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Validation of a quantitative real-time PCR assay for HTLV-1 proviral load in peripheral blood mononuclear cells

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A B S T R A C T

The objective of this study was to validate a TaqMan real-time PCR assay for HTLV-1 proviral load detection in peripheral blood mononuclear cells. TARL-2 cells were used to generate a standard curve. Peripheral blood mononuclear cell gDNA from 27 seropositive and 23 seronegative samples was analyzed. The sensitivity, specificity, accuracy, precision, dynamic range of the standard curve and qPCR efficiency were evaluated. All of the positive samples amplified the target gene. All of the negative samples amplified only the control gene (*β-actin*). The assay presented 100% specificity and sensibility. The intra- and inter-assay variability was 2.4% and 2.2%, respectively. The qPCR efficiency, slope and correlation coefficients (r^2) were all acceptable. The limit of detection was 1 copy/rxn. This assay can reliably quantify HTLV-1 proviral load.

1. Introduction

The human T-cell lymphotropic virus type 1 (HTLV-1) was the first retrovirus described in humans (Poiesz et al., 1980). HTLV-1 infection remains asymptomatic in most infected individuals. However, the virus may cause a neurological chronic disorder referred to as HTLV-1-associated myelopathy (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). HTLV-1 infection is also related to adult T-cell leukemia/lymphoma (Poiesz et al., 1980).

The laboratory diagnosis of this viral infection is based on immunoassays such as ELISA and Western blot. These techniques have several disadvantages, as in cases with indeterminate results and their impossibility of being used in immunosuppressed patients or in neonatal infections. These immunoassays, which are based on antibody detection, may also yield false-negative results for recent infections (Tamegão-Lopes et al., 2006). In such cases, molecular biology techniques are an alternative (Andrade et al., 2010). Beyond qualitative results, real-time PCR can quantify proviral load, which may help to assess disease progression (Besson and Kazanji, 2009; Nagai et al., 1998; Olindo et al., 2005). Although real-time PCR has a high sensitivity and specificity, the method is still an in-house technique. Thus, prior validation is essential before the implementation of this technique in routine laboratory (Kamihira et al., 2010). The present study aimed to validate a TaqMan real-time PCR assay for HTLV-1 proviral load detection in peripheral blood mononuclear cells (PBMCs) based on a conserved region of the *pX* gene.

2. Materials and methods

2.1. Clinical samples

Samples of frozen PBMCs were obtained from 27 HTLV-1-seropositive patients seen at the Neuroinfection Outpatient Clinic, Hospital Universitário Gaffrée e Guinle (HUGG/UNIRIO). These samples were tested by ELISA (Murex HTLV-I + II, Diasorin, United Kingdom). The presence of anti-HTLV-1 antibodies was confirmed by Western blotting. In total, 23 seronegative individuals (screened by ELISA) were included in the study. The genomic DNA (gDNA) was extracted using a QiAmp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

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Table 1
Description of primers and probes used in real time PCR.

Primers and probes	Sequence	Position
pX (HTLV-1)		
pX Forward primer	ACAAAGTTAACCATGCTTATTATCAGC	7299–7325
pX Reverse primer	ACACGTAGACTGGGTATCCGAA	7378–7357
pX TaqMan probe	FAM-TTCCCAGGGTTTGGACAGAGTCTTCT-TAMRA	7330–7355
β-actin		
Actin Forward primer	CACATCGTGCCCATCTACGA	2146–2165
Actin Forward primer	CTCAGTGAGGATCTTCATGAGGTAGT	2250–2225
Actin TaqMan probe	FAM-ATGCCCTCCCCCATGCCATCCTGCGT-TAMRA	2171–2196

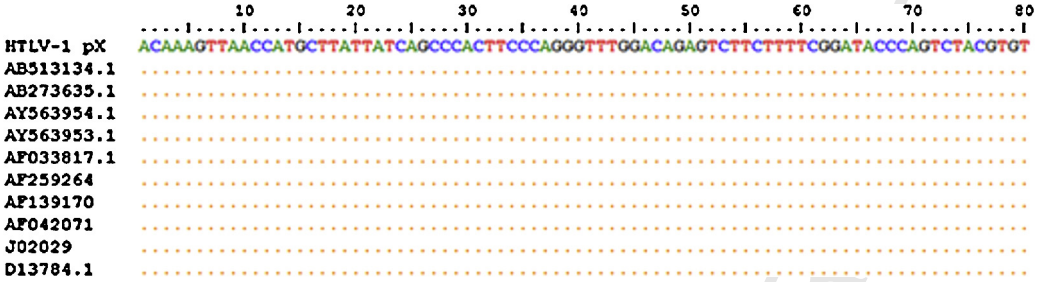

Fig. 1. Sequence alignments of target sequence used in real-time PCR.

Table 2
Reproducibility experiments: intra- and inter-assay reproducibility of TARL-2 cells DNA standard dilutions analyzed in triplicate on four different assays.

	CT Mean	Intra-assay variability								Inter-assay
		Experiment 1		Experiment 2		Experiment 3		Experiment 4		
		SD	CV (%)	SD	CV (%)	SD	CV (%)	SD	CV (%)	
pX										
50,000	22.7	0.1	0.6	0.1	0.3	0.1	0.3	0	0.1	1.5
50,00	26.0	0	0.2	0.1	0.2	0.1	0.3	0.1	0.4	1.5
500	29.5	0.1	0.4	0.1	0.2	0	0	0.1	0.5	1.2
50	33.0	0.2	0.6	0.2	0.6	0	0	0.2	0.7	0.7
5	36.3	0.6	1.7	0.1	0.3	0.1	0.2	0.8	2.2	2.1
β-actin										
66,600	23.4	0	0.1	0	0.2	0	0	0	0.2	2.2
13,320	24.3	0.1	0.5	0.1	0.5	0.1	0.3	0.1	0.6	2.2
2664	25.0	0.2	0.9	0.2	0.6	0.1	0.4	0.2	0.7	1.9
532	25.6	0.1	0.5	0.1	0.4	0.1	0.2	0.1	0.3	1.8
106.6	26.8	0.1	0.4	0.1	0.3	0.1	0.2	0.1	0.3	2.0

2.2. Cell line description

The gDNA of TARL-2 cells was kindly provided by National Institutes of Health (USA). TARL-2 cells are a rat T-cell line that contains a single copy of HTLV-1 provirus (Tateno et al., 1984).

2.3. Primers and probes

The primers and probes used were described previously (Nagai et al., 1998). Briefly, for the HTLV-1 *pX* region, the primer set was 5′-ACAAAGTTAACCATGCTTATTATCAGC-3′ and 5′-ACACGTAGACTGGGTATCCGAA-3′ (positioned at 7299–7325 and 7378–7357, respectively; GenBank: J02029.1). The TaqMan fluorescent probe used was FAM- TTCCCAGGGTTTGGACAGAGTCTTCT – TAMRA (positioned at 7330–7355; GenBank: J02029.1). The *β-actin* gene was used as an internal control and to determine the input cell number to avoid variation due to differences in DNA input into the reaction. The primer set was 5′-CACATCGTGCCCATCTACGA-3′ and 5′-CTCAGTGAGGATCTTCATGAGGTAGT-3′. The *β-actin* probe was FAM- ATGCCCTCCCCCATGCCATCCTGCGT – TAMRA (Table 1). The BioEdit program was used for sequence alignments (Fig. 1).

2.4. TaqMan real-time quantitative PCR

Real-time PCRs were conducted using a 7500 ABI (Applied Biosystems). The DNA of HTLV-1-infected TARL-2 cells was used for the standard curve. Serial 10-fold dilutions were analyzed from 5×10^4 to 5 copies of *pX*. The DNA of PBMCs from non-infected individuals was used for the *β-actin* standard curve. The series represented a five-fold dilution of up to 6.6×10^4 copies of *β-actin* (Table 2). Both standard curves were repeated during each experiment. Because there is a wide range of proviral loads among patients, to evaluate the intra- and inter-assay variation, each standard curve dilution was analyzed in triplicate in four different assays. Moreover, one sample was tested 15 times on two consecutive days. Five clinical samples with different proviral loads (0.1; 1.8; 18.8; 24.5; 109.2 copies/100PBMCs) were selected and also analyzed in triplicate on two different days. To evaluate the limit of detection, a sample containing only one copy of the target gene was added and analyzed in duplicate in three consecutive days. The real-time PCR mixtures consisted of 12.5 μL of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.9 μM of forward and reverse primers and 0.25 μM of probe brought up to 2.5 μL with nuclease-free water and 10 μL of gDNA (10 ng/μL clinical samples or standard template), totaling 25 μL

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