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Development of a nested Real Time PCR/ High Resolution Melting assay for human T-cell lymphotropic viruses types 1 and 2 (HTLV-1 and 2) identification

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Running title: Rapid screening of HTLV-1 and 2

Significance and Impact of Study section

HTLV-1 and HTLV-2 are present in different high-risk populations; pathogenic consequences and clinical manifestations of these two viruses differ significantly, demanding proper diagnosis. A Nested Real-time PCR technique for rapid identification of HTLV-1 and 2 followed by High Resolution Melting was designed with a sensitivity assay of at least 1 viral copy of HTLV-1 or 1.5 viral copy of HTLV-2.

ABSTRACT

HTLV-1 and HTLV-2 are present in different high-risk populations, such as sexual workers and injecting drug users (IDUs). HTLV-1 is endemic in areas of Middle East, Southern Japan and Latin America, whereas HTLV-2 infection is endemic among some Native Americans and some Central African tribes. The pathogenic consequences and clinical manifestations of these two viruses differ significantly, demanding an adequate identification so proper diagnosis of HTLV-1 and 2 infection is crucial. To get a final diagnosis of HTLV-1 or 2 infection, it is recommended that positive serologic samples should be confirmed by PCR assays or Western blot (WB) analysis. Thus, the aim of this study was to develop and implement a simple reaction for rapid identification of HTLV-1 and 2. Nested Real-time PCR technique followed by High Resolution Melting was performed based on *tax/rex* sequences of HTLV-1 (M2) and HTLV-2 (MoT) cell lines perfectly discriminating between HTLV-1 from HTLV-2, by distinct melting curve profiles. The sensitivity assay of this method revealed that at least 1 viral copy of HTLV-1 or 1.5 viral copy of HTLV-2 could be amplified. Later, this method was validated using 200 blood samples from corpses. In agreement with previous epidemiological, the HTLV-1 and 2 prevalence was 1.5% (CI 95%: 0.31-4.3) and 0.5% (CI 95%: 0.013 – 2.75), respectively. The strategy proposed herein has some advantages over other PCR-based tests because it not only reduces considerably time and the costs of the total diagnosis, but also allows detection and discrimination of HTLV-1 and 2 in the same reaction.

Keywords

Retrovirus, HTLV-1, HTLV-2, nested PCR, Real Time PCR, high resolution melting, screening, viral diagnosis

INTRODUCTION

Human T-cell lymphotropic viruses type 1 (HTLV-1) and type 2 (HTLV-2) constitute a highly related complex of retroviruses that promote excessive T cell activation, and induces cellular transformation and immortalization in cells cultures and persist in infected individuals (Feuer and Green, 2005). HTLV-1 and 2 are present in different high-risk populations, such as sexual workers or injecting drugs users (IDUs), and are globally spread, being endemic in diverse places worldwide. While HTLV-1 endemism is detected in Southern Japan, the Caribbean islands, tropical Africa, Latin America, some restricted areas of Middle East and Melanesia; HTLV-2 infection is endemic among some Native American and Central African tribal groups (Proietti et al., 2005). In Argentina, HTLV-1 is endemic among Natives American descendants inhabiting highest altitude areas at northwestern regions (e.g., Puna Jujeña) (Dipierri et al., 1999), whereas HTLV-2 is endemic among aboriginal groups in northwestern Argentina (Bouzas et al., 1994).

Clinical manifestations of these two viruses differ significantly. HTLV-1 preferentially targets and transforms CD4+ T-cells and is the etiologic agent of adult T-cell leukemia (ATL) (Yoshida et al., 1982) and HTLV associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Osame et al., 1986). HTLV-2 is much less pathogenic but has been associated with a few cases of atypical hairy cell leukemia and neurological disease (Hjelle et al., 1993; Kalyanaraman et al., 1982).

The proper diagnosis of HTLV-1 and 2 infections is crucial because these retroviruses can be transmitted by blood products and by organ transplantation. It has been recommended to confirm serologic results by PCR assays instead of Western blot (WB) analysis, particularly in HTLV-1 and 2 endemic areas where subjects serologically reactive but indeterminate by WB are highly prevalent and HTLV isolates could diverge from the ones employed in commercial WB-based diagnostic tests (Mangano et al., 2004; Berini et al., 2010; Morimoto et al., 2007;

Jacob et al., 2008). So far, highly sensitive nested-PCR protocols of *pol* and *tax* genes are used for detection and identification of HTLV-1 and 2 (Vandamme et al., 1997; Liu et al., 1999; Thorstensson et al., 2002; Campos et al., 2017). However, faster, and more sensitive PCR protocols are still lacking.

We present herein a novel, faster and cost-efficient method for detection and discrimination between HTLV-1 and HTLV-2 which consists of a nested PCR reaction followed by high resolution melting analysis.

Results and Discussion

In HTLV-1 and 2 endemic areas, subjects serologically reactive but indeterminate by WB are highly prevalent. It has been shown that the diagnostic test WB 2.4 (HTLVblot 2.4, Genelabs Diagnostics, Science Park, Singapore) yields large number of WB sero-indeterminate results and does not accurately detect HTLV-1 and 2 in truly infected patients (Morimoto et al., 2007; Jacob et al., 2008). The same results have been observed in Argentinean high-risk populations (Mangano et al., 2004; Berini, 2010). These specimens produced indeterminate results with two commercial WB kits, but immunofluorescence (IFA) and PCR assays confirmed HTLV-2 infection. Although IFA showed high sensitivity and specificity to confirm HTLV-1 and 2 infection, this methodology is time-consuming and technically challenging, making it less useful for routine practice. Furthermore, it has been recently described a strategy for screening and confirmation of the HTLV-1/2 diagnosis with high sensitivity, but the algorithm includes the use of at least four serologic methods and two confirmatory assays which significantly increase the cost of diagnosis (Ji et al., 2020). Moreover, some studies reported the existence of HTLV-1 and 2 infected subjects who are serologically negative (Ishak et al., 2007; Campos et al., 2017).

To establish proper epidemiological data, a highly sensitive and specific molecular assay is needed as a complementary technique in the confirmatory diagnosis of the viral infection. So far, highly sensitive nested-PCR protocols of *pol* and *tax* genes are used for detection and identification of HTLV-1 and 2 (Heneine et al., 1992; Tuke et al., 1992; Vandamme et al., 1997; Liu et al., 1999; Thorstensson et al., 2002; Campos et al., 2017; Gomes et al., 2020). Some of them proposed a generic outer PCR followed by discriminatory inner PCRs (Heneine et al., 1992; Vandamme et al., 1997), whereas other used restriction enzyme digestion of nested PCR products for further typing of HTLV-1 and 2 (Tuke et al., 1992) or ^{32}P end-labeled oligonucleotides (Heredia et al., 1996). Recently, Gomes et al. designed a loop-mediated isothermal nucleic acid amplification (LAMP), with similar to superior performance than PCR-based assays but it requires agarose gel electrophoresis (Gomes et al., 2020). A one-step, multiplex, real-Time PCR assay was previously described to discriminate HTLV-1, 2 or 3 with an excellent dynamic range of 106 to 100 copies per assay (Besson et al., 2009). However, the use of molecular beacons in a screening technique could be expensive if a large number of samples need to be routinely processed. Therefore, cheaper, faster, and more sensitive PCR protocols are still lacking.

The assay proposed herein discriminates accurately between HTLV-1 and HTLV-2, by distinct melting curve profiles with temperatures of 84.2°C±0.04 and 84.53°C±0.02 respectively using the PIS-POA (primer inner-sense; primer outer-antisense) combination (Figure 1A), or melting temperatures of 83,63± 0,02 or 84,97°C± 0,02 after using the alternative primer combination (primer outer-sense; primer inner-antisense) (Figure 1B). The dissimilar melting temperatures between HTLV-1 and 2 are explained by the presence of non-identical sites located in the amplified PCR fragment: 18 sites in the 144 bp product amplified by the combination of

primers POS and PIA, and, 6 sites in the 94 bp product amplified by the PIS-POA combination (Figure 2).

The nucleotide sequence analysis and posterior alignment with HTLV-1 and 2 GenBank reference sequences demonstrated 100% homology with them. The analytical sensitivity of the nested PCR technique described herein revealed that partial amplification of the HTLV-1 and 2 *tax/rex* region was possible starting from 1 viral copy of HTLV-1 MT2 and 1.5 viral copies of HTLV-2 MoT using the combination of primers PIS-POA. The alternative design (combination POS-PIA) showed that 2 viral copies of HTLV-1 MT2 and 3 viral copies of HTLV-2 MoT could be detected and discriminated. The PCR conditions using these sets of primers allow the amplification of both viruses although non-identical sites are present in the annealing region of HTLV-2 genome (2 sites in the 144 bp product and 4 sites in the 94 bp amplicon; Figure 2). This last, could be the explanation of the slightly less HTLV-2 sensibility respect HTLV-1.

The screening in the 200 cadaveric samples using the method described in this study detected the presence of HTLV-1 and 2 genomes in 4 samples. Three of them were ascribed to HTLV-1 and one to HTLV-2. The HTLV-1 prevalence was 1.5% (CI 95%: 0.31-4.3) and 0.5% to HTLV-2 (CI 95%: 0.013 – 2.75). HIV was detected in 73 out of 175 tested samples (36.5%), whereas the prevalence of HBV and HCV serological markers was 4.5% (9 out of 27 samples) and 9.5% (19 out of 37 samples), respectively. From the individuals carrying HTLV-1 and 2, three of them were co-infected with HIV, HBV and/or HCV and the fourth, was a cocaine addict that belongs to at risk population, as well. Viral co-infections are frequent because of their shared pattern of transmission (sexual or parenteral) (Berini et al., 2010; Pando et al., 2006; Dos Ramos Farias, 2011). Data reported by other groups revealed that the HTLV-1 prevalence is 2,0% and 1,0% for HTLV-2 in the HIV, HBV and/or HCV infected population (Berini et al., 2012). These prevalence rates are in agreement with the results obtained in this

study showing that the sensitivity of this method is comparable to other diagnostic techniques used in previous studies (Berini et al., 2012). The typing results obtained from the PCR followed by high resolution melting (PCR-HRM) completely matched those of the phylogenetic analysis in all samples (Figures 3 and 4). The three HTLV-1 positive samples were classified to the Cosmopolitan subtype transcontinental subgroup (Aa) of HTLV-1 (Figure 3), whereas the HTLV-2 positive sample was ascribed to the viral subtype b (Figure 4). These HTLV-1 and 2 subtypes are the most prevalent in Argentina, according to previous studies (Eirin et al., 2010; Dos Ramos Farias, 2011; Berini et al., 2012). One limitation of the present study is that the presence of HTLV-1 and 2 could not be corroborated in the 200 analysed samples or in positive clinical samples by "gold standard" screening techniques, such as serologic and WB or PCR assays, due to their unavailability at the time of the study. It would be of great interest to compare the diagnostic metrics of the "gold standard" techniques and the method presented herein.

We believe that the strategy proposed herein has advantages over other PCR-based tests, in terms of safety, cost and simplicity. The proposed method not only detects and discriminates HTLV-1 and HTLV-2 viruses with high sensitivity, but also it eliminates the need of agarose gel electrophoresis and ethidium bromide staining, and thus it is a fast, cost effective, and rapid assay.

Materials and Methods

Primers targeting the HTLV-1 and HTLV-2 *tax/rex* region (Matsumoto et al., 1990; Tuke et al. 1992; Heneine et al., 1992) were designed based on the nucleotide sequences of HTLV-1 and 2 reference sequences deposited in GenBank: J02029.1 (HTLV-1) and M10060.1 (HTLV- 2) (Table 1). Self and heterodimers, potential hairpins and secondary structures were checked using OligoAnalyzer software (http://www.idtdna.com/annalyzer/applications/oligoanalyzer). Prediction of melting temperature was done by the online software uMelt (Dwight et al., 2011). We used DNA extracted from cell lines infected with HTLV-1 (MT2) and HTLV-2 (Mo [Mo T], ATCC® CRL-8066™) gently supported by Dr. Andrea Mangano from the Cellular Biology and Retrovirus Laboratory; Molecular Epidemiology and Virology Unit from the Pediatric Hospital "Prof. Dr. Juan P. Garrahan" as positive controls to optimize the PCR reaction. DNA-free samples and blanks were also included as controls.

The first amplification round was performed using primers POS and POA (Table 1). PCR reaction mixture was: 5 ng total genomic DNA (quantified as described by Ginart et al., 2015), 20pM of each primer, 0.16mM dNTPs, 25mM syto9, 1U of GoTaq® polymerase (Promega, Madison, WI, USA) and 5X Go Taq reaction buffer in a total volume of 25µl. The cycling conditions were: 2 min denaturation at 95°C; 30 cycles consisting of 30 sec at 94°C, 40 sec at 50°C and 20 sec at 72°C; followed by 2 min extension at 72°C in a Real Time PCR equipment RotorGene 6000 (Corbet Inc., Sidney, Australia). The second amplification round was performed using the inner/sense primer (PIS) and the same antisense primer of the first amplification round (POA) (Table 1; Tuke et al., 1992), with a final PCR product of 94 base pairs (bp). Alternatively, the second amplification round was performed using the same sense primer from the first amplification round (POS) and the inner antisense primer (PIA) described by Tuke et al., 1992 (Table 1) leading to a final PCR product of 144 bp. The PCR reaction mixture and cycling conditions were the same as those described above, except from the fact

that 1µl of the product of the first round was added to the mixture and only 12 cycles were carried out in the cycling conditions. Finally, high resolution melting was completed between 81°C and 87°C to discriminate between HTLV-1 and HTLV-2. Appropriated precautions and procedures were strictly followed to avoid cross-contamination in every step of DNA amplification and detection (Kwok and Higuchi, 1989).

Recombinant HTLV-1 and HTLV-2 plasmids containing the PCR product of the first PCR round (159 bp) were prepared in order to determine the sensitivity of both PCR protocols. The purified amplicons were ligated to the pDrive cloning vector (QIAGEN, GmbH, Hilden, Germany) and used to transform Escherichia coli DH5α competent cells (Subcloning Efficiency™ DH5α Competent Cells (ThermoFisher Scientific, Waltham, MA, USA). Recombinant plasmids were sequenced using Big-Dye Termination chemistry system (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA, USA) and compared with HTLV-1 and HTLV-2 reference sequences retrieved from the GenBank database. The DNA concentrations of the pure preparations of these recombinant plasmids were determined by fluorometry (Quantus™ Fluorometer, Promega, Madison, WI, USA), as follows: 0.84ng/µl, which corresponds to 1.97E+08 copies/ml of the HTLV-1 recombinant plasmid and 0.63ng/µl, which corresponds to 1.48E+08 copies/ml of the HTLV-2 recombinant plasmid (https://www.idtdna.com/pages/education/decoded/article/calculations-converting-from-nanograms-to-copy-number). Serial dilutions of these plasmid solutions were amplified using both PCR protocols, and the analytical sensitivity of these techniques was considered the last dilution exhibiting a melting peak distinguishable. To confirm the specificity of the obtained products, all PCR-amplified fragments were bi-directionally sequenced using BigDye® Terminator v3.1 Cycle Sequencing and sequencing amplicons were separated by using an ABI-3500 Sequencer equipment (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA, USA). The sequences were analysed with the software Sequencher® v. 5.3 (Gene Codes

Corporation, Ann Arbor, MI, USA) and compared with the HTLV-1 and HTLV-2 reference sequences from GenBank.

To validate the reaction, we randomly selected stored blood samples from 200 corpses. The presence of HIV, HBV and HCV serological markers was requested by the forensic team in 175, 27 and 37 out of the 200 autopsies. Sampling was approved by the Department of Teaching, Research and Bioethics of the Forensic Medical Examiner's Office of the National Justice of Argentina. All samples were treated anonymously during the study. DNA was extracted from 500µl of cadaveric blood by CTAB method (Corach et al., 1995). Total genomic DNA quantification was performed as described by Ginart et al., 2015. The presence of the HTLV-1 or HTLV-2 genome was determined in all samples by the Real Time PCR protocol followed by High resolution Melting described above.

The HTLV-1 and HTLV-2 subtypes were confirmed in all positive samples by 3' long tandem repeat (LTR) amplification and phylogenetic analysis. Briefly, 3'LTR was amplified by previously described nested PCR protocols (Switzer et al., 1995; Meertens et al., 2001) and bi-directionally sequenced by Big-Dye Termination chemistry (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA, USA). Multiple sequence alignments of the HTLV-1 and HTLV-2 3'LTR sequences obtained in this study and reference sequences ascribed to all known HTLV-1 and 2 subtypes deposited at GenBank, were performed using BioEdit version 7.2.5 (Hall, 1999). Phylogenetic trees were constructed using the Neighbor-joining method with software MEGA 7 (Kumar et al., 2016). Group support was evaluated by a standard bootstrap procedure (1000 pseudo-replicates). GenBank/EMBL/DDBJ accession numbers of the HTLV-1/2 3'LTR sequences reported in this study are:OM974700-OM974703.

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CONFLICT OF INTEREST

No conflict of interest declared.

REFERENCES

Besson G, Kazanji M. One-step, multiplex, real-time PCR assay with molecular beacon probes for simultaneous detection, differentiation, and quantification of human T-cell leukemia virus types 1, 2, and 3. J Clin Microbiol. 2009 Apr;47(4):1129-35. doi: 10.1128/JCM.02006-08. Epub 2009 Feb 11. PMID: 19213697; PMCID: PMC2668303.

Berini C. A, Maria E. Eirin, Cecilia M. Delfino, Mercedes Weissenbacher, and Mirna M. Biglione. Predominance of Human Lymphotropic T Cell Virus Type 2 Subtype b in Urban Populations of Argentina. AIDS RESEARCH AND HUMAN RETROVIRUSES Volume 28, Number 00, 2012. Mary Ann Liebert, Inc. DOI: 10.1089/aid.2011.0311.

Berini CA, Gendler SA, Pascuccio S, Eirin ME, McFarland W, Page K, Carnevali L, Murphy E, Biglione MM. Decreasing trends in HTLV-1 and 2 but stable HIV-1 infection among replacement donors in Argentina. J Med Virol. (2010); 82(5):873-7.

Bouzas MB, Zapiola I, Quiruelas S, Gorvein D, Panzita A, Rey J, Carnese FP, Corral R, Perez C, Zala. HTLV type I and HTLV type II infection 150 among Indians and natives from Argentina. AIDS Res Hum Retroviruses. (1994); 10(11):1567-71.

Campos, K.R.; Gonçalves, M.G.; Costa, N.A.; Caterino-de-Araujo, A. Comparative performances of serologic and molecular assays for detecting human T lymphotropic virus type 1 and type 2 (HTLV-1 and HTLV-2) in patients infected with human immunodeficiency virus type 1 (HIV-1). Braz. J. Infect. Dis. 2017, 21, 297–305.

Corach D., Penacino G., Sala A. Cadaveric DNA Extraction Protocol Based on Cetyl Trimethyl Amonium Bromide (CTAB). In: Mangin P., Ludes B. (eds) Acta Medicinæ Legalis Vol. XLIV 1995. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-79523-7_8

Dipierri JE, Tajima K, Cartier Robirosa L, Sonoda S: A seroepidemiological survey of HTLV-I/II carriers in the Puna Jujena.Medicina (B Aires) 1999;59:717-720.

Dos Ramos Farias 2011 https://bibliotecadigital.exactas.uba.ar/download/tesis/tesis_n5007_DosRamosFarias.pdf

Dwight Z, Palais R, Wittwer CT. uMELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application. Bioinformatics. 2011 Apr 1;27(7):1019-20. doi: 10.1093/bioinformatics/btr065. Epub 2011 Feb 7. PMID: 21300699.

Eirin ME, Berini CA, Jones LR, Dilernia DA, Puca AA, Biglione MM. Stable human T-cell lymphotropic virus type 1 (HTLV- 1) subtype a/subgroup a endemicity in Amerindians from Northwest Argentina: a health problem to be resolved. J Med Virol. 2010 Dec;82(12):2116-22.

Feuer G, Green PL. Comparative biology of human T-cell lymphotropic virus type 1 (HTLV-1) and HTLV-2. Oncogene. 2005 Sep 5;24(39):5996-6004. doi: 10.1038/sj.onc.1208971. PMID: 16155606; PMCID: PMC2659530.Berini CA, Pando MA, Bautista CT, Eirin ME, Martinez-Peralta L, Weissenbacher M, Avila MM, Biglione MM. HTLV-1 and 2 among high-risk 144 groups in Argentina: molecular diagnosis and prevalence of different sexual transmitted infections. J Med Virol. (2007); 79(12):1914-20

Ginart S., E. Alechine, M. Caputo, H. Cano, D. Corach, A. Sala. Development and validation of a human DNA quantification and sex determination approach based on real time PCR followed by high resolution melting analysis. Forensic Sci. Int. Gene. Suppl. (2015), http://dx.doi.org/10.1016/j. fsigss.2015.09.107.

Gomes, Y., Caterino-de-Araujo, A., Campos, K., Gonçalves, M. G., Leite, A. C., Lima, M. A., Araújo, A., Silva, M. T., Espíndola, O. (2020). Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid and Accurate Confirmatory Diagnosis of HTLV-1 and 2 Infection. Viruses, 12(9), 981. https://doi.org/10.3390/v12090981

Hall WW, Takahashi H, Liu C, Kaplan MH, Scheewind O, Ijichi S, Nagashima K, Gallo RC. Multiple isolates and characteristics of human T-cell leukemia virus type II. J Virol. 1992;66:2456-63.

Heredia A, Soriano V, Weiss SH, Bravo R, Vallejo A, Denny TN, Epstein JS, Hewlett IK. Development of a multiplex PCR assay for the simultaneous detection and discrimination of HIV-1, HIV-2, HTLV-I and HTLV-II. Clin Diagn Virol. 1996 Nov;7(2):85-92. doi: 10.1016/s0928-0197(96)00255-3. PMID: 9137864.

Heneine W, Khabbaz RF, Lal RB, Kaplan JE. 1992. Sensitive and specific polymerase chain reaction assays for diagnosis of human Tcel lymphotropic virus TypeI (HTLV-I) and HTLV-II infections in HTLV-I/II-seropositive individuals. J Clin Microbiol 30:1605–1607.

Hjelle B, Wilson C, Cyrus S, Bradshaw P, Lo J, Schammel C, Wiltbank T,Alexander S: Human T-cell leukemia virus type II infection frequently goes undetected in contemporary US blood donors. Blood 1993;81:1641-1644.

Ishak, R., Vallinoto, A. C., Azevedo, V. N., Vicente, A. C., Hall, W. W., Ishak, M. O. (2007). Molecular evidence for infection by HTLV-2 among individuals with negative serological screening tests for HTLV antibodies. Epidemiology and infection, 135(4), 604–609. https://doi.org/10.1017/S0950268806006984

Jacob F, Santos-Fortuna E, Azevedo RS, Caterino-de-Araujo A. Serological patterns and temporal trends of HTLV-1 and 2 infection in high-risk populations attending Public Health Units in São Paulo, Brazil. J Clin Virol. 2008 Jun;42(2):149-55. doi: 10.1016/j.jcv.2008.01.017. Epub 2008 Mar 17. PMID: 18346935.

Ji H, Chang L, Yan Y, Jiang X, Sun H, Guo F, Wang L. A Strategy for Screening and Confirmation of HTLV-1/2 Infections in Low-Endemic Areas. Front Microbiol. 2020 Jun 3;11:1151. doi: 10.3389/fmicb.2020.01151. PMID: 32582093; PMCID: PMC7283491.

Kalyanaraman VS, Sarndadharan MG, Robert-Guroff M, Miyoshi I, Blayney D, Golde D, Gallo RC. A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. Science. (1982); 218: 571-3.

Kwok S, Higuchi R. Avoiding false positives with PCR. Nature. 1989 May 18;339(6221):237-8. doi: 10.1038/339237a0. Erratum in: Nature 1989 Jun 8;339(6224):490. PMID: 2716852.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33:1870-4.

Liu H, Shah M, Stramer SL, Chen W, Weibler BJ, Murphy EL 1999. Sensitivity and specificity of human T-lymphotropic virus (HTLV) types I and II polymerase chain reaction and several serologic assays in screening a population with a high prevalence of HTLV-II. *Transfusion 39*: 1185-1193.

Mangano AM, Remesar M, del Pozo A, Sen L: Human T lymphotropic virus types I and II proviral sequences in Argentinian blood donors with indeterminate Western blot patterns. J Med Virol 2004;74:323-327.

Matsumoto, C., Mitsunaga, S., Oguchi, T., et al. (1990) Detection of human T-cell leukemia virus type I (HTLV-I) provirus in an infected cell line and in peripheral mononuclear cells of blood donors by the nested double polymerase chain reaction method: comparison with HTLV-I antibody tests. J. Virol. 64, 5290-5294.

Meertens L, Rigoulet J, Mauclère P, Van Beveren M, Chen GM, Diop O, Dubreuil G, Georges-Goubot MC, Berthier JL, Lewis J, Gessain A. Molecular and phylogenetic analyses of 16 novel simian T cell leukemia virus type 1 from Africa: close relationship of STLV-1 from Allenopithecus nigroviridis to HTLV-1 subtype B strains. Virology. 2001;287(2):275-85.

Morimoto HK, Morimoto AA, Reiche EMV, Ueda LT, Matsuo T, Reiche FV, et al. Difficulties in the diagnosis of HTLV-2 infection in HIV/AIDS patients from Brazil. Comparative performances of serologic and molecular assays, and detection of HTLV-2b subtype. Rev Inst Med Trop Sao Paulo 2007;49:225–30.

Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M. HTLV-I associated myelopathy, a new clinical entity. Lancet (1986); 1(8488):1031-2.

Pando MA, Berini C, Bibini M, Fernandez M, Reinaga E, Maulen S, Marone R, Biglione M, Montano SM, Bautista CT, Weissenbacher M, Sanchez JL, Avila MM: Prevalence of HIV and other sexually transmitted infections among female commercial sex workers in Argentina. Am J Trop Med Hyg 2006;74:233-238.

Proietti FA, Carneiro-Proietti AB, Catalan-Soares BC, Murphy EL. Global epidemiology of HTLV-I infection and associated diseases. Oncogene.(2005); 24(39):6058-68.

Switzer WM, Pieniazek D, Swanson P, Samdal HH, Soriano V, Khabbaz RF, Kaplan JE, Lal RB, Heneine W. Phylogenetic relationship and geographic distribution of multiple human T-cell lymphotropic virus type II subtypes. J Virol. 1995;69(2):621-32.

Thorstensson R, Albert J, Andersson S: Strategies for diagnosis of HTLV-I and -II. Transfusion 2002;42:780-791.

Tuke PW, Luton P, Garson JA. Differential diagnosis of HTLV-I and HTLV-II infections by restriction enzyme analysis of „nested" PCR products. J Virol Methods (1992); 40:163–173.

Vandamme AM, Van Laethem K, Liu HF, Van Brussel M, Delaporte E, de Castro Costa CM, Fleischer C, Taylor G, Bertazzoni U, Desmyter J, Goubau P: Use of a generic polymerase chain reaction assay detecting human T-lymphotropic virus (HTLV) types I, II and divergent simian

strains in the evaluation of individuals with indeterminate HTLV serology.J Med Virol 1997;52:1-7.

Yoshida M, Miyoshi I, Hinuma Y: Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. Proc Natl Acad Sci U S A 1982;79:2031-2035.

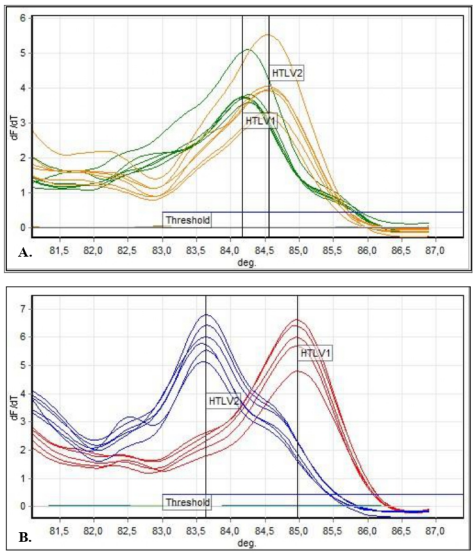
AUTHOR CONTRIBUTION

All authors listed contributed to this work and are entitled to authorship. Dr. Caputo directed and coordinated this study. Dr. Corach, Dr. Trinks and Dr. Caputo provided conceptual and technical guidance for all the aspects of the project. Azcurra, Bsc. collected the biological samples. Azcurra, Bsc. and Dr. Caputo designed the SBE primers, conducted the performance testing of the technique and analysed the data. Dr. Trinks obtained the recombinant plasmids for the sensitivity assay. Dr. Caputo and Dr. Trinks drafted the manuscript. Dr. Corach supervise the project. Dr.Corach and Dr. Trinks provided financial resources. All authors were involved in manuscript editing and approved the version submitted for publication.

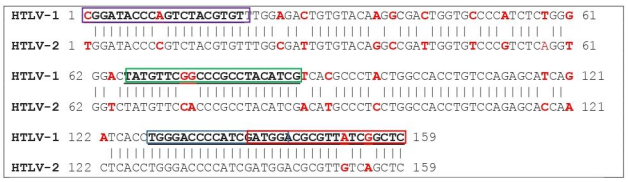
Table 1. Primers used in this study

Primer name	Sequence (5'- 3')	Amplicon (bp)
Outer/sense (POF)*	CGGATACCCAGTCTACGTGT	
Outer/antisense first round (POR)*	GATGGACGCGTTATCGGCTC	POF-POR 159 bp
Inner/sense second round (PIF)	TATGTTCGGCCCGCCTACATCG	PIF-POR 94 bp
Inner/antisense second round (PIR)*	CCATCGATGGGGTCCCA	POF-PIR 144 bp

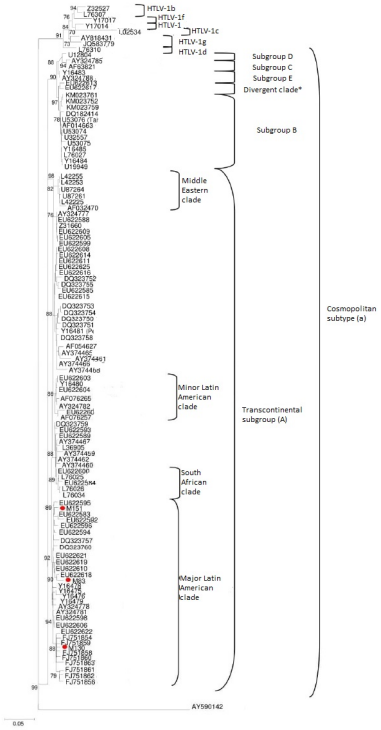
* described by Tuke et al. 1992



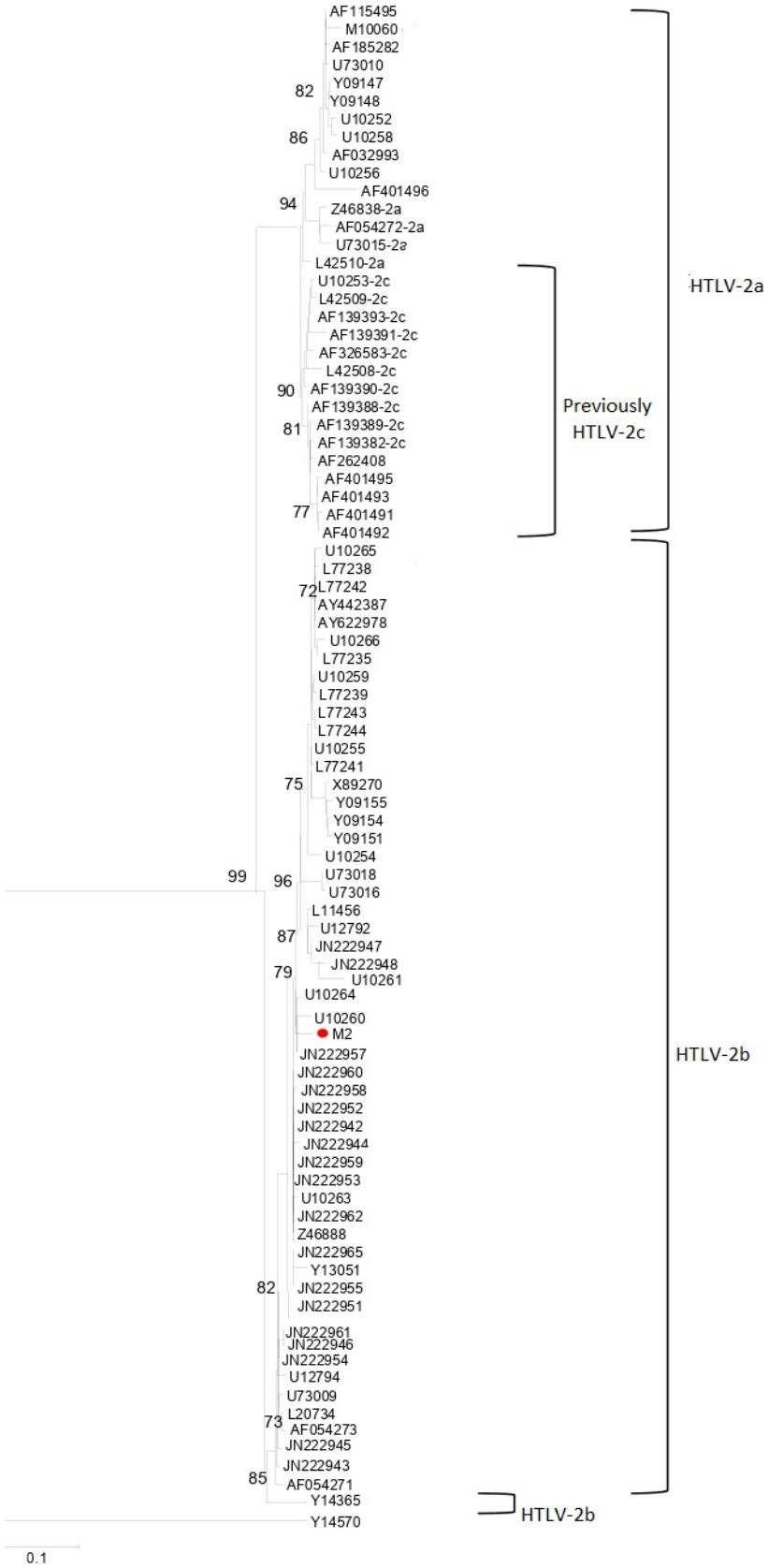
LAM_13752_FIGURE 1.tif



LAM_13752_FIGURE 2.tif



LAM_13752_FIGURE 3.tif



LAM_13752_FIGURE 4.tif