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HTLV-1 Proviral Load Absolute RT-qPCR Development for Assessing on Clinical Outcomes in HAM/TSP Patients

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Abstract

Background: The significance of HTLV-1 proviral load as a prognostic biomarker in HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) has been a subject of controversy. This study aims to assess the impact of HTLV-1 proviral load (PVL) on the clinical outcome in patients with HAM/TSP.

Methods: An absolute quantitative HTLV-1 PVL RT-qPCR, TaqMan method was developed with 100% sensitivity and specificity. Then, from 2005-2018, the HTLV-1 PVL of 90 eligible newly diagnosed HAM/TSP patients were assessed for demographic, clinical symptoms and their associations with HTLV-1-PVL.

Results: The quality control of the designed RT-qPCR showed a sensitivity and specificity of 100%. Spasticity in lower limbs in 58.9% and urinary symptoms in 17.8% of HAM/TSPs were observed. Using this designed RT-qPCR, the HTLV-1-PVL strongly affected spasticity and sphincter disturbance (p=0.05). The multivariate logistic test showed that only the beginning of lower limb weakness along with tremor was associated with PVL (OR: 2.78. 95% CI (0.99-1.02) and p=0.05). Urinary incontinence was prevalent among these patients; however, no association was identified with the HTLV-1 proviral load (PVL).

Conclusion: The absolute RT-qPCR developed for measuring HTLV-1 proviral load (PVL) demonstrated reliable results. Despite a high prevalence of urinary incontinence in these patients, no association was observed with the PVL. Consequently, it appears that HTLV-1 proviral load is specifically associated with developing spasticity in HAM/TSP.

Keywords: Clinical manifestations, HTLV, Neuroinflammatory disease, Proviral load, TaqMan.

Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is the etiological agent of two diseases; adult T cell leukemia/lymphoma (ATLL) and a neuroinflammatory disorder called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (1-3). It is estimated that more than 10 million people are currently suffering from HTLV-1 (4). HTLV-1 induces a latent infection, which in the majority of the subjects does not induce any noticeable disorder throughout their lifetime, and they remain asymptomatic carriers (ACs) (5). Although HTLV-1 is distributed globally, the Middle East is a known endemic area (6, 7). HTLV-1 is disseminated in the northeast of Iran with a rate of 2.12% for subtype A (6, 8, 9).

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a neuro-

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inflammatory disease with one or more neurological manifestations, such as lower extremity muscle weakness, spastic paraparesis of lower extremities, urinary bladder disturbance, and sensory disturbance (10). The onset of clinical symptoms in patients with HAM/TSP occurs several decades after infection. Two risk factors of gender and age significantly affect the manifestation of the symptoms (11, 12).

Studies introduced the HTLV-1 provirus copies as a prognostic risk factor in HAM/TSP development and progression (13, 14), while others did not find any association (15-17). Therefore, in the current cross-sectional study, the impact of HTLV-1 proviral load (PVL) as a virological factor on clinical manifestations of 90 newly diagnosed HAM/TSP patients was evaluated.

Materials and Methods

Designing and developing an Absolute RT-qPCR

Designing primers and probes

The highly conserved region of HTLV-1-*Tax* gene and human *albumin* was obtained from the nucleotide database of NCBI (www.NCBI.NLM.NIH/nucleotide) and aligned by the Clustal W2 program. Primers and Probes were selected using the Beacon Designer (version 7) software program and then assessed by NCBI BLAST software. The primers and probes were submitted to BioNeer in South Korea for synthesis. The fluorogenic probes were labeled with FAM and BHQ1 as the fluorochrome and quencher. The conventional PCRs were carried out on PBMCs DNA of HTLV-1 patients for *Tax*- and *albumin* using the designed primers. Briefly, genomic DNA, extracted with the Roche Genomic extraction kit (Düren, Germany), was subjected to PCR amplification, PCR products (10 µL) were gel-electrophoresed and visualized by ethidium bromide staining. Then PCR products were sent for sequencing to validate the design (BioNeer, Korea). The oligonucleotide of primers and probes for both DNA fragments, *Tax*, and *albumin* are shown in Table 1.

Table 1. The sequences of all primers and probes were used in this study.

			Length (bp)	TM (°C)	GC (%)	Sequence (5' to 3')	Product size (bp)
HTLV-1	Quantitation Primers	Forward	20	58.2	60	5́-CGGCTCAGCTCTACAGTTCC-3́	95
		Reverse	20	58.5	60	5́-GAGTGATTGGCGGGGTAAGG-3́	
	Prob		24	68.6	70.8	FAM-CGACTCCCCTCCTTCCCCACCCAG3BHQ!	
	Qualification Primes	Forward	19	48.8	42.1	5́_AGCGAATAGAAGAACTCC_3́	190
		Reverse	20	49	40	5́_CGGTAAATGTCCAAATAAGG_3́	
Albumin	Alb-S Primer	Forward	22	58	52	5́_GCTGTCATCTCTTGTGGGCTGT_3́	90
	Alb-AS Primer	Reverse	22	68	53	5́_AAACTCATGGGAGCTGCTGGTT_3́	
	Alb TaqMan Probe	Prob	21	70	49	FAM-CCTGTCATGCCCACACAAATCTCTCC-TAMRA	

Construction of plasmids

To design a reliable absolute, quantitate kit, the fragments of interest from HTLV-1*Tax*, and the human albumin gene was separately amplified with their specific primers. Then each PCR product TA is cloned into PTZ 57R/T (Fermentas, Lithuania). All the engineered constructs were sequenced with M13 primers to check the sequence of the inserts and the number of inserts in multiple cloning sites). After confirming the Tax-bioengineered and human bioengineered plasmids, the concentration was assessed using a Nanodrop (Thermo Fisher Scientific, Canada) and stored in siliconized tubes at −20 °C.

Quality control

For quality control of the designed absolute RT-qPCR kit, the HTLV-1 infected HUT-102 cell line (ATCC-CRM-TIB-16.2) with 4-6 copy numbers per cell. This cell line was kindly gifted by Professor Bazarbashi from the American University in Beirut, Lebanon. Moreover, the patients had a PVL measurement at their admission to the Clinic of HTLV-1 using a commercial HTLV-1 PVL assessment kit (Novingene, Iran).

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The DNA isolated from peripheral blood mononuclear cells (PBMCs) of HTLV-1 infected subjects were applied to our homemade kit for assessing HTLV-1-PVL. The designed bioengineered plasmids stocks were 10-fold-serial diluted to construct 6-point standard curves for each gene, Tax, and albumin. After optimizing this homemade kit and confirming the quality, the PVL of HAM/TSP patients in the study was measured.

Study population

A retrospective cohort study is running in Mashhad University of Medical Sciences (MUMS). After, designing and producing the HTLV-1 homemade kit. The effect of PVL, was evaluated on clinical manifestations of HAM/TSP patients.

Ninety eligible patients diagnosed with HAM/TSP, who were admitted to the referral HTLV-1 Clinic at Ghaem Hospital affiliated to MUMS Iran, between 2005 and 2018, were included in the study. The Urinary Disturbance Score (UDS) and the World Health Organization (WHO) criteria (18) were used for the classification of the patients (19, 20). All participants were seropositive for HTLV-1, 2 antibodies, tested by a commercial ELISA kit (Dia. Pro, Italy). These data were confirmed by PCR, as previously described (20). Additionally, all patients underwent thorough examination and assessment by two neurologists and the characteristics of neurological manifestations were systematically examined. Clinical symptoms in patients with HAM/TSP were assessed in two categories: sensory symptoms and motor signs. Motor dysfunctions, including abnormal gait, spasticity in lower limbs, sensory disturbance, and urinary disorders, were assessed in patients with HAM/TSP.

Assessment of HTLV-1 Proviral Load

Blood samples of HAM/TSP patients were collected, and the peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient (Sigma, Schnelldorf, Germany). A DNA extraction kit (QIAamp; Qiagen, Germany) was applied for genomic extraction of PBMCs. The real-time PCR assay was then carried out on extracted DNA, using the designed TaqMan quantification kit in a Rotor-gene Q 6000 machine (Qiagen, Germany). Tax-specific primers and a fluorogenic probe were utilized to measure copy number of HTLV-1 in PBMCs. The PVL in HTLV-1 infected HUT cell line, and PVL-defined samples were calculated by a Rotor-Gene software (Qiagen, Germany) according to the standard curve, as previously described (20). Briefly, the normalized value of the HTLV-1 provirus in each subject was measured according to this equation: HTLV-1 PVL per 10^4 PBMCs= number of HTLV-1 DNA copies (Tax gene)/number of albumin DNA copies/2) $\times 10^4$.

Statistical analysis

The SPSS software version 11.0 (SPSS Inc, USA) was used to analyze the patients' data. The distribution of the variables was assessed with the Kolmogorov–Smirnov test. The One-way ANOVA and student's t-test were then used for variables with normal and the Kruskal-Wallis and Mann‑Whitney tests for variables with non-normal distributions. Spearman's tests were used to assess correlations among the studied variables. Multivariate logistic regression tests were also employed for risk factor assessments. A P-value less than or equal to 0.05 was considered statistically significant.

Results

Absolute HTLV-1 RT-qPCR

The bioengineered *Tax* plasmid stock concentration was 10^{11}, and *albumin* was 10^{10}. The 6 standards points from 10-10^6 were prepared. The sensitivity and specificity of the designed kit were for HTLV-1 diagnosis was 100%. The lower detection limit for HTLV-1 copies was around 17 per 10^4 PBMC cells. The controls were HTLV1 positive HUT and the commercial kit (Novin Gene Gostar, Iran).

Study population

Of 90 HAM/TSP subjects, 31 (12%) were men, and 68 (88%) were women. The average age of participants was 45 years. There was no significant difference in gender and age between men and women (Table 2).

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Table 2. Characteristics and HTLV-1-PVL of HAM/TSP patients.

Characteristics	Genders Number (%)	
	Women	Men
Subjects n (%)	62 (68.88%)	28 (31.12%)
Age (mean years)	43.21±13.14	47.54±15.74
Proviral load*	670.55±95.62	687.21±130.44
Minimum proviral load	12	42
Maximum proviral load	4883	2838

*Mean± SE of copy number per 10000 PBMCs.

HTLV-1 proviral load

The average of PVL (Mean±SEM) was 674±76.9 copies in 10^4 PBMCs, while the PVL average in women was (670.55±95.62) and in men 687.21±130.44 copies in 10^4 PBMCs. Maximum and minimum HTLV-1-PVL were 4883 and 12 copy number/10^4 PBMCs, respectively (Table 2).

Clinical findings

The clinical assessment showed that primary mobility dysfunction in HAM/ TSP patients was 58.9% (Table 3). There was no significant correlation between clinical disorders and age. Urinary symptoms have been observed in 22.1% of HAM/TSP cases. In a more detailed examination, it can be classified into incontinence (8.90%), increased urinary frequency (4.40%), dysuria (1.10%), the sensation of incomplete emptying (1.10%), nocturia (1.10%), increased urinary frequency (1.10%) and nocturia (1.10%), increased urinary frequency and incontinence (4.4%) (Table 4).

Table 3. The distribution of disorders in HAM/TSP patients.

Movement disorder, Number (%)	Women	Men	Total
Abnormal gait and spasticity in lower limbs	36(58.1%)	17 (60.7%)	53(58.9%)
urinary symptoms	11(17.7%)	5(17.9%)	16(17.8%
Abnormal gait and urinary symptoms	6(9.7%)	3(10.7%)	9(10%)
Asymptomatic	9(14.5%)	2(10.7%)	11(13.3%)

Table 4. The distribution of urinary and movement symptoms.

Urinary symptoms	Number (%)		
	Women	Men	Total
incontinence	5(8.1%)	3(10.7%)	8(8.9%)
increased urinary frequency	3(4.8%)	1(3.6%)	4(4.4%)
dysuria	1(1.6%)	0(0%)	1(1.1%)
sensation of incomplete emptying	0(0%)	1(3.6%)	1(1.1%)
nocturia	0(0%)	1(3.6%)	1(1.1%)
increased urinary frequency and nocturia	0(0%)	1(3.6%)	1(1.1%)
increased urinary frequency and incontinence	4(6.5%)	0(0%)	4(4.4%)
Movement symptoms			
Gait disturbance	32(53.2%)	21(67.6%)	(53)56.9%
Back pain	18(29.1%)	5(17.9%)	23(25.5%)
Asymtomatics	8(13%)	3(12%)	11(12%)

The assessment of sensory symptoms (16.6%) in HAM/TSP patients included distal tactile hypoesthesia, unusual mild numbness, and distal impairment of vibration with a lower sense of position were observed in 11.30% of patients (Table 5). In comparison to individuals without symptoms, those who reported sensory disturbances exhibited a statistically significant difference in viral load (p=0.003).

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Table 5. The distribution of sensory symptoms.

Sensory symptoms, Number (%)	Women	Men	Total
Mild distal impairment of vibration and sense of position and numbness	7(11.3%)	3(10.7%)	10(11.1%)
Numbness	2(3.2%)	1(3.6%)	3(3.3%)
Mild distal impairment of vibration and sense of position	0(0%)	2(7.1%)	2(2.2%)
Total	9(14.5%)	6(21.4%)	15(16.6%)

Mann-Whitney U-Independent Test showed that the PVL in HAM/TSP patients with abnormal gait and urinary symptoms was high, although it was not statistically significant at 95% confidence intervals (p=0.09, CI=91%).

Data analysis by multivariate logistic regression showed lower limb weakness and sphincter disturbance as early prognosis signs among HAM/TSP patients (p=0.06 and p=0.009, respectively) (Table 6). Furthermore, the logistic regression analyses for feeling lower limb weakness, tremor, paraparesis, and tetra paraparesis showed that only the PVL was associated with lower limb weakness (OR=2.7, 95% CI; 0.99–1.02). Gender was a risk factor for the development of paraparesis (OR= 4.37, 95% CI; 0.02–0.88), and the older age was a risk factor for tetra paraparesis (OR=4.067, 95% CI; 1.05–33.85) (Table 7). n HAM/TSP development and progression, women were more susceptible to paraparesis and older subjects to tetra paraparesis.

Table 6. The data for HTLV-1 proviral load and clinical symptoms in early phase of HAM/TSP in newly diagnosed patients.

Clinical symptom		n (%)	Proviral load (Mean ±SD)	p-value
Flushing and heart palpitations	Yes	2 (3.2%)	2712±3066.01	0.23
	No	60 (96.8%)	602.40±529.50	
Constipation	Yes	13(21%)	1006.08±1313.08	0.38
	No	49(79%)	581.53±500.80	
Lower limb weakness	Yes	2 (3.2%)	1058±458.20	0.05
	No	60 (96.8%)	657.63±759.80	

Table 7. Logistic regression for feeling lower limb weakness and tremor, paraparesis, and tetra paraparesis.

Logistic regression for feeling lower limb weakness and tremor					
	B	SE	P	Multivariate	
				OR	95%Cl
Pro Viral Load	0.011	0.007	0.05	2.783	0.99-1.02
Gender	4.580	2.883	0.11	2.524	0.00-2.91
Age	0.351	0.202	0.08	3.024	0.95-2.10
Hb	0.047	0.326	0.88	0.020	0.50-1.80
Neutrophil	1.388	0.887	0.11	2.447	0.04-1.42
Lymphocyte	1.428	0.909	0.11	2.466	0.04-1.42
Logistic regression for paraparesis					
	B	SE	P	Multivariate	
				OR	95%Cl
Viral Load	0.001	0.001	0.37	0.786	0.99-1.00
Gender	0.563	0.946	0.03	4.385	0.02-0.88
Age	0.015	0.038	0.90	0.015	0.93-1.08
Hb	0.273	0.249	0.27	1.210	0.46-1.23
Neutrophil	0.146	0.158	0.35	0.855	0.63-1.17
Lymphocyte	0.048	0.157	0.76	0.093	0.70-1.29
Logistic regression for tetra paraparesis					
	B	SE	P	Multivariate	
				OR	95%Cl
Viral Load	0.006	0.004	0.11	2.492	0.99-1.01
Gender	0.124	0.090	0.17	1.873	0.94-1.35
Age	1.786	0.886	0.04	4.067	1.05-33.85
Hb	1.117	0.675	0.09	2.735	0.08-1.23
Neutrophil	1.177	0.702	0.09	2.811	0.07-1.22

B: Regression estimated coefficient, SE: Standard Error, P: Probability.

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Discussion

The HTLV-1 PVL as a prognostic or even diagnostic factor for HAM/TSP onset has been very controversial among different studies (13-17). Therefore, to explain the relationship between HTLV-1-PVL and clinical symptoms, 90 newly diagnosed HAM/TSP patients were evaluated in this study. Our findings showed that HTLV-1 PVL might be a prognostic factor for spasticity symptoms but no other signs and symptoms in the HAM/TSP onset. Furthermore, in the present study, according to the strong correlation of PVL with frequent lower limb weakness and tremors, it can be suggested that frequent feelings of lower limb weakness and tremors could be a prognostic factor for HAM/TSP manifestation in HTLV-1 ACs subjects. In other words, in HAM/TSP, the clinical events begin with the emergence of HTLV-1 from latency to active replication and consequently increase the PVL. Clinical-molecular epidemiologic studies have revealed that reactivation of HTLV-1 is an essential pathogenic step in multiple virus-associated diseases such as HAM/TSP and ATLL. However, to escape from host immune responses, the *Tax* is suppressed and virus backs to the latency, and PVL decreased during disease progression. On the other hand, the presence of chronically active HTLV-1-specific $CD8^+$ cytotoxic T cells to HTLV-1 antigens in all infected subjects, regardless of their PVL, argues against the total latency of the virus *in vivo* (21, 22).

HTLV-1 was discovered around 30-year ago, but its pathophysiology remains to be elucidated (3, 23). Previous studies have shown that increased PVL and inflammatory conditions predispose the spinal cord lesion (24, 25). The increased PVL in HAM/TSP shows the presence of viral factors such as *Tax* and HTLV-1 bZIP factor (HBZ), implicating the replication of HTLV-1 *in vivo* and disease development (24). In contrast, Lezin *et al.* found a lower rate of HTLV-1-PVL in the ACs compared with HAM/TSP patients. The values between the two groups overlapped, making this factor unsuitable as a prognostic or diagnostic criterion (26). Recently, Grassi *et al.* suggested a value of 498 copies/10000 PBMCs, with high reliability as the best PVL cut-off point to differentiate HAM/TSP subjects from ACs (27). However, in a cohort study, Furtado *et al*. argued that increased PVL in the CSF and not the blood of ACs was an essential feature for the onset of neurological symptoms in HAM/TSP patients. They have highlighted the importance of monitoring the spinal cord damage and mobility in HAM/TSP patients (28). However, the clinical consequences and the frequency of occurrences in HAM/TSP vary dependent on the duration of the disease, the host genetic background, and the nature of the virus (29, 30). Brazilian HAM/TSP patients have mainly suffered from the lower limbs, low back pain, motor disability, moderately sensory deficits, hand numbness, low frequency of foot numbness, increased tendon jerks in upper limbs, and sphincter problems(31). In Japanese infected individuals, the main symptoms were gait impairment and moderate disorders, including urinary disturbance, numbness of the lower legs, and lower frequency of lumbago and constipation (19). However, in Iranian HAM/TSP patients, the main annoying symptoms were fatigue and spasticity in the lower limbs, paresthesia, constipation, gait impairment, and urinary disturbance (32). Like the previous studies, most HAM/TSP patients suffered from walking difficulties due to gait disturbances, spasticity in the lower limbs, urinary disorders, dysfunction and deficiency in the lower extremities, and urinary problems.

The main common urinary symptoms in Brazilians have been reported as urinary incontinence, nocturia, and increased frequency (33); however, in this study among HAM/TSP patients, the prevalence of urinary incontinence was more than the other urinary disorders. In line with previous studies, the current findings showed that women were more susceptible to HTLV-1 infection (34, 35). These studies suggested that the impact of a higher rate of PVL on clinical symptoms

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in women, particularly on sphincter disturbance, was significant.

According to these studies and our present findings, frequent feelings of lower limb weakness and tremors could be the introductory sign in ACs who may develop HAM/TSP. Therefore, assessing HTLV-1 PVL in this stage can be a diagnostic factor to differentiate from other neurological or psycho-neurological abnormalities in HTLV-1 infected subjects.

The evaluation of sensory complaints in the present study showed that viral load in individuals who had sensory disorders was significantly higher than in others with no symptoms. Some other authors reported the sensory symptoms, such as a mild distal impairment of vibration, cutaneous paresthesia, distal tactile hypoesthesia, sense of position around, and pinprick hypoesthesia in their patients (36). Furthermore, impairment of vibration sense and pain in the lower limbs have also been reported (37, 38)

In this study, the HTLV-1-PVL did not significantly relate to the gender and age of the HAM/TSP patients, which confirms the results of the Vakili *et al.* survey in Iranian HAM/TSP patients (32). However, the relationship between PVL and age has been controversial. Although, Furtado *et al*.(28) did not discover any association between PVL and age at occurrence or duration of disease. Matsuzaki *et al*. (39) demonstrated a statistically meaningful association between PVL and the disease manifestation at>65 years.

The present study showed that there was not any significant association between PVL and urinary incontinence. Data from a clinical study conducted on Iranian HAM/TSP patients in our research centers suggested that not only triple therapy (Pegylated IFN-α, Prednisolone, and Sodium Valproate) had a positive effect on patients' clinical symptoms but also decreased the HTLV-1-PVL. Although symptoms of spasticity were noticeably improved in that study, the urinary symptoms were not highly affected by the triple therapy. The study also confirmed that there was a strong association between PVL with spasticity rather than urinary problems (40).

The correlation between genetics, viral factors, environmental condition, socioeconomic situation, and PVL should be taken into account to understand the pathogenesis of HTLV-1 better, inducing diseases. It can be suggested that feeling frequent lower limb weakness and tremors may associate with increasing activities of HTLV-1 and is an early/initiation sign for the development of HAM/TSP, in which women are more susceptible to an older age to tetra paraparesis. The newly diagnosed HAM/TSP patients have been studied in this study; therefore, it has limitations. The sample size was reduced because many cases had been misdiagnosed and taken medications when referred to the HTLV-1 clinic. The spastic and urinary scores at the beginning of the disease manifestation were low to have a statistic analysis with PVL. Although the HTLV-1 is recently brought to the attention of the WHO, it is still a neglected disease in terms of epidemiology, medical and general education in an endemic area, and proper treatment. These issues highlight the importance of the slowly increasing number of HTLV-1 infections globally. Thus, other molecular epidemiology and host-virus interactions (epigenetics) studies should be conducted to find proper prognosis and prevention methods.

In conclusion, the findings revealed that HTLV-1 manipulation of the host immune system toward HAM/TSP is a complicated condition in which HTLV-1-PVL can only act as a prognostic factor in the very early step of spastic signs and symptoms, not urinary or sensory complications. Therefore, the HTLV-1 associated diseases development and progression must be considered in terms of virus-host interactions and their consequences with an assessment of virus regulatory factors such as Tax and the primary host immune reaction to this immune-dominant HTLV-1 Ag, maybe IL-2Rα.

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Statement of Ethics
Mashhad University of Medical Sciences Biomedical Ethics Committee confirmed the study (IR.MUMS.fm.REC1388.574). Before the sampling, written informed consent was obtained from all patients.

Conflict of Interest
The authors have no conflicts of interest to declare.

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