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Artigo / Article

Evaluation of real time PCR technique to diagnosis of human T-lymphotropic virus type I (HTLV-I) in patients in the Hematologia da Fundação Hemope Hospital, in Northeastern Brazil

Avaliação da técnica de PCR em tempo real para o diagnóstico do virus linfotrópico tipo I de células T humana (HTLV-I) em pacientes do Hospital de Hematologia da Fundação Hemope, no Nordeste do Brasil

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*As a high degree of homology exists between the proviral genomes of HTLV-I and HTLV-II, there is significant cross-reactivity. Therefore although detection of HTLV antibodies is characteristic of viral infection, it is not sufficient to confirm the presence of the viral type. Molecular tests used to diagnose the HTLV-I/II viruses are based on investigations of proviral genomic sequences, and allow for an infection to be diagnosed prior to the appearance of any sign or symptom. The HTLV proviral load in infected individuals can be determined using real-time PCR, a faster method with less risk of contamination than simple or nested PCR. We analyzed 63 samples from the Hemope Hospital, of which 33 were from HTLV seropositive individuals and 30 from blood donors, to determine the type of virus and the proviral load. The sensitivity of qualitative PCR in comparison to ELISA was 87.5% (95% IC: 70.1 - 95.9%) and the specificity was 100% (IC 95%: 85.9 - 100.0%). The sensitivity and specificity of real-time PCR in comparison to the serological test (ELISA) were 100% (95% IC: 86.7 - 100.0%) and 96.67% (95% IC: 80.9 - 99.8%) respectively. The proviral load in the seropositive individuals ranged from 13 to 343820 copies/106 PBMC cells. Our study also observed that individuals with TSP/HAM had a higher proviral load than those who showed no symptoms. The use of real time PCR for routine clinical testing of infected individuals will play a significant role in identifying the virus type and determining the proviral load, thereby providing more appropriate treatment. Rev. Bras. Hematol. Hemoter. 2008;**30**(5):384-389.*

Key words: *Human T-cell lymphotropic virus; HTLV-I infections; molecular diagnosis; polymerase chain reaction.*

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Introduction

Screening tests for HTLV-I and II were first introduced at blood centers in Japan in 1986, in the United States in 1988, and in Brazil only in 1993. The purpose of this was to prevent the transmission of these agents during hemotherapy. Since then a significant number of asymptomatic carriers of these viruses have been identified.[1] According to Catalan-Soares *et al*.[2] in Brazil, the mean prevalence rate of infection varies considerably; from 0.4/1000 donations in the State of Santa Catarina, to 7.5/1000 donations in the State of Pernambuco and 10/1000 donations in the State of Maranhão (the highest incidence recorded). This differs from the figures found in other countries, such as Canada, where the incidence of positive results is 0.48/100000 individuals/year.3 Laboratory diagnosis of HTLV-I and HTLV-II infection may be required in a variety of clinical situations: i) when a patient exhibits symptoms of the illnesses caused by these retroviruses to corroborate the hypothesis of the clinical diagnosis; ii) during diagnostic screening of individuals who have been exposed to HTLV-I or HTLV-II, such as, for example, the sexual partners of asymptomatic carriers and family members; iii) as part of the compulsory screening for bone marrow and blood donors.[1]

Diagnosis of HTLV-I/II infection is usually carried out using serological tests based on antibodies generated to combat virus antigens in the serum of infected individuals. Several tests for the serological diagnosis of HTLV infection are currently available on the market, and include: i) latex particle or gelatin tests; ii) ELISA - Enzyme-Linked Immunosorbent Assay; iii) RIPA-Radioimmunoprecipitation; iv) IIF-Indirect immunofluorescence and v) Western Blot. The last two are normally used as tests to confirm a previous diagnosis.

Due to the high degree of homology between the two types of HTLV, although the detection of anti-HTLV-I/II antibodies using conventional serological tests is characteristic of cases of viral infection, it is not sufficient to distinguish between type I and type II. The discriminatory serological tests are themselves based on research into antibodies produced to combat specific epitopes of each HTLV type. For this reason, immunonenzymatic assays, which contain synthetic peptides which are type-specific antigens,[4] or Western blot modified assays, which incorporate recombinant antigens which are type-specific to viral lysate,[5] are used. However, it should be remarked that, in some cases, even the discriminatory serological tests are not always capable of identifying the type of HTLV responsible for the infection, which makes it more difficult to provide individuals with the appropriate medical advice.[1,6]

Furthermore, two new types of HTLV have recently been discovered: HTLV-3 and HTLV-4. When analyzed using the confirmatory serological assays currently available, they gave HTLV-I indeterminate or false-positive or HTLV-II false-positive results.[7,8]

The molecular assays used for diagnosis of HTLV-I/II infection are based on research into the gene sequences of lysates of mononuclear peripheral blood cells. Unlike other retroviruses, the viral load circulating in the blood of HTLV-infected individuals is very small. Therefore, its detection by molecular assays is carried out using DNA as the target nucleic acid. Conserved regions of the viral genome (pol or tax) have normally been used for this, along with consensual primers, which are capable of amplifying both sequences of HTLV-I and HTLV-II. The HTLV proviral load in infected individuals can be determined using real time PCR, which is a quicker technique and less prone to contamination than simple or nested PCR, since amplification and measurement can be carried out in a single closed reaction tube.[9] Furthermore, this allows detection to be visualized during amplification, and is used to determine the HTLV proviral load, due to its speed, precision and accuracy.

In this study, we evaluated the performance of real time PCR in the diagnosis of the HTLV-I, with a view to its routine use in the laboratory of the Hematologia da Fundação de Hematologia e Hemoterapia de Pernambuco Hospital, in Brazil (Hemope Hospital).

Methodology

Study population

The population was a convenient non-probabilistic sample, selected between November 2006 and April 2007, made up of individuals whose serum tested positive for anti-HTLV antibodies (n=33), attending an outpatients clinic at Hemope Hospital, in Recife, the capital of the State of Pernambuco, in the Northeast of Brazil, as well as a similar number of blood donors (n=30) from the same institution. After standard clinical-laboratory tests had been carried out, individuals whose serum tested positive using ELISA (Murex Biotech Limited, UK), were selected to perform qualitative PCR. Blood donors who tested negative for the virus were used as a control. The study was approved by the Research Ethics Committee of Hemope Hospital and by the Research Ethics Committee of the Aggeu Magalhães Research Center/ Fiocruz. The patients were evaluated by the doctor responsible for the Hematology Unit of Hemope Hospital, with the assistance of a psychologist, a specialist in medical genetics and a social worker. Those who tested positively for HTLV were referred for counseling.

Obtaining the PBMC

The blood, collected in a tube containing EDTA, was first centrifuged at 300 x g for 7 minutes and the plasma removed. The remaining blood was diluted in an equal quantity of 0.01 M PBS, pH 7.2 (1:1). Five mL of the blood diluted in PBS was carefully put in a 15 mL tube containing 2 mL of Ficoll (Amersham Pharmacia Biotehc, Uppsala, Sweden). The tube was centrifuged for 30 min at 800 x g, the

leucocytary ring formed immediately, and was collected and transferred to another 15 mLltube, where it was washed twice in PBS, being centrifuged for 5 minutes at 300 x g between washings. The cells were re-suspended in 1 to 3 ml of PBS and counted in a Neubauer chamber. Tubes containing 10^6 cells were separated, centrifuged and the pellet-stored at -20°C.

Extraction of the DNA from PBMC cells

The extraction of DNA was carried out using the "DNA Purification and Extraction GFX Column" kit (Amersham Pharmacia Biotehc, Uppsala, Sweden), in accordance with the manufacturer's instructions.

Qualitative Nested PCR

The DNA was submitted to qualitative PCR carried out using a 2-phase amplification. For the first amplification of HTLV-I, the following primers were used: SK110-pol: 5 - CCC TAC AAT CCA ACC AGC TCA G - 3 and SK44-tax: 5 - GAG CCG ATA ACG CGT CCA TCG - 3. For the second amplification the primers were: SK248-env: 5 - CAT GTC GAC GCT CCA GGA TAT GAC C - 3 and SK249-env: 5 - CAG ACC GCC ACC GGT ACC GCT CGG C - 3. The primers were obtained by GenBank (access J02029). The amplification reaction mixture for HTLV-I, with a final volume of 20 mL contained 20 µM Tris-HCL (pH 8.4), 50 mM KCl, 1.25 mM $MgCl_2$, 5 pmol of each primer, 0.2 mM DNTP (final concentration of 200 µols for each dnucleotide), 0.5 U of Taq DNA Polimerase and 2 µL of the extracted DNA. The first amplification was performed at 95ºC for 7 min, 35 cycles of 55ºC for 1.5 min, 72ºC for 2 min and 95ºC for 1 min, and finally an additional 10 min at 72ºC. For the second amplification the same reaction mixture was used, changing only the primers used and the product of the first amplification diluted at 1:100 as a template. The amplification protocol involved denaturation at 94ºC for 5 min, 30 cycles at 58ºC for 1 min, 72ºC for 2 min and 94ºC for 1 min, followed by an extension at 72ºC for 10 min[10] (modified). The final result was a 466 bp product.

In the case of the HTLV-II, the first amplification used the following primers: ET401-env/tax: 5 - CTC CAT TTC TGG GAA CAA GGG GGT TT - 3 SK44. The second amplification used ET403 env/tax: 5 - GGC TGG GGA CTA AAC TGG GAT CCT GG - 3 and ET404 env/tax: 5 - CCA AAC ACG TAG ACG GGG GAT CC - 3. The primers were obtained by GenBank (access NC001488). The amplification reaction mixture for the HTLV-II, also with a final volume of 20 µL, contained 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 3.0 mM $MgCl_2$, 5 pmol of each primer, 0.2 mM DNTP (final concentration of 200 µols for each dnucleotide), 0.2 mM DMSO (Merck, Darmstadt, Germany), 0.5 U of Taq DNA Polimerase and 2 mL of the extracted DNA as a template. The first and second amplifications followed the same cycling protocol as the first amplification for HTLV-I. The second amplification used the same reaction mixture changing only the primers and using the product of the first amplification diluted at 1:100 as a template[10] (modified). The final result was an 821 bp product.

Real time PCR

The plasmid used to plot the standard curve was provided by the LASP - Laboratório Avançado de Saúde Pública of the Centro de Pesquisas Gonçalo Moniz in Bahia, Brazil. It is a clone with double insertion containing a portion of the intron 12 of the albumin gene and the pol region of the HTLV-I genome, lying between positions 4708 and 4953.[11] The fluorescence dye chosen was the Sybr Green (Applied Biosystem/Foster City, U.S.A.).

Measurement of HTLV-I in the samples

All the samples were amplified in duplicate using the following primers: SK 110: 5´- CCC TAC AAT CCA ACC AGC TCA G -3´ and SK 111: 5´- GTG GTG AAG CTG CCA TCG GGT TTT - 3´.[11] For the HTLV-I and albumin, the following amplification protocol was used: 2 min at 50ºC, 10 min at 95ºC, 45 cycles of 15 sec at 95ºC and 1 min at 65ºC and were amplified in parallel in the Rotor Gene 3000 (Corbett Life Science, Sydney, Australia).

Measurement of the albumin in the samples

All the samples were amplified in duplicate using the following primers: Alb-S: 5´- GCT GTC ATC TCT TGT GGG CTG T - 3´ and Alb-The: 5´- AAA CTC ATG GGA GCT GCT GGT T - 3´.[11] The main mixture used in the two reactions utilized 2 mL of the DNA, 10 µL of each first and Green Master Mix, to a total of 25 mL of reaction. The HTLV-I proviral load was calculated as the ratio between the mean number of copies of HTLV-I and the mean number of copies of albumin x 2 x 10^6 and expressed as the number of copies/ 106 PBMC cells.[11]

Data analysis

The real time PCR DNA detection limit for the quantification of the HTLV-I proviral load was analyzed by means of serial dilutions (10^0 to 10^5). Win Episcope 2.0 software[12] was used to calculate the values for the sensitivity and specificity of the tests. The confidence interval (CI) was established at 95%.

Results

Sixty-three samples were analyzed: 33 samples tested serologically positive for HTLV and 30 samples tested negative. The positive group contained 27 women with a mean age of 47 years, and 6 men with a mean age of 48 years. The group of individuals testing negative (the Control Group) comprised 26 men with a mean age of 35 years and 4 women with a mean age of 35 years.

Qualitative PCR for HTLV-I and II

The results of the analysis of the qualitative HTLV PCR are shown in Figure 1 and Table 1. All of the individuals who tested serologically negative were also negative in the qualitative PCR and only one was HTLV-II positive. The group of individuals testing positive presented 4 negative results using qualitative PCR. The sensitivity of qualitative PCR was 87.9% (95% CI: 70.1-95.0%) and the specificity 100% (95% CI: 85.9-100.0%).

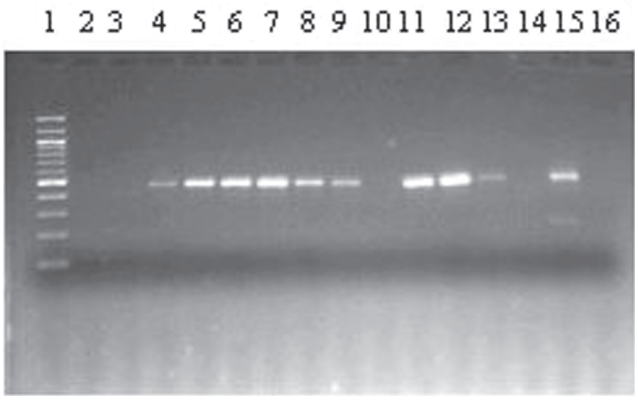

Figure 1. Representative sample of qualitative PCR for HTLV-I. Line 1 – standard molecular weight of 100pb, lines 2, 3, 10 and 14 – samples testing negative. Lines 4 to 9,12 to 13 – samples testing positive, line 15 – control testing positive and line 16 – control testing negative

Table 1. Results of Qualitative PCR for HTLV

	HTLV Serological	Reaction Test Result
Qualitative PCR	Positive	Negative
Positive	29	0
Negative	4	30

The HTLV-I Proviral Load

Once the standard curve had been calculated, the software automatically quantified the number of DNA of HTLV-I or of albumin, reporting the Ct of the sample to the Ct of the standard curve (Figures 2 and 3). The HTLV-I proviral load was then calculated using the following formula: (mean number of HTLV-I copies / mean number of albumin copies) x 2 x 10^6. The results were expressed as the number of copies/10^6 PBMC cells. The proviral load of the individuals testing serologically positive, as measured using real-time PCR, ranged between 13 copies/10^6 PBMC cells and 343,820 copies/10^6 PBMC cells (Figure 4). Of the 30 individuals who tested negative, one showed a proviral load of 308 copies/10^6. The sensitivity and specificity of real-time PCR were 100% (95% CI: 86.7 - 100.0%) and 96.67% (95% CI: 80.9 - 99.8%), respectively. Based on the analysis of the CI, the qualitative PCR and the real-time PCR tests did not show significantly different sensitivities and specificities.

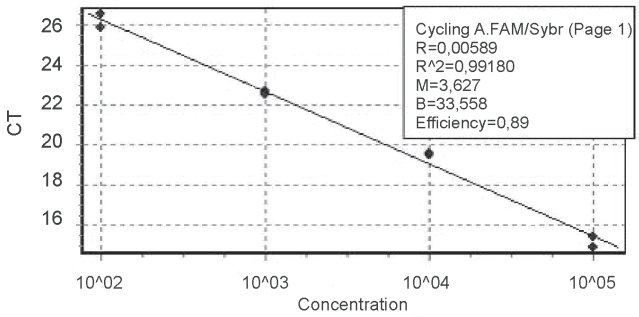

Figure 2. Linear regression of standard HTLV-I curve

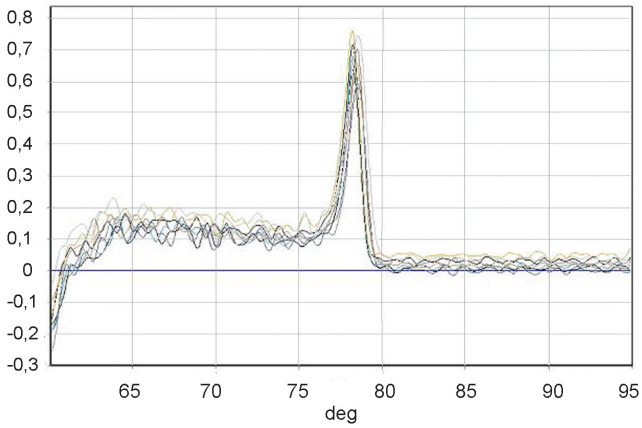

Figure 3. Curve showing deviation from HTLV-I standards

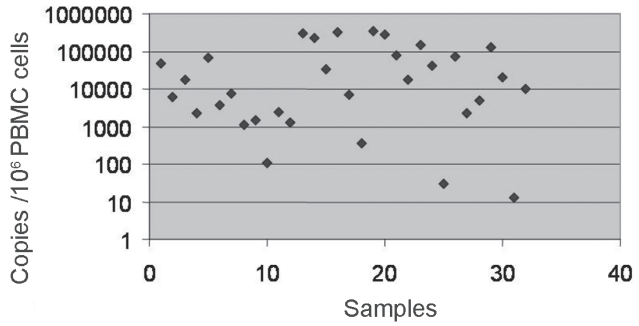

Figure 4. Proviral load dispersion by real time PCR from HTLV-I positive patients

Discussion

Laboratory screening/diagnosis of HTLV infection is carried out using serological assays, principally ELISA, with the Western blot being used to confirm the diagnosis and to determine the type of the virus (I or II). In cases where Western blot proves inconclusive, molecular approaches should be used[8,13,14] which show, especially when PCR is used, a higher degree of specificity.[15] Real-time PCR allows the detection and quantification of target sequences by continuously measuring the fluctuations in fluorescence during reaction

amplification. This is carried out in a closed system, thereby diminishing the risk of contamination and eliminating the gel phase of standard PCR.[16] During real-time PCR, or, more precisely, during the annealing phase of the primers and the extension of the target sequence, larger quantities of SYBR Green are linked to the double strands of DNA, which leads to an increase in the emission of fluorescence. When the DNA is once again denaturized during the PCR cycle, the SYBR Green detaches itself, thereby diminishing the emission of fluorescence.[17]

According to Gunson *et al*.[16] the property of SYBR Green linking itself to double strands of DNA has both advantages and disadvantages. It may produce annealing base errors during the extension phase, but it is, nevertheless, capable of cross-linking itself and emitting fluorescence. This is a highly valuable property when studying viruses, because even small sequence mutations or changes in conserved regions can lead to annealing probe errors, causing false-negative results. With SYBR Green this possibility still exists, but it is limited when compared to assays that use probes. However, this very same property reduces the specificity of the reaction. Fluorescence emission may occur even when the sample is negative, owing to the connection of the SYBR Green to primer dimmers.[16,17] To avoid errors in analysis, we used the dissociation curve, which allows amplicon sizes to be distinguished from primer dimers.

The use of SYBR Green in our study was based on the fact that it is a cheaper assay and does not require a specific probe. The primers are used to amplify the pol region of the HTLV-I virus, because this is a conserved region and has already been used in several studies involving viruses.[11,18,19]

The HTLV-I standard curve was plotted using serial dilutions of the plasmid from 10^0 to 10^5. After carrying out real-time PCR with the dilutions in triplicate, the detection limit observed was 10 copies/reaction, where the dilution of 10^3 copies had a Ct of 22.59, confirming the results obtained by Lee *et al*.[10]

In the group which tested serologically negative for HTLV-I, i.e. the Control Group, all 30 individuals also showed up negative when qualitative PCR was used. However, one individual exhibited a proviral load of 30^8 copies / 10^6 PBMC cells when real-time PCR was used. Both the qualitative PCR and real-time PCR tests were repeated and the same results were produced, suggesting a possible cross-over contamination. The results for the proviral load in the individuals who tested serologically positive ranged between 13 and 343,820 copies/10^6 PBMC cells, corroborating the results of other studies.[10,20,21] Like other studies,[19,20,21] our study also observed that individuals with PET/MAH had a higher proviral load than those who exhibited no symptoms. However, it remains to be scientifically proven that an inverse relation exists; that HTLV-I carriers with a high proviral load, in whom no clinical illness has been identified, are more susceptible to develop the illnesses associated with the virus. For this reason, as suggested by Montanheiro *et al*.,[19] there is a need for a long term follow-up involving asymptomatic carriers.

Although the present study did not show any statistical difference between the two molecular approaches to the identification of the HTLV-I, the occurrence of false-negative results, at almost 10%, was observed in the qualitative PCR results. If this observation is reproduced in a larger sample, real-time PCR could be seriously considered as a means of identifying the virus and simultaneously determining the proviral load.

If it is established that HTLV-I carriers with a high proviral load more frequently develop leukemia and neurological illnesses when compared to those with a low proviral load, this would raise questions regarding the current Ministry of Health guidelines on follow-up procedures and provide them with some degree of uniformity. In this case, a different clinical follow-up strategy should be adopted for asymptomatic carriers, in the expectation that earlier diagnosis and treatment of clinical illnesses may result in a more optimistic prognosis.

Resumo

Como os genomas provirais do HTLV-I e HTLV-II exibem grande homologia, há uma expressiva sororeatividade cruzada. Assim, a detecção de anticorpos anti-HTLV-I/II embora caracterize infecção viral, não permite estabelecer distinção entre os agentes. Os testes moleculares empregados para o diagnóstico dos vírus HTLV-I/II, baseiam-se na pesquisa de seqüências genômicas provirais permitindo o diagnóstico da infecção antes de aparecer sinal ou sintoma. A carga proviral de HTLV pode ser determinada através da utilização da PCR em tempo real, uma técnica rápida e com menor risco de contaminação que a PCR simples ou nested PCR. Analisamos, 63 amostras do Hospital HEMOPE, das quais 33 foram de indivíduos com sorologia reagente para HTLV e 30 de doadores de sangue, para determinar o tipo de vírus e a carga proviral. A sensibilidade da PCR qualitativa em relação ao ELISA foi de 87,9% (IC 95%: 70,9-96,0%) e a especificidade foi de 100% (IC 95%: 85,9-100,0%). A sensibilidade e especificidade da PCR em tempo real foram de 100% (IC 95%: 86,7-100,0%) e 96,67% (IC 95%: 80,9-99,8%), respectivamente. A carga proviral variou entre 13 cópias/10^6 células PBMC e 343820 cópias/10^6 células PBMC. Nosso estudo também observou que os indivíduos com PET/MAH tiveram carga proviral mais elevada que a dos indivíduos assintomáticos. A utilização da PCR em tempo real na rotina clínica dos indivíduos infectados poderá desempenhar um papel relevante na identificação do vírus e na determinação da carga proviral, contribuindo para direcionar um tratamento adequado. Rev. Bras. Hematol. Hemoter. 2008; 30(5):384-389.

Palavras-chave: *Vírus linfotrópico T humano; infecções por HTLV-I; diagnóstico molecular; reação em cadeia da polimerase.*

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