# HTLV-1/2 Viral Load and Immunologic Parameters among HIV-1 Co-infected Individuals

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## Introduction

HIV/HTLV-1 and HIV/HTLV-2 co-infections occur frequently in metropolitan areas where injection drug use is a common mode of transmission. Previous studies have observed some HIV-HTLV-1 co-infected subjects with elevated CD4 counts and HIV-HTLV-2 subjects with elevated CD8 counts (Nadler et al 1996, Beilke et al 2004). Other studies have associated a higher HTLV-1 viral load with HTLV-associated disease (Yamano et al 2001), and with HIV-1 co-infection (Beilke et al 1997). The objective of the current study was to describe factors associated with high HTLV-1/2 proviral loads in patients with HIV co-infection.

## Methods

The New Orleans HIV Outpatient Clinic is the largest provider of HIV treatment in the Gulf south. From this clinic population a prospective cohort study was established to examine clinical outcomes among patients with HIV/HTLV co-infection. Ninety seven (97) HTLV-1/2 ELISA-positive subjects were evaluated at baseline enrollment into the study. Laboratory studies included T cell subsets, HIV viral loads, HTLV-1/2 Western Blotting analysis, peripheral blood mononuclear cell (PBMC) cultures for detection of HTLV-1/2 p19 antigens, and real time PCR to quantify HTLV-1/2 proviral copy number. Antiretroviral therapy administration was available for each subject.

Real Time PCR Assay: Cryopreserved PBMC aliquots (1 x $10^6$ cells) were used to extract DNA for real time PCR amplification. Consensus primers and probe used were: HTV-F5(7359): 5'-CGG ATA CCC IGT CTA CGT GTT T-3' ; HTV-R4 (7519): 5'CTG AGC IGA IAA CGC GTC CA-3' ; Probe P-HTV: 5'-Reporter dye-FAM-ATC ACC TGG GAC CCC ATC GAT GGA-3'-TAMRA-Quencher dye (Miley et al., 2000). Each 25ul reaction contained from 1 x $10^1$ to 1 x $10^6$ copies of viral standard, or 200ng purified genomic PBMC DNA, and the following in sterile water: 1X TaqMan buffer A, 200uM each dATP, dCTP, dGTP; 400 uM dUTP, 3.5 mM MgC12, and 300 mM each of the two HTLV primers (HTV-F5, HTV-R4), 200 nM of HTLV probe (P-HTV), 0.625 U AmpliTaq Gold, and 0.25 U uracil N-glycosylase and were amplified in duplicate, in 96-well arrays of optical-grade PCR plates. Reactions were performed in the Applied Biosystems (ABI) Prism 7700 Sequence Detection System (Foster City, CA). After 2 minutes at 58°C for uracil N-glycosylase digestion as a contamination control for 10 minutes, at 95°C for 15 seconds, and 60°C for 60 seconds, the cycle was repeated for a total of 40 cycles. Real Time PCR Amplification of RNaseP were run in parallel on the same patient DNA samples (not shown). The RNase P gene is a single-copy gene with two copies per diploid cell and was used as an endogenous DNA reference to normalize sample variability and allow accurate quantitation of cell equivalents. RNase P primers, TaqMan probe, reagents and standards were from a commercially available kit (ABI). Universal thermal cycling parameters were used, and performed on standards ($10^1$ to $10^4$ copies) and patient samples as described above. RNAse P results ranged from 2.1 X $10^4$ to 5.3 x $10^5$ copies per sample. Values were divided by two to calculate cell number for each of the samples for results ranging from 1.05 X $10^4$ to 2.65 x $10^5$ cells. Each patient results were normalized by dividing viral copy number by cell number to give viral copies per cell and then multiplied by $10^6$ to give a final value of viral copies per $10^6$ PBMCs.

Statistical analysis: HTLV proviral load, a non-normally distributed continuous variable was dichotomized into **≤ 20,000** and **>20,000** categories for ease of comparison. CD4 and CD8 were categorized into three ordinal categories, using well-established cut-points. Frequencies were computed and chi-squared statistics or Fisher's Exact test were used to test for differences between groups. Hypothesis tests were two-sided and SAS version 8.2 was used to conduct statistical analysis. Additionally, Cochran-Armitage tests of trend were used to test for trend in CD4 and CD8 ordinal categories, with respect to the dichotomous variables of interest.

## Results

Of 97 patients studied, 85 were African American, 70 male, 41 were drug users, mean age was 46.98 (s.d. 9.22), 33 had > 10,000 HIV viral load, 24 had positive P19 Qualitative culture results. Mean CD4 and CD8 were 384.37 (s.d. 423.05) and 854.26 (s.d. 712.91), respectively. HTLV-1/2 viral copies per 1 x $10^6$ PBMCs were: 43$\leq$ 20,000 and 18 > 20,000. The factors associated with HTLV-1/2 viral load > 20,000 were the P19 Qualitative assay result, HTLV type (1,2 or both), as proxied by Western Blot assay, CD4 count and CD8 count. Factors associated with a positive or negative P19 qualitative assay result were CD4 count, CD8 count and HTLV group, as proxied by Western Blot assay. Other factors considered but not found to be associated were age, race, gender, drug use within the last three months, antiretroviral therapy and HIV viral load (>10,000 vs. $\leq$ 10,000).

## Conclusions

There appears to be differential immunophenotypic expression for HIV-1 subjects co-infected with HTLV-1 vs. those co-infected with HTLV-2. Higher CD4 counts seem to be associated with higher HTLV viral load in HTLV-I co-infected patients. HTLV-2 co-infected patients with lower CD8 cell numbers tended to have higher HTLV viral loads. However, caution must be taken in interpreting these results because of relative small sample sizes. HTLV-2 subjects had lower HTLV viral load, while HTLV-1 subjects had higher HTLV viral load. A positive P19 result was more frequently associated with a Western blot result of HTLV-1, while a negative P19 result was associated with HTLV-2. Further study is needed to determine the clinical significance of these laboratory findings.

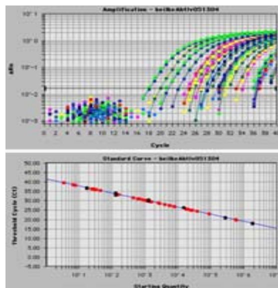

**Figure 1. Real Time PCR Amplification of HTLV- *tax* from New Orleans clinic patients PBMC DNA.**
**Upper Graph (A)** shows the amplification fluorescence emissions (Rn) plotted per cycle for each HTLV PCR reaction. Threshold cycle (Ct) were calculated for each reaction and plotted as a function of input template copy number and a least squared regression performed with the Prism 7700 software. The amplification plot showed virus standards (pHPB3.5) ranging from $10^1$ to $10^6$ copies that resulted in threshold cycle (Ct) values from 36.72 to 17.84, respectively **(black)**.
**Lower Graph (B):** Standard curve that resulted when Ct values were plotted against the log of target input. Results were linear over five orders of magnitude with a correlation coefficient of 0.996. Copy numbers for unknown patient clinical samples were determined by interpolation of the Ct value obtained onto the control standard regression curve (red). The Ct results produced for patient samples ranged from 40 to 23.01, and calculated to 0 to 8.8 X $10^4$ viral copies per sample tested

**Western blotting analysis and HTLV-1/2 p19 qualitative culture results**

| Freq(%) | HTLV-1/2 viral copies per 1 x $10^6$ PBMCs (N=61) | | |
|---|---|---|---|
| **Western Blot** | **≤ 20,000** | **>20,000** | **p-value** |
| HTLV 1 | 3(6.98) | 11(61.11) | <.001 |
| HTLV 2 | 30(69.77) | 6(16.67) | |
| HTLV-1 and -2 | 1(2.33) | 0(0) | |
| Negative | 4(9.30) | 0(0) | |
| Indeterminate | 5(11.63) | 1(5.56) | |

**T cell subsets for HIV/HTLV-1 Co-infected (N=14)**

| Freq(%) | HTLV-1/2 viral copies per 1 x $10^6$ PBMCs | | |
|---|---|---|---|
| | **≤ 20,000** | **>20,000** | **p-value** |
| **$CD4/mm^3$** | | | |
| <200 | 2(66.77) | 2(18.18) | .21 |
| 201-499 | 1(33.33) | 3(27.27) | |
| 500 | 0(0) | 6(54.55) | .13(trend) |
| **$CD8/mm^3$** | | | |
| <200 | 1(33.33) | 1(9.09) | .55 |
| 201-499 | 0(0) | 1(9.09) | |
| 500 | 2(66.77) | 9(81.82) | .85(trend) |

**T cell subsets for HIV/HTLV-2 Co-infected (N=36)**

| Freq(%) | HTLV-1/2 viral copies per 1 x $10^6$ PBMCs | | |
|---|---|---|---|
| | **≤ 20,000** | **>20,000** | **p-value** |
| **$CD4/mm^3$** | | | |
| <200 | 9(30.00) | 5(83.33) | .07 |
| 201-499 | 11(36.67) | 1(16.67) | |
| 500 | 10(33.33) | 0(0) | .025(trend) |
| **$CD8/mm^3$** | | | |
| <200 | 1(3.33) | 3(50.00) | .002 |
| 201-499 | 5(16.67) | 2(33.33) | |
| 500 | 24(80.00) | 1(16.67) | .002(trend) |

**HTLV-1/2 p19 qualitative culture results**

| Freq(%) | Positive | Negative | p-value |
|---|---|---|---|
| **$CD4/mm^3$ N=79** | | | |
| <200 | 7(29.17) | 26(47.27) | .09 |
| 201-499 | 6(25.00) | 17(30.91) | |
| 500 | 11(45.83) | 12(21.82) | .039(trend) |
| **$CD8/mm^3$ N=79** | | | |
| <200 | 2(8.33) | 12(21.82) | .060 |
| 201-499 | 1(4.17) | 10(18.18) | |
| 500 | 21(87.50) | 33(60.00) | .040(trend) |
| **Western Blot, N=77** | | | |
| HTLV 1 | 15(65.22) | 3(5.56) | <.0001 |
| HTLV 2 | 7(30.43) | 39(72.22) | |
| HTLV 1/2 | 0(0) | 1(1.85) | |
| Negative | 0(0) | 4(7.41) | |
| Indeterminate | 1(4.35) | 7(12.96) | |
| **HTLV-1/2** viral copies per 1 x $10^6$ PBMCs**, N=59** | | | |
| **≤ 20,000** | 6(35.29) | 35(83.33) | <.001 |
| **>20,000** | 11(64.71) | 7(16.67) | |