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SYBR Green–based quantitation of human T-lymphotropic virus type 1 proviral load in Peruvian patients with neurological disease and asymptomatic carriers: Influence of clinical status, sex, and familial relatedness

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To evaluate the human T-lymphotropic virus type 1 (HTLV-1) proviral DNA load in patients with HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and asymptomatic HTLV-1 carriers, a SYBR Green–based real-time quantitative polymerase chain reaction (qPCR) assay was developed. HTLV–1 proviral DNA in peripheral blood mononuclear cells (PBMCs) was quantified using primers targeting the pX region and the HTLV-1 copy number normalized to the amount of ERV-3 (Endogenous Retrovirus 3) cellular DNA. Thirty-three asymptomatic HTLV-1 carriers (ACs) and 39 patients with HAM/TSP were enrolled. Some participants were relatives of HAM/TSP cases (16 ACs and 7 patients with HAM/TSP). On multiple linear regression analysis, the authors found a significant association between clinical status and HTLV-1 proviral load ($P < .01$), but only among women. ACs showed a median proviral load of 561 copies per 10^4 PBMCs (interquartile range: 251–1623). In HAM/TSP patients, the median proviral load was 1783 (1385–2914). ACs related to HAM/TSP patients presented a relatively high proviral load (median 1152); however, the association between relatedness to a HAM/TSP patient and proviral load was not significant ($P = .1$). In HAM/TSP patients, no association was found between proviral load and disease duration, progression or severity. The fact that the effect of HAM/TSP upon the HTLV-1 proviral load differed between sexes and the finding of a high proviral load among asymptomatic relatives of HAM/TSP patients suggest that not yet identified genetic or environmental factors influence the pathogenesis of HTLV-1 infection. *Journal of NeuroVirology* (2006) **12,** 456–465.

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Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1) is a poorly recognized infection, although it affects an estimated 10 to 20 million people worldwide (de Thé and Bomford, 1993). The areas of highest prevalence are southern Japan, intertropical Africa, the Caribbean, and South America (Proietti *et al*, 2005). In South America, HTLV-1 is endemic in Brazil, Colombia, and Peru, where 2% to 10% of the healthy adult population are infected (Gotuzzo *et al*, 2000). HTLV-1 is associated with severe diseases, such as adult T-cell leukemia/lymphoma (ATLL) (Hinuma *et al*, 1981; Poiesz *et al*, 1980) and a progressive inflammatory disease of the central nervous system called HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain *et al*, 1985; Osame *et al*, 1986). It is estimated that these diseases affect less than 5% of the infected people, but the associated morbidity and mortality is substantial (Kaplan *et al*, 1990; Murphy *et al*, 1989).

Increased HTLV-1 viral burden in peripheral blood mononuclear cells (PBMCs) (Kira *et al*, 1991; Kubota *et al*, 1993; Nagai *et al*, 1998), measured as the proviral load (number of HTLV-1 DNA copies per fixed number of PBMCs), and exacerbated immune responses to HTLV-1 (Jacobson *et al*, 1990; Kira *et al*, 1992; Parker *et al*, 1992) have been reported in patients with HAM/TSP, compared to asymptomatic HTLV-1 carriers (ACs), suggesting a role of viral and host factors in the pathogenesis of HAM/TSP.

The HTLV-1 proviral load (PVL) remains stable over time in infected individuals (Kubota *et al*, 1993). However, this equilibrium "set point" of PVL can vary considerably between individuals, and appears to be associated with the risk of disease: the prevalence of HAM/TSP increases exponentially once the PVL exceeds 1% of PBMCs (Nagai *et al*, 1998). However, it is still not known whether a high HTLV-1 proviral load is a cause or an effect of the disease process.

The wide variation in PVL levels at equilibrium has been explained by individual differences in the efficiency of the $CD8^+$ T-cell response to HTLV-1 (Asquith *et al*, 2005a; Vine *et al*, 2004). Host genetic factors contribute in determining the HTLV-1 PVL *in vivo*. First, a higher PVL in ACs genetically related to HAM/TSP patients compared to non–HAM/TSP-related ACs has been reported in a Japanese population (Nagai *et al*, 1998). Second, the presence of certain class I HLA alleles (e.g., *HLA-A*02* and *HLA-Cw*08*) was associated with a lower HTLV-1 PVL and a lower risk of HAM/TSP in the same population (Jeffery *et al*, 1999; 2000).

At present, there are no biological or clinical markers available to assess the risk of disease or prognosis in HTLV-1–infected people. The HTLV-1 PVL has been proposed to be such a marker (Matsuzaki *et al*, 2001; Nagai *et al*, 1998; Olindo *et al*, 2005; Takenouchi *et al*, 2003; Taylor *et al*, 1999), but its clinical value has yet to be validated in follow-up studies. First, a high PVL is associated with HAM/TSP, but there is wide overlap in PVL between HAM/TSP patients and ACs (Nagai *et al*, 1998). Second, PVL alone has a weak predictive value as a marker of disease progression among HAM/TSP patients (Olindo *et al*, 2005). Other candidate surrogate markers include the amount of viral expression (HTLV-1 *tax* mRNA and Tax protein levels), the ratio *tax* mRNA/proviral DNA load in PBMCs (Asquith *et al*, 2005b; Yamano *et al*, 2002), and the $CD8^+$ cell–mediated antiviral response measured as the lysis rate of Tax-expressing cells (Asquith *et al*, 2005a; 2005b).

Several procedures for the quantitative assessment of PVL have been described, but until recently, these methods presented certain limitations. They were difficult to compare, mainly due to poor standardization and inaccuracy of the post–polymerase chain reaction (PCR) analysis (Ishihara *et al*, 1994; Kira *et al*, 1991; Kubota *et al*, 1993). They had a limited dynamic range or were too laborious to be used in a routine setting (Cimarelli *et al*, 1995; Albrecht *et al*, 1998). The development of real–time quantitative PCR (qPCR) technology has greatly simplified proviral load measurements (Kamihira *et al*, 2003). qPCR-based methods allow a reliable quantification of the amplicon produced during the exponential phase of amplification, present a broad dynamic range, and low intra- and inter-assay variability, allow a high sample throughput, and avoid the need of post-PCR manipulation (Mackay *et al*, 2002). The TaqMan qPCR system based on fluorescently labeled probes has been applied to the quantitation of HTLV-1 *tax* and *pol* genes (Miley *et al*, 2000; Nagai *et al*, 1998; Estes and Sevall, 2003; Dehée *et al*, 2002). Recently, an alternative qPCR assay based on the intercalating dye SYBR Green has been successfully applied to the quantitation of HTLV-1 and HTLV-2 proviral load in a clinical cohort (Lee *et al*, 2004) and for diagnostic confirmation (Vitone *et al*, 2006).

In this study, we established and validated a SYBR Green–based qPCR assay to quantitate the HTLV-1 PVL in PBMCs. The choice of SYBR Green over the more established TaqMan system was based on two reasons: first, SYBR Green chemistry is less expensive than labeled probes, making of it an affordable test for large-scale testing of clinical samples in resource-limited settings; second, this assay is insensitive to nucleotide variations within the amplified region, which results in a low rate of false-negative results (Papin *et al*, 2004). We describe the features of our assay and report on its use in a cross-sectional analysis of the HTLV-1 proviral load in patients with HAM/TSP and ACs.

Results

qPCR assay for HTLV-1 proviral load determination
Sensitivity and dynamic range of the assay: The sensitivity of our SYBR Green–based qPCR assay

for HTLV-1 DNA quantitation was evaluated by end point titration. Dilutions of p4.39 plasmid DNA corresponding to 10, 1, and 0.1 copies per reaction were prepared and 94 replicates of each dilution point were amplified. Ten copy equivalents of HTLV-1 proviral DNA were detected in 100% of replicates (94 out of 94 reactions); single-copy detection was achieved in 33% of replicates (31 out of 94 reactions) and 0.1 copies were detected in 7% of replicates (7 out of 94 reactions). The dynamic range of the assay for HTLV-1 (pX) quantitation encompassed at least four orders of magnitude (10^5 to 10^1 copies), with a strong linear relationship ($r^2 > .995$) between the Ct values and the $\log_{10}$ of the input template copy number.

Intra- and inter-assay reproducibility: The intra-assay reproducibility of HTLV-1 and ERV-3 quantitation was assessed for four replicates of each standard dilution, run in the same qPCR assay. For HTLV-1 (pX), the coefficient of variation (CV) of C_t values was $\leq$1.8% for all dilution points tested (10^5 to 10^1 copies). For ERV-3, the CV of C_t values was $\leq$1% in the assay range (6×10^4 to 4.8×10^2 copies).

The inter-assay reproducibility was evaluated using different aliquots from a single stock of the respective standards, run in 10 independent assays and analyzed with the same fluorescence threshold. The inter-assay CV of C_t values was $\leq$1.5% for HTLV-1 DNA quantitation and $\leq$0.5% for ERV-3 DNA quantitation, for all dilutions analyzed. Both standard curves (i.e., for HTLV-1 and ERV-3 quantitation) were consistently characterized by correlation coefficients (r^2) $\geq .995$ and slopes ≥ -3.59, indicating a high and comparable amplification efficiency ($\geq$ 90%).

In a next stage, we assessed the intra- and inter-assay reproducibility of the HTLV-1 proviral load measurement in one clinical sample. For this purpose, eight independent DNA extractions of PBMCs from a HAM/TSP patient were prepared. The analysis of four DNA extractions of this sample within the same qPCR run (intra-assay) showed a variation of 33% for pX reactions (2043 $\pm$ 670 copies) and 27% for ERV-3 reactions (24650 $\pm$ 6562 copies). This variability was reduced to 22% when the HTLV-1 (pX) copy number was normalized to the ERV-3 copy number (1670 $\pm$ 367 copies of pX per 10^4 PBMCs). The inter-assay reproducibility was evaluated on all eight independent DNA extractions of this control sample, each included in four or five separate runs (comprising a total of 36 runs). The copy number of pX was 2269 $\pm$ 618 (27% CV) and of ERV-3 was 22175 $\pm$ 5796 (26% CV). This variability was maintained after normalization using ERV-3 quantitation results (2123 $\pm$ 571 copies of pX per 10^4 PBMCs; 27% CV).

As positive control of the proviral load assay, DNA from the MT-2 cell line was included in each run. The number of HTLV-I pX (*tax*) DNA copies in MT-2 cells measured by our qPCR assay was 15.4 $\pm$ 3.0/cell (range 9.7–20.1; 20% inter-assay CV).

Measurement of the HTLV-1 proviral load in clinical samples

Study population: We enrolled 33 ACs and 39 patients with HAM/TSP. The motives for HTLV-1 screening among the ACs were (i) blood donation in 15 individuals; (ii) 1 was the father of a blood donor; (iii) 1 woman was pregnant during a stay in Japan and participated in the local antenatal HTLV-1 screening program; and (iv) 16 individuals were asymptomatic family members (4 siblings, 6 children and 6 spouses) of HAM/TSP patients. Sex and ages of the subjects were as follows: HAM/TSP, 10 men and 29 women, 22 to 86 (mean 51) years old; ACs, 16 men and 17 women, 20 to 67 (mean 42) years old. The age was statistically different between both groups ($P < .01$, *t* test), but did not differ according to sex ($P = .7$, *t* test). Regarding the ethnic origin of the participants, 57% ($n = 21/37$) of HAM/TSP patients and 41% of ACs ($n = 13/32$) were of Andean origin ($P = .4$, chi-squared test). With regard to the clinical presentation of HAM/TSP, the most frequent signs and symptoms in the lower limbs were spasticity and hyperreflexia (39/39), other pyramidal signs (i.e., clonus and/or Babinski reflex: 37/39), paresthesia (35/39), weakness (33/39), and diminished vibratory sensation (31/37). In addition, most of the patients had urinary complaints (34/39), constipation (29/38), and lumbar pain (24/38). In 32 patients, disease severity was assessed using the Barthel Index: the median score was 73 (interquartile range [IQR]: 63–90). The Expanded Disability Status Scale (EDSS) was established in 26 patients: the median score was 5 (IQR: 2–6). Six out of 14 patients who presented rapid progression of HAM/TSP received treatment with lamivudine and/or zidovudine.

When the sample for PVL measurement was taken, the HAM/TSP patients had a median disease duration of 7 years (IQR: 3–11) and a median time of HAM/TSP diagnosis of 2 years (IQR: 0–5 years). Seven HAM/TSP patients had another family member with HAM/TSP (five blood relatives and two spouses).

Comparison of the HTLV-1 proviral load between HAM/TSP patients and ACs: Proviral DNA was detectable in the samples of all HTLV-1–seropositive subjects evaluated in the present study ($n = 72$). The lower limit of detection of the assay was 1 copy of HTLV-1 DNA per 10^4 PBMCs. The proviral load, reported as the copy number of HTLV-1 proviral DNA per 10^4 PBMCs, varied from 142 to 8641 in HAM/TSP patients and from 1 to 4773 in ACs. The mean $\pm$ standard deviation (median) of the proviral load was 2187 $\pm$ 1588 (1783) in HAM/TSP patients and 1009 $\pm$ 1083 (561) in ACs.

The effect of clinical status (HAM/TSP *versus* ACs) on proviral load was evaluated in a multiple linear regression analysis. Although the fit of the model was not perfect, the residual distribution showed little deviation from normality and the model seemed to

Table 1 Multiple linear regression model[a]: predictors of HTLV-1 proviral load

Factor, condition	*P*	*Partial eta squared*	*B*	*Standard error of B*
A. Base model: Rsq = 0.315 (adj Rsq = 0.240), p Levene = 0.015.				
Intercept	0.00	0.71	7.70	0.68
Clinical status	0.001	0.15	−1.72	0.66
Family contact with HAM/TSP	0.11	0.04	−0.28	0.56
Andean ethnical origin	0.25	0.02	0.37	0.32
Sex	0.93	0.00	− 0.75	0.48
Age	0.96	0.00	− 0.001	0.01
Interaction between clinical status and sex	0.03	0.08	1.55	0.68
Interaction between clinical status and family contact with HAM/TSP	0.41	0.01	−0.59	0.71
B. Second step: Rsq = 0.300 (adj Rsq = 0.247), p Levene = 0.034.				
Intercept	0.00	0.96	7.74	0.49
Clinical status	0.001	0.14	−1.52	0.64
Family contact with HAM/TSP	0.12	0.04	−0.21	0.53
Sex	0.93	0.00	−0.63	0.46
Interaction between clinical status and sex	0.05	0.06	1.31	0.64
Interaction between clinical status and family contact with HAM/TSP	0.34	0.01	−0.66	0.69
C. Final model: Rsq = 0.258 (adj Rsq = 0.225), p Levene = 0.013.				
Intercept	0.00	0.96	7.57	0.24
Clinical status	.001	0.15	−1.85	0.39
Sex	0.76	0.001	−0.62	0.47
Interaction between clinical status and sex	0.03	0.07	1.44	0.65

Note. P: *P* value; partial Eta squared: proportion of the total variability that is attributable to a factor; B: regression coefficients.
[a]Multiple linear regression model, UNIANOVA procedure. The dependent variable is the natural logarithm of the HTLV-1 proviral load. (A) Base model. (B and C) In two steps, the least significant variables (age, ethnic background, and relatedness to a HAM/TSP patient) were discarded from the model.

explain 26% of the variation in proviral load (Table 1). The proviral load was different by clinical status ($P = .001$) and by the interaction term Clinical Status x Sex ($P = .03$), which indicates that the effect of the HAM/TSP status upon HTLV-1 proviral load differed between male and female patients (Table 1). The median proviral load in female HAM/TSP patients was 2081 copies per 10^4 PBMCs compared to 410 in female ACs (Mann-Whitney *U* test: $P < 0.001$; Figure 1). In men, there was no effect of the HAM/TSP condition upon PVL: 1166 in male patients with HAM/TSP *versus* 870 in male ACs (Mann-Whitney *U* test: $P = .5$; Figure 1). In addition, there was no significant difference in PVL according to sex in ACs ($P = .3$, Figure 1), but among HAM/TSP patients, the difference in PVL between men and women was significant ($P = .04$, Figure 1).

The median PVL of ACs related to HAM/TSP patients was 1152 copies per 10^4 PBMCs compared to 362 in ACs not related to a HAM/TSP patient. However, in the multiple regression analysis, the association between proviral load and relatedness to a HAM/TSP patient was not significant ($P = .1$, Table 1,A).

HTLV-1 proviral load in relation to clinical status in HAM/TSP patients: Among 24 HAM/TSP patients with slow disease progression, the median PVL was 1829 copies per 10^4PBMCs. Among rapid progressors ($n = 14$), the median PVL was 2013 (Mann-Whitney *U* test: $P = .9$; Table 2). In the rapid progression group, the PVL in HAM/TSP patients receiving antiretroviral treatment (median 1617) was similar to that in patients not undergoing antiretroviral treatment (median 2516) (Mann-Whitney *U* test: $P = .4$; Table 2).

The HTLV-1 PVL among HAM/TSP patients did not show a significant correlation with age at disease onset (Spearman's rho = −.2, $P = .2$) or disease duration (Spearman's rho=.3, $P = .08$; Figure 2). Finally, HTLV-1 proviral DNA also did not correlate with disease severity; the latter was assessed by the Barthel Index (Spearman's rho = −.03, $P = .9$; $n = 32$) and/or EDSS (Spearman's rho = .06, $P = .8$; $n = 26$).

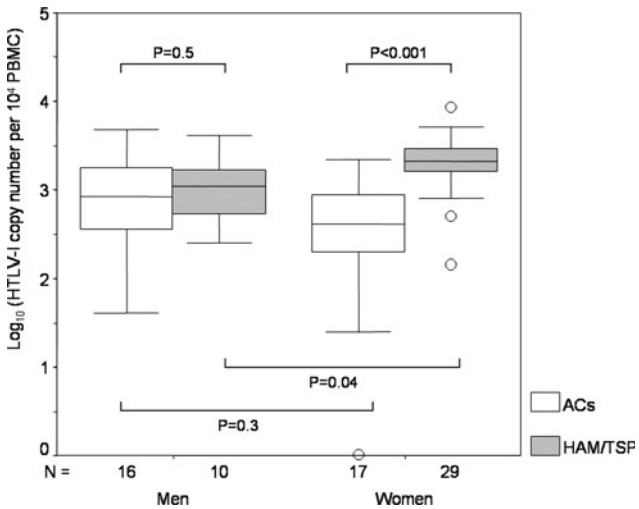

Figure 1 HTLV-1 proviral load according to sex and clinical status. HTLV-1 copy number per 10^4 PBMCs is represented on a logarithmic scale.

Table 2 HTLV-1 proviral DNA levels in HAM/TSP patients in relation to disease progression

	Proviral load[a]		
HAM/TSP progression[b]	*Median*	*IQR*	*Range*
Slow; $n = 24$	1829	1448–2872	142–5086
Rapid; $n = 14$	2013	540–2915	250–8641
Rapid (after start ARV treatment)[c]; $n = 6$	1617	503–2859	475–2914
Rapid (before start ARV treatment)[d]; $n = 8$	2516	1072–3218	250–8641

Note. *n* number of subjects; IQR: interquartile range (25th percentile–75th percentile); ARV: antiretroviral treatment (lamivudine and/or zidovudine).
[a]HTLV-1 copy number per 10^4 PBMCs.
[b]One patient without a definite progression course was excluded from the analysis.
[c]Blood sample for proviral load measurement was taken after start of antiretroviral treatment.
[d]Blood sample for proviral load measurement was taken before start of antiretroviral treatment.

Discussion

We have used a quantitative real-time PCR assay based on SYBR Green chemistry to measure the HTLV-1 proviral load in PBMCs from HTLV-1–infected people. The HTLV-1 DNA PVL has been studied previously using TaqMan (Dehée *et al*, 2002; Estes and Sevall, 2003; Miley *et al*, 2000; Nagai *et al*, 1998; Olindo *et al*, 2005) and SYBR Green (Lee *et al*, 2004; Vitone *et al*, 2006) real-time PCR detection formats. The latter method is less expensive than TaqMan probes and has been shown to be a simple and effective approach for the quantitative detection of HTLV-1 PVL in a large clinical cohort (Lee *et al*, 2004) and in individuals with serological indeterminate results (Vitone *et al*, 2006).

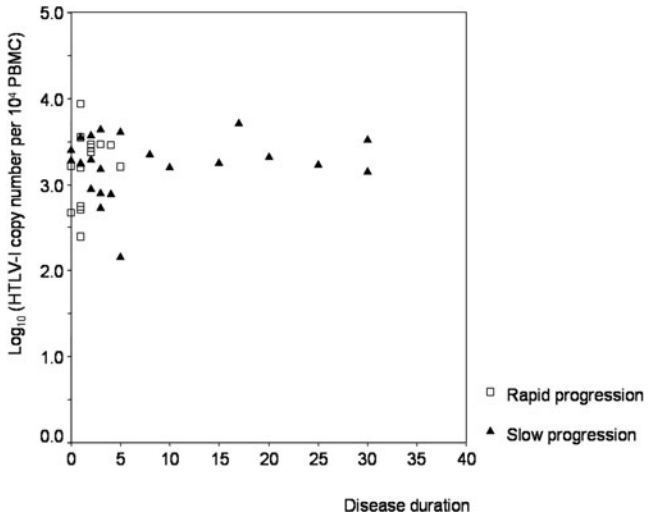

Figure 2 HTLV-1 proviral load in HAM/TSP patients according to disease duration and progression. HTLV-1 copy number per 10^4 PBMCs is represented on a logarithmic scale.

A segment within the pX (*tax/rex*) region of the HTLV-1 genome, highly conserved among viral isolates, was the target for amplification in our qPCR assay. All HTLV-1–seropositive subjects enrolled in the present study had detectable levels of HTLV-1 proviral DNA. The lowest PVL was 1 copy of HTLV-1 provirus per 10^4 PBMCs for an asymptomatic HTLV-1 carrier, coincident with the lower limit of detection of the assay. Our assay showed 100% sensitivity for 10 copy equivalents/reaction. The same sensitivity has been reported with the use of the TaqMan system (Nagai *et al*, 1998).

Because the qPCR assay aimed at quantifying the cell-associated HTLV-1 viral burden, we performed the parallel quantitation of cellular genes (β-actin and ERV-3). These measurements allowed to adjust for variations in DNA recovery and amplification efficiency between samples. The intra-assay CV of HTLV-1 proviral load quantitation, estimated with our SYBR Green–based qPCR assay for one clinical sample, was 22% and the inter-assay CV for the same sample 27%. The observed reproducibility is in agreement with the intra-assay CV of 24% for PBMCs reported by Dehée *et al* (2002), and the mean inter-assay CV of 25% for PBMCs reported by Miley *et al* (2000), using TaqMan qPCR assays.

Next, we assessed the clinical applicability of our established qPCR assay by evaluating its performance in HTLV-1–seropositive patients with HAM/TSP and ACs. The median proviral load in HAM/TSP patients was higher than in ACs, a finding consistent with previous cross-sectional studies performed in other HTLV-1–infected populations (Dehée *et al*, 2002; Kira *et al*, 1991; Kubota *et al*, 1993; Montanheiro *et al*, 2005; Nagai *et al*, 1998; Olindo *et al*, 2005). This supports the view that increased replication of HTLV-1 in individuals with HAM/TSP may be related to the pathogenesis of the disease (Nagai *et al*, 1998).

In comparison with previous reports from Brazil (Montanheiro *et al*, 2005), Japan (Nagai *et al*, 1998), and the Caribbean (Olindo *et al*, 2005), we found a higher median PVL both in HAM/TSP patients and ACs. This might be related to the standards used in the quantitation of HTLV-1 proviral DNA (plasmid templates *versus* HTLV-1–transformed cell lines) and the qPCR system employed (SYBR Green detection in our study compared to the TaqMan system in the other studies). It is also possible that there are actual differences in proviral load between populations from different geographical regions. The fact that different prevalences of HAM/TSP and risk factors for developing HAM/TSP have been documented for some HTLV-1–endemic regions (Gotuzzo *et al*, 2000; Kaplan *et al*, 1990; Proietti *et al*, 2005), supports this view.

Interestingly, we observed a significant influence of sex on HTLV-1 proviral load in HAM/TSP patients, in that female patients showed a higher PVL. This finding is in agreement with the results of a Japanese cohort study (Nagai *et al*, 1998). The reason for this sex difference is unknown; however, three facts can be pointed out. First, epidemiological studies have documented a preponderance of female over male patients with HAM/TSP (Gessain and Gout, 1992; Gotuzzo *et al*, 2004; Lima *et al*, 2005; Maloney *et al*, 1998). Second, in most HTLV-1–endemic areas, HTLV-1 seroprevalence increases with age and is higher in women (Dumas *et al*, 1991; Ouattara *et al*, 1989; Maloney *et al*, 1998; Murphy *et al*, 1991; Hanada *et al*, 1989). Third, sex-related differences regarding the clinical course of HAM/TSP have also been described. A study conducted in Brazil (Lima *et al*, 2005) reported a faster and more severe progression of HAM/TSP in female as compared to male patients, especially if disease onset occurs before the menopause, suggesting a potential role of female hormones in the pathogenesis of HAM/TSP (Lima *et al*, 2005). The latter hypothesis is supported by the beneficial effects of danazol, an attenuated androgenic drug, in the treatment of some patients with HAM/TSP (Harrington *et al*, 1991). On the other hand, some arguments do not fit with this hypothesis. Olindo *et al* (2005) reported a higher HTLV-1 proviral load in male HAM/TSP patients in Martinique, and this was associated with high disability scores and a rapid progression course of the disease. In our cohort of HTLV-1–infected people in Peru, we have not found differences in disease progression between male and female patients with HAM/TSP (Gotuzzo *et al*, 2004). Another argument against a direct effect of sex upon HTLV-1 proviral load is the fact that we did not find a difference in proviral load between male and female ACs. This has also been described in the Japanese cohort (Nagai *et al*, 1998).

In this study, the ACs related to HAM/TSP patients had relatively high HTLV-1 proviral loads, which were comparable to those of the HAM/TSP cases. However, the association between relatedness to a HAM/TSP patient and proviral load did not reach significance in the multiple linear regression model, possibly due to lack of statistical power. A Japanese study revealed a nine-fold higher median proviral load in ACs genetically related to HAM/TSP patients as compared to non-HAM/TSP-related ACs (Nagai *et al*, 1998). Taken together, these findings suggest that relatedness to a person with HAM/TSP is a risk factor for high proviral load among HTLV-1 carriers and point to a contribution of host genetic factors to the replication of the HTLV-1 provirus *in vivo*.

The HTLV-1 proviral load in PBMCs did not correlate with the clinical parameters studied among HAM/TSP patients, i.e., disease duration, progression, and severity. Consistent with our results, previous studies also did not find a correlation between the duration of HAM/TSP disease and the HTLV-1 proviral load in PBMCs (Nagai *et al*, 1998; Olindo *et al*, 2005; Takenouchi *et al*, 2003). On the other hand, a correlation has been found between shorter HAM/TSP disease duration and progressive stage of the disease and an increased ratio of HTLV-1 proviral load in cerebrospinal fluid (CSF) cells to that in PBMCs (Takenouchi *et al*, 2003), indicating that monitoring proviral load in both compartments (PBMCs and CSF) could be useful to estimate disease activity.

Regarding disease progression, we describe our findings in 24 HAM/TSP patients with slow progression and 14 patients with rapid progression. There was extensive overlap in HTLV-1 proviral load levels between these groups and we did not find a significant difference. Olindo *et al* (2005) conducted a study in Martinique with a sample size of 100 HAM/TSP patients and found a higher HTLV-1 proviral load in rapid progressors compared to slow progressors. In that study, the definition of progression rate was based on the degree of walking disability and on disease duration at the time of sampling. Clearly, their definition differs from ours, which is based on the first moment the HAM/TSP patients become aware of their symptoms in the lower limbs (Gotuzzo *et al*, 2004).

Despite the finding of a higher HTLV-1 proviral load in rapid progressors, Olindo *et al* considered that the association between proviral load and disease progression was not strong enough to use proviral load as a surrogate prognostic marker of HAM/TSP progression in clinical practice. Previous studies demonstrated a weak correlation between HTLV-1 proviral load in PBMCs and motor disability (Matsuzaki *et al*, 2001) or disease progression (Takenouchi *et al*, 2003) among HAM/TSP patients. The lack of such correlation could be explained by the fact that the HTLV-1 proviral load in PBMCs does not represent the actual viral activity (Olindo *et al*, 2005). Indeed, an earlier study showed that the amount of expressed antigen (HTLV-1 *tax* mRNA levels) and the ratio *tax* mRNA/proviral load in PBMCs (*tax* mRNA levels after adjusting for proviral load) better reflected the viral activity and could serve as predictive markers of disease progression in HAM/TSP (Yamano *et al*, 2002). Further evaluation of these candidate markers of disease progression in HAM/TSP patients is necessary, as proviral load has a weak predictive value and surrogate prognostic markers are not available.

In summary, this is the first report on the measurement of the HTLV-1 proviral load in Peru. One of the values of the present study is that it shows the feasibility and reliability of the SYBR Green-based qPCR test system in an endemic area with restricted resources. Clearly, this type of assay is a prerequisite to enable large prospective studies in such areas. On a more general level, our results confirm that high levels of HTLV-1 proviral load are associated with HAM/TSP. The observation of a differential effect of the HAM/TSP condition upon the HTLV-1 proviral load according to sex and the finding of a high

proviral load among ACs in families of HAM/TSP patients suggest that not yet identified factors contribute to HTLV-1 pathogenesis.

Materials and methods

Clinical samples and cells

All participants were selected from the HTLV-1 cohort at the Institute of Tropical Medicine Alexander von Humboldt in Lima, Peru. From April 2004 to April 2005, blood samples were collected from 72 HTLV-1–infected individuals: 39 HAM/TSP patients and 33 asymptomatic HTLV-1 carriers (ACs). In addition, blood samples from 12 HTLV-1–seronegative subjects were obtained and tested in the proviral load quantitation assays as negative controls. Written informed consent was obtained from all participants. The study protocol was approved by the Research Ethics Committee of the Universidad Peruana Cayetano Heredia. HTLV-1 serologic status was determined by enzyme-linked immunosorbent assay (ELISA) (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France; Bio–Rad Laboratories, Hercules, CA, USA; or Cambridge Biotech, Worcester, MA, USA) and confirmed by Western blot (Genelabs Diagnostics, Singapore) or line immunoassay (INNOLIA HTLV I/II Score; Innogenetics, Ghent, Belgium).

HAM/TSP patients were included if the following criteria were met: clinical diagnosis of HAM/TSP (based on WHO guidelines; Osame, 1990) and known duration of symptoms. Patients were divided into two groups based on disease progression: slow progressors and rapid progressors. We considered as rapid disease progression a course of HAM/TSP in which the time between the first lower limb symptoms and the inability to walk without the aid of two walking sticks was 2 years or less (Gotuzzo *et al*, 2004). All other patients were defined as having slow disease progression. Disease severity was measured by using the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) and/or the Barthel Index (Mahoney and Barthel, 1965). Patients were excluded if they had concomitant diagnoses of neoplasia or opportunistic infections.

HTLV-1–seropositive ACs were not included if they had a clinical diagnosis of a known HTLV-1–associated disease at any moment, history of tuberculosis in the last 10 years, first-degree relatedness to a person with leukemia/lymphoma, and any HTLV-1–related abnormality found in general clinical, neurological, and/or ophthalmologic examinations.

The HTLV-1–infected human T-lymphoblastoid cell line MT-2 (ECACC 93121518; Miyoshi *et al*, 1981) was used as positive control for the proviral load assay.

Sample preparation

We isolated PBMCs from EDTA-treated blood samples by Ficoll-Hypaque (Pharmacia Biotech, Sweden) gradient separation. Dry pellets of 5×10^6 cells were prepared and stored at -70°C until use. Genomic DNA was extracted using QIAamp blood DNA mini kit (QIAGEN, Hilden, Germany) and adjusted to a concentration of 20 ng/μl.

Standard curves

Standard curves for HTLV-1 proviral DNA quantitation were generated using 10-fold serial dilutions (10^5 to 10^1 copies/reaction) of the HTLV-1 plasmid p4.39 (kindly provided by Thérèse Astier-Gin, Université Victor Ségalen Bordeaux 2, France). This plasmid contains 1 copy of the HTLV-1 proviral genome, which was cloned from the HTLV-1 cell line 2060 (Nicot *et al*, 1993).

Human β-actin and ERV-3 (Endogenous Retrovirus 3) DNA quantitation was performed in parallel on all samples in order to determine the actual input amount of cellular DNA and were used as endogenous references to normalize variations in DNA recovery and amplification efficiency (Nagai *et al*, 2001; Miley *et al*, 2000). Human genomic DNA (gDNA) extracted from PBMCs of healthy HTLV-1–seronegative donors served as the standard. As ß-actin and ERV-3 are single-copy genes with two copies per diploid cell (Nagai *et al*, 2001; Yuan *et al*, 2001), 3.3 pg of human gDNA contain 1 copy of both genes. This equivalence was used for the preparation of the calibration curves in a range of 6×10^4 to 4.8×10^2 copies/reaction (five-fold serial dilutions).

During the experimental setup of the qPCR assay for HTLV-1 DNA quantitation, we assessed the impact of background gDNA on assay performance (i.e., sensitivity and amplification efficiency), as to mimic the background matrix of the clinical samples. For this purpose, we compared standard curves based on serial dilutions of the p4.39 plasmid DNA (10^5 to 10^1 copies/reaction) in nuclease-free water *versus* the same dilution series performed in a background of human gDNA equivalent to 1.5×10^4 PBMCs per reaction (PBMCs were obtained from HTLV-1–negative donors). The sensitivity of HTLV-1 quantitation was not affected in the presence of background gDNA, as similar C_t values were obtained in both conditions, within a CV (coefficient of variation) of 2% for all tested dilutions of the standard plasmid DNA (data not shown). However, the use of gDNA lowered the PCR efficiency (86% compared to 95% in the absence of background gDNA). Therefore, for subsequent experiments, water alone was used. This choice was also based in the comparative results of a previous study (Dehée *et al*, 2002).

Quantitation of HTLV-1 proviral load

HTLV-1 proviral load was determined by quantitative real-time PCR (qPCR) using the SYBR Green I dye as the fluorescent reporter. The primers used for amplification of the HTLV-1 proviral DNA targeted a conserved sequence of 80 bp within the pX (*tax/rex)*

region of the HTLV-1 proviral genome and were: forward: 5′-ACAAAGTTAACCATGCTTATTATCAGC-3′ (positioned at 7299 to 7325) and reverse: 5′-ACACGTAGACTGGGTATCCGAA-3′ (positioned at 7378 to 7357) [nucleotide positions numbered according to the prototype HTLV-1 sequence, GenBank accession no. J02029], as previously described (Nagai *et al*, 2001). The primers targeting ß-actin amplified a segment of 105 bp; forward: 5′-CACACTGTGCCCATCTACGA-3′ (positioned at 3933 to 3952) and reverse: 5′-CTCAGTGAGGATCTTCATGAGGTAGT-3′ (positioned at 4037 to 4012) (GenBank accession no. AY582799) (Nagai *et al*, 2001). The target sequence for ERV-3 quantitation was a segment of 135 bp within the envelope region of the endogenous provirus. The primers used were PHP10-F (5′-CATGGGAAGCAAGGGAACTAATG-3′, positioned at 1601 to 1623) and PHP10-R (5′-CCCAGCGAGCAATACAGAATTT-3′, positioned at 1735 to 1714) (GenBank accession no. M12140), as previously reported (Yuan *et al*, 2001).

qPCR reactions were performed in a 25-μl reaction mixture consisting of 5 μl of DNA sample (100 ng gDNA in the case of clinical samples, roughly equivalent to 1.5×10^4 PBMCs), 0.2 μM each primer, 0.2 mM each dNTP, 3mM $MgCl_2$, 0.3 U of iTaq hot-start DNA polymerase, 10 nM fluorescein, and 1x SYBR Green containing buffer (iQ SYBR Green Supermix reagent; Bio-Rad). Reactions were run on an iCycler iQ instrument (Bio-Rad) using the following thermal cycling conditions: an initial denaturation step at 95°C for 3 min, followed by 45 cycles at 95°C for 30 s and 60°C for 1 min. Data collection and analysis were performed with the iCycler iQ Optical System Software, version 3.0a (Bio-Rad). After each PCR amplification, a melting curve analysis was performed by gradually raising the temperature (0.5°C/10 s up to 95°C) in order to ascertain if the expected product was amplified and no nonspecific products or primer dimers (which could bias the quantification) were formed.

As quantitation controls, DNA from the MT-2 cell line and from a clinical sample of a HAM/TSP patient were included in each run. In addition, DNA samples from healthy HTLV-1–seronegative donors were tested randomly. All samples were processed blindly.

All standard dilutions, controls and clinical samples were amplified in triplicate for each gene (pX and ERV-3) in an optical 96-well reaction plate (Bio-Rad). The HTLV-1 proviral DNA load was calculated as follows: [(pX average copy number)/(ß-actin or ERV-3 average copy number/2)] $\times 10^4$ and expressed as the number of HTLV-1 copies per 10^4 PBMCs, assuming one provirus per HTLV-1–infected cell. Results after normalization using ß-actin quantitation data showed a good correlation with those of ERV-3 (Pearson correlation coefficient:.98; data not shown). This allowed us to analyze the proviral load data on the basis of one reference gene. We chose ERV-3 because a higher reproducibility of the standard curves and a better and consistent amplification efficiency were obtained.

Statistical analysis

Laboratory data were entered into Excel spreadsheets from which they were read into SPSS (version 13; SPSS) for a multiple linear regression analysis using the UNIANOVA procedure. Proviral load was computed as [(CoTax/(CoERV/2)) $\times$ 10000], where CoTax (number of copies of *tax*) and CoERV (number of copies of ERV-3) were the instrument readings. The dependent variable in the analysis was the natural logarithm of the proviral load. The candidate predictor variables in the base model were clinical status (HAM/TSP or AC), relatedness to a HAM/TSP patient, ethnic background (Andean or other), sex, and age in years, as well as the interaction terms Clinical Status $\times$ Sex and Clinical Status $\times$ Relatedness to a HAM/TSP patient. In two subsequent steps, age, ethnic background and relatedness to a HAM/TSP patient were discarded from the model, based upon the significance level. The ethnic background of a participant was defined as Andean if both parents or the four grandparents were born in the Andean region. The HTLV-1 proviral load was analyzed separately for men and women using the Mann-Whitney *U* test. Among the patients with HAM/TSP, we evaluated the association between HTLV-1 proviral load and the clinical parameters of HAM/TSP studied: progression course (Mann-Whitney *U* test), disease duration, and severity (Spearman's rank correlation test).

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