of HTLV-I/II in Argentina. However, other studies should be conducted using a larger number of samples which would be more representative. Finally, the uselfuness of this assay as a tool for the molecular diagnosis of HTLV-I/II infections should be evaluated for its use in other developing countries.

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