LINE IMMUNOASSAY FOR CONFIRMATION AND DISCRIMINATION OF HUMAN T-CELL LYMPHOTROPIC VIRUS INFECTIONS IN INCONCLUSIVE WESTERN BLOT SERUM SAMPLES FROM BRAZIL

LINE IMMUNOASSAY TO CONFIRM HTLV-1/2 INFECTIONS

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**ABSTRACT**

Difficulties to confirm and discriminate human T-cell lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) infections by serological Western Blotting (WB) assay (HTLV Blot 2.4, MP Biomedicals) has been reported in Brazil, mainly in HIV/AIDS patients, with a large number of WB-indeterminate and WB-positive but HTLV untypeable results. Nonetheless, the line immunoassay (LIA) (INNO-LIA HTLV-I/II, Fujirebio) was pointed to enhance specificity and sensitivity for confirming HTLV-1/2 infections. To add information concerning the improved ability of LIA in relation to WB when applied in samples of individuals from different risk-groups from Brazil, we performed the present study. Three groups were analyzed: group 1 [G1], 62 samples from HIV/AIDS patients from São Paulo-SP (48 WB-indeterminate + 14 HTLV); group 2 [G2], 24 samples from patients with hepatitis B or hepatitis C from São Paulo (21 WB-indeterminate + 3 HTLV; 17 HIV-seropositive), and group 3 [G3], 25 samples from HTLV out-patients clinic from Salvador-Bahia (16 WB-indeterminate + 9 HTLV; all HIV-seronegative). Overall, the LIA confirmed HTLV-1/2 infection (HTLV-1, HTLV-2 or HTLV) in 66.1% [G1], 83.3% [G2], and 76.0% [G3] of samples. Interestingly, the majority of WB-indeterminate results were confirmed by LIA as HTLV-2 in G1 and G2, but not in G3, in which the samples were defined as HTLV-1 or HTLV positives. These results agree with the virus types that circulate in such patients of different regions in Brazil, and emphasize the LIA as the best serological test for confirming HTLV-1 and HTLV-2 infections, independently of being applied in HTLV-monoinfected or HTLV-coinfected individuals.

**KEYWORDS** diagnostic, HTLV, confirmatory serologic tests, LIA, WB
INTRODUCTION

Despite regional variations, Brazil is one of the largest endemic areas for human T-cell lymphotropic virus type 1 (HTLV-1) in the world (1). Higher rates of HTLV-1 infection have been detected in general populations in the north and northeast regions, while human T-cell lymphotropic virus type 2 (HTLV-2) is endemic among indigenous communities in the north, as well as in injectable drug users in urban areas, mostly in southeastern in Brazil (2).

Difficulties in confirming the diagnosis of HTLV-1 and HTLV-2 by serological assay (Western Blot – WB, HTLV Blot 2.3 and 2.4) have been reported in Brazil, due to a large number of WB-indeterminate and HTLV-positive but untypeable results, mainly in patients truly infected with HTLV-2 and/or HIV (3-5). Consequently, molecular assays have been employed to detect proviral DNA segments of HTLV-1 and HTLV-2 (pol, LTR, env and tax) in peripheral blood mononuclear cells using nested-PCR, PCR-hybridization, and/or PCR-RFLP assays (6-11). Nonetheless, there was no consensus regarding the criteria to consider a blood sample real infected by HTLV-1/2 using these techniques; if positive for one or at least two DNA proviral segments of the HTLV-1/2 (6, 10, 11). Subsequently, another molecular assay, real-time PCR or quantitative PCR (qPCR), was proposed as a confirmatory HTLV-1/2 molecular assay; however, low sensitivity was found when applied in HIV blood samples and in those from Brazilian patients infected with HTLV-2, which could be due to low HTLV-2 proviral load (12-15).

In 1998, a new HTLV-1/2 serologic confirmatory assay employed a line immunoassay (LIA) with nylon membrane sensitized with the most relevant antigens, recombinant proteins or synthetic peptides of HTLV-1 and HTLV-2 (16). This new
immunoassay (INNO-LIA HTLV) demonstrated better results than WB, with improved
sensitivity in the confirmation of HTLV-1 and HTLV-2 infections, thereby reducing
numbers of WB-indeterminate results (16). However, the high cost of INNO-LIA
prevented its routine adoption in Brazil. Consequently, few studies have compared the
performance of LIA and WB in Brazil, one employing blood bank samples (17) and two
in serum samples from HIV/AIDS patients (14, 15). These studies indicated that LIA was
the best assay to confirm or rule out HTLV-1/2 infection.

In Brazil, molecular biology laboratories are not widely available due to
differences in socioeconomic conditions. Thus the use of a confirmatory serologic assay
of high performance is essential and necessary for HTLV-1/2 diagnosis. Accordingly, the
present study aimed to evaluate the use of INNO-LIA for clarifying WB-indeterminate
and WB-HTLV untypeable serum samples.

MATERIALS AND METHODS

Samples

The serum samples employed in the present study were obtained from the
biorepositories of the HTLV Research Laboratory (LPHTLV), Department of
Immunology, Adolfo Lutz Institute (IAL), located in São Paulo, Brazil, and the Integrated
and Multidisciplinary HTLV Center (CHTLV), located at the Bahiana School of Medicine
and Public Health (EBMSP) in Salvador-Bahia, Brazil. Briefly, the samples from São
Paulo were collected between 2012 and 2016 in the course of previous studies
designed to detect the prevalence of HTLV-1/2 in HIV-infected individuals, as well as in
patients with hepatitis B or hepatitis C in the state of São Paulo-Brazil (results published
elsewhere) (14, 15, 18-20). The samples from Salvador-Bahia were obtained from routine diagnostic procedures at an out-patient clinic in Salvador (CHTLV) from 2015 to 2017; these samples were additionally used to assess the performance of four commercially available HTLV serological screening tests in Brazil (21). Table 1 lists the characteristics of the samples collected from these two biorepositories (number of samples per sex, age), as well as HTLV-1/2 screening results and confirmatory Western Blot (WB) assay results (Blot HTLV 2.4, MP Biomedicals). Only samples that were WB-inconclusive (WB-indeterminate or HTLV untypeable) were selected for analysis in the present investigation.

Of the 145 samples with WB-inconclusive results, a volume sufficient for immunoassaying was present in 111; these were used to determine the ability of the line immunoassay (LIA) (INNO-LIA HTLV, Fujirebio) to confirm and discriminate HTLV-1/2 specific antibodies. The evaluated samples originated from three groups of patients: Group 1 [G1], 62 samples (48 WB-indeterminate and 14 HTLV untypeable) obtained in the course of routine HTLV diagnosis at the Adolfo Lutz Institute or from the São Paulo Sexually Transmitted Disease/AIDS Reference and Training Center (CRT DST/AIDS-São Paulo), all of which were obtained from individuals who were known to be HIV-seropositive; Group 2 [G2], 24 samples (21 WB-indeterminate and 3 HTLV untypeable) were collected from patients who were initially seen at Gastroenterology Centers in São Paulo; 14 had hepatitis B (HBV), 10 had hepatitis C (HCV), and 17 of these were HIV-seropositive (10 [HBV] and 7 [HCV]); G3, 25 HTLV-monoinfected samples from patients seen at the CHTLV outpatient clinic in Salvador, Bahia (16 WB-indeterminate and 9 HTLV untypeable).
Screening assays

The samples from São Paulo were screened for the presence of HTLV-1/2 antibodies by two enzyme immunoassays (EIA): Murex HTLV-I+II (DiaSorin S.p.A., Dartford, UK) and Gold ELISA HTLV-1/2 (REM Indústria e Comércio LTDA, São Paulo, Brazil). The samples from Salvador were initially screened by the Ortho® HTLV-1/HTLV-2 Ab-Capture ELISA Test System (Ortho-Clinical Diagnostic, Raritan, USA), as well as by four other HTLV-1/2 screening tests commercially available in Brazil: three EIA [Murex HTLV-I+II (DiaSorin S.p.A., Dartford, UK), anti-HTLV-1/2 SYM Solution (Symbiosis Diagnóstica LTDA, Leme, Brazil) and Gold ELISA HTLV-1/2 (REM Indústria e Comércio LTDA, São Paulo, Brazil)], as well as one chemiluminescence assay (CLIA) kit (Architect rHTLV-1/2, Abbott Diagnostics Division, Wiesbaden, Germany). All assays were performed according to the manufacturer’s instructions, which were also used to interpret results. Cutoff values and gray zone were calculated for each assay, and samples considered reactive or inconclusive in screening were submitted to a confirmatory assay.

Confirmatory assays

The Western blot (WB) assay (HTLV Blot 2.4, MP Biomedicals Asia Pacific Pte. LTD, Singapore) was used as confirmation of HTLV-1 and HTLV-2 infection in all the previous studies that generated the samples analyzed herein, and these results were interpreted according to the stringent criteria provided by the manufacturer. Briefly, HTLV-1-positive serum samples were defined as the presence of gag (p19 with or without p24) and two env (GD21 and rgp46-I) bands. HTLV-2-positive samples were defined as demonstrating reactivity to gag (p24 with or without p19) and two env (GD21
and rgp46-II) bands. Samples that showed the presence of antibodies to both gag (p19 and p24) and env (GD21) were defined as HTLV positive, but were considered untypeable. Any other pattern of bands was deemed to be indeterminate.

The present study employed the line immunoassay (LIA) (INNO-LIA HTLV I/II, Fujirebio, Europe N.V, Belgium) in an attempt to confirm and/or discriminate samples with inconclusive results under WB (i.e., WB-indeterminate or HTLV positive but untypeable). The strips used in LIA contain antigens for validation, confirmation and discrimination. For validation, the line marked by each sample was compared to the control line, and a score ranging from +/- to +3 was assigned. The confirmatory antigens included gag p19 I/II, gag p24 I/II, env gp46 I/II, and env gp21 I/II. No bands or the occurrence of a single band (gag p19 I/II, gag p24 I/II or env gp46 I/II) denoted a negative result. The presence of one band (env gp21 I/II) or two bands (except env gp21 I/II) indicated indeterminate results, while two bands (env gp21 I/II and gag p19 I/II, gag p24 I/II or env gp46 I/II) indicated HTLV positivity. Three discriminatory bands (gag p19-I, env gp46-I, and env gp46-II) were considered as follows: HTLV-1 positivity was indicated by reactivity to gag p19-I and/or env gp46-I, while HTLV-2 positivity was found when samples showed env gp46-II or higher intensity of the env gp46-II band than gag p19-I and env gp46-I.

**Statistical analyses**

Differences in the number of males and females in each group were evaluated statistically using the Chi-square test. GraphPad Prism software version 5.03 (San Diego, CA, USA) was used for age comparisons between groups using the Kruskal-
Wallis ANOVA test, complemented with Dunn's Multiple Comparison Test. Results with a p-value ≤0.05 were considered statistically significant.

Ethical considerations
The present research protocol was approved by the Institutional Review Board of the Adolfo Lutz Institute (IAL) in São Paulo, Brazil (protocols no. 106D/2012, 62H/2015, and 21I/2016), and by the Institutional Research Board (IRB) of the Bahiana School of Medicine and Public Health (EBMSP) in Salvador, Bahia-Brazil (protocol no. 464.286). All procedures were performed in accordance with the principles established in the Declaration of Helsinki and its subsequent revisions.

RESULTS
The characteristics of the patients (sex and age) and the distribution of WB-inconclusive results in each study group are presented in Table 2. More males were found in the HBV/HCV infected patients in G2, with significant differences in relation to the HIV/AIDS patients in G1 and the HTLV patients in G3 (p=0.0048). A comparative analysis of age among the groups showed no significant differences, although the individuals in G2 were older overall. Concerning the distribution of WB-inconclusive samples, the three groups contained more WB-determinate than HTLV untypeable samples: G1 (77.4%), G2 (87.5%) and G3 (64.0%).

LIA provided confirmation of HTLV-1/2 infection (HTLV-1, HTLV-2, or HTLV) in 66.1% [G1], 83.3% [G2] and 76.0% [G3] of the samples analyzed. Interestingly, most WB-indeterminate results in G1 and G2 were confirmed as HTLV-2 by LIA, but this was
not the case in G3. In G3 only HTLV-1 (40.0%) and HTLV (36.0%) positive samples were detected in both WB-indeterminate and HTLV untypeable samples (Fig. 1).

Table 3 shows the WB-indeterminate profiles detected in the present study, the number of samples that presented each profile, and the number and percentage of samples with confirmed HTLV-1/2 infection. These results indicate that the WB patterns showing env bands (GD21 and/or rgp46-I or –II) plus p19 or p24 were confirmed as HTLV-1/2 infection by LIA. The overall patterns from the WB inconclusive samples (n=111) and the patterns returned by LIA are presented in Table 4, revealing that the majority of indeterminate WB profiles which not confirmed as HTLV-1/2 infection by LIA presented only gag bands in G1, only GD21 in G2, and one of three bands (GD21, rgp46-II, p24) in G3. Noteworthy, among 26 HTLV positive but untypeable samples (14 in G1, three in G2 and nine in G3), after LIA analysis, 28.6% confirmed as HTLV-1, 28.6% as HTLV-2, and 42.8% remains HTLV untypeable in G1. In G2, 66.7% confirmed as HTLV-1 and 33.3% as HTLV-2, and in G3, 66.7% confirmed as HTLV-1, and 33.3% remains HTLV untypeable (Fig. 1, Table 4).

DISCUSSION

HTLV-1- and HTLV-2-seroindeterminate WB results are prevalent worldwide, with rates fluctuating according to country and study-group (endemic or non-endemic geographic areas and populations). Several attempts have been made to improve WB sensitivity and specificity, such as adding HTLV-1 and HTLV-2 recombinant envelope proteins and transmembrane protein to the HTLV-1 viral lysate. These include rgp46-I, rgp46-II and GD21, the latter which blocks the cross-reactivity of gp21 with Plasmodium falciparum infections in regions endemic for malaria (22, 23). In spite of these efforts,
WB version 2.4 continues to yield high rates of WB-indeterminate and/or untypeable HTLV results (4-8, 10, 11, 13-17, 21). The present study found similar results, and disclosed that in populations presenting a high risk of acquiring viral infections (G1 and G2), as well in the general population (G3) of Brazil, a large number of WB-inconclusive results were detected. Several hypothesis were taken into consideration for these WB-inconclusive results, such as low HTLV-1 and HTLV-2 proviral loads, mutations in the provirus (defective particles), low production of viral antigens leading to consequently low specific antibody production, seroconversion period, cross-reactivity with other antigens or viruses, coinfection with HIV and the use of antiretroviral therapy, among others (5, 12-15, 24-27).

Interestingly, the majority of WB-indeterminate results that not were confirmed by seroconversion or PCR assays were due to cross-reactivity with gag antigens. For instance, the WB-indeterminate pattern exhibiting p19, p26, p28, p32, p36 and p53, termed HTLV-1 Gag indeterminate profile (HGIP), has been detected in epidemiological studies, mostly in Cameroon and in the Caribbean, but was not associated with true HTLV-1 infection (23, 24). This pattern was not frequently described in Brazilian WB-indeterminate samples (5, 13, 15, 26), and was also not observed in any samples analyzed in the present study. In spite two samples herein presented an HGIP incomplete pattern (without p32 and p36 bands), only the p19 I/II band was detected by LIA. Thus they were considered HTLV negative according to LIA manufacturer criteria. Corroborating the LIA result, the same profile (only p19 I/II band) was detected when we analyzed samples from five patients attended at HTLV-outpatients clinics in São Paulo, during the years 2000 to 2006, which exhibited p19, p26, p28, and p36 WB bands (5).
Moreover, we conducted a retrospective analysis of 108 well-characterized blood samples from patients from São Paulo [tested by two serological (WB and LIA) and two molecular assays (qPCR pol and PCR-RFLP tax), and confirmed a series of more than 10 HTLV-1 and 10 HTLV-2 as positive by both WB and LIA criteria, and they were concordant. In addition, we revised data from two samples HTLV-1+2 WB positive, one of which was confirmed HTLV-1 and HTLV-2 by LIA and molecular assays (15). Additionally, we tested other two HTLV-1+2 WB positive samples by LIA, and only HTLV-1 was confirmed, taking into account the intensity of the discriminatory bands (p19-I and gp46-I, and gp46-II). Note, the WB-indeterminate profile (strong GD21 and p28 bands) described in Pygmies and Bantus living in the southern Cameroonian rainforest (28), has not been detected in any study conducted in São Paulo (5, 13, 15) and herein.

Although PCR assays presented lower sensitivity than WB in detecting true HTLV-1/2 infection in HIV/HTLV-coinfected individuals in São Paulo, Brazil, the molecular assays were able to confirm and discriminate between HTLV-1 and HTLV-2 in some WB-indeterminate and HTLV untypeable cases indicating that both serological and molecular assays are useful for HTLV diagnosis (13, 15). Due to the presence of large numbers of WB-indeterminate samples, coupled with the high cost of obtaining WB and LIA assays in Brazil, we recently proposed an algorithm that employs qPCR to confirm HTLV infection, followed by testing any PCR-negative samples with WB or LIA. This strategy was shown to reduce costs and improve the diagnostic accuracy of HTLV-1/2 (13, 15). Nonetheless, due to highly divergent socioeconomic conditions among different regions in Brazil, in laboratories without the means to perform molecular assays, high-performance serological testing presents an acceptable alternative.
Some studies of HTLV diagnosis conducted in blood donors in Latin America (considered endemic area for HTLV-1/2) have reported differing numbers of WB-indeterminate samples that were subsequently confirmed as positive by PCR (29-31). Also in blood donors from another endemic area in Northeast Iran, WB-indeterminate samples were found to be positive by PCR, and the most prevalent WB bands presenting variable combinations of rgp46-I, GD21 and gp21 (32).

In corroboration with these findings, the majority of serum samples herein that presented WB patterns GD21 and/or gp46-I or –II plus p19 or p24 were subsequently confirmed as positive under LIA. These types of WB patterns were observed in G1, and PCR assays demonstrated HTLV-1 or HTLV-2 positivity (data not shown, published elsewhere) (15). In addition, the majority of serum samples that presented only gag bands in WB analysis were negative for HTLV-1/2 infection by LIA. Of note, one blood sample in G1 that showed a faint GD21 band in WB analysis tested negative for HTLV-1/2 by both LIA and PCR. Another serum sample presenting a GD21, rgp46-I and rgp-46-II WB pattern was found to be negative under LIA; unfortunately, this sample could not be analyzed by PCR because only serum was sent to the laboratory for analysis. However, retesting of this serum sample by WB and LIA confirmed the discrepant results. It is interesting to note that, in serum samples (n=14) that tested HTLV untypeable by LIA, PCR confirmed HTLV-1 in five samples and HTLV-2 in another two samples from G1 (data not shown), emphasizing the need for employing molecular assays to conclude HTLV diagnosis in patients with HIV/HTLV coinfection.

In G2, the majority of WB-indeterminate patterns presented either GD21 alone or this protein in association with one gag or envelope band. LIA confirmed HTLV-2 in 11/21 (52.4%) of WB-indeterminate samples. The high number of HTLV-2 positive
samples in G2 leads us to suppose that these patients acquired HBV and HCV, as well as
HTLV-1/2 and HIV, at the same time, probably by parenteral route and prior to when
serological testing for HIV and HBV (1989), and subsequently for HTLV and HCV
(1993), became mandatory in blood banks throughout Brazil; in addition, when
intravenous drug addiction was more frequent in this country, as was previously
described (18-20). Corroborating this hypothesis, the older age and male sex
predominated in G2. Regarding the lack of WB in diagnosing HTLV-2 truly infected
samples, we have been described this difficulty since the years of 2000 (8, 13, 15), and
hypothesized that the rgp46-II (K55) present in the WB strip is not as sensitive to detect
antibodies to the HTLV-2 strains that circulate in Brazil (HTLV-2a subtype, variant -2c)
(33). This seems to be not the case of the gp46-II present in the LIA strip.

Concerning the two WB-indeterminate samples in G2 with negative HTLV
seropositivity by LIA, both presented reactivity for GD21 in WB analysis, and one of the
samples showed a faint band. Curiously, LIA demonstrates the best performance in this
group of patients, with 20/24 (83.3%) of HTLV positivity detected among the WB-
inconclusive samples. Unfortunately, only plasma/serum samples were available for
analysis from these patients, which did not allow for the use of PCR to perform a
comparative analysis of serological and molecular results. Nonetheless, associations
between HTLV-1/2 and hepatitis B and C have been reported in several studies
conducted in Brazil and elsewhere (18-20, 24, 29, 34).

The WB-inconclusive patterns in G3 were quite different from those in the other
groups analyzed. Several of the HTLV untypeable samples demonstrated the presence
of almost all bands corresponding to HTLV-1 viral lysate, without reactivity to rgp46-I,
and six of nine were subsequently confirmed as HTLV-1 by LIA. Twelve out of the 25
WB-inconclusive samples that could be submitted to PCR (nine WB-indeterminate and three HTLV), 11 were confirmed as HTLV-1-infected, six of which were HTLV untypeable by LIA analyses (data not shown). In addition, the serum samples that tested negative by LIA presented three different WB-indeterminate patterns: (i) GD21, (ii) p24 (faint band), and (iii) rgp46-II (faint band). Only one of these samples could be tested by PCR and presented HTLV-negative result (data not shown). In summary, the samples in G3 were confirmed as HTLV-1 or HTLV, but not HTLV-2 infection. This finding could be partially related to the ethnic origin of the included individuals (African descendants), the lack of HIV infection in this group, and the characteristics of the patients seen at HTLV out-patient clinics in Salvador-BA (35).

Of note, the reasons previously described to explain WB-inconclusive results could also be applied to the PCR-negative results in truly HTLV-1/2-infected individuals, including the low proviral load in HIV/AIDS patients undergoing antiretroviral therapy in G1, and in some cases in G2 (13-15); HTLV-2 infection is known to show low proviral loads (11, 12, 25); the presence of defective provirus not detected by the primers employed in the PCR assays (27); infection with other viruses, such as HTLV-3 or HTLV-4, which can only be detected using specific primers (36, 37).

Noteworthy, since the discovery of HTLV-3 and HTLV-4 in central Africa (36, 37), studies were conducted to ascertain that assays commercially available and employed in the HTLV-1/2 diagnosis were able to detect these new HTLV (38-40). The results obtained confirmed HTLV-1/2 screening assays as sensitive to detected antibodies to HTLV-3 and HTLV-4 (38), and disclosed discordant and misclassified results in relation to confirmatory serologic assays (WB and LIA) (36-41).
In fact, we could not rule out HTLV-3 and HTLV-4 infections in Brazil, since populations in central Africa migrated from Africa and Australia to the American continent previously of Asiatic population migration, and their descendant, such as the Amerindians could maintain or spread to the general population such viruses, which could justify the frequent presence of WB-indeterminate results in Amerindians, as previously described (42).

In relation to the LIA, although presented the best performance in diagnosing HTLV-1 and HTLV-2, we could not exclude misclassified positive results, as occurred in HTLV-3 and HTLV-4 infected individuals, which could be erroneously diagnosed as infected with HTLV-2 (36-38).

Of note, despite the fact that LIA demonstrated better performance than WB in both HTLV-1 and HTLV-2 serological diagnosis, additional considerations are warranted for both assays. With respect to WB, we consider the lack of an ability to score the intensity of a positive band to be a problem, since it is not known when a faint band should be truly considered positive. The criteria (band profiles) established by the manufacturer to confirm HTLV-1 and HTLV-2 infections in WB assays are excessively stringent and deserve a review. Taking into account the results obtained herein, we suggest that samples presenting only one gag band (p19 or p24) plus GD21 and rgp46-I or rgp-46-II should be considered as HTLV-1 and HTLV-2 positive, respectively, since samples that demonstrated p24, GD21 and rgp46-I bands were confirmed as HTLV-1 positive by LIA and PCR. By contrast, we detected true HTLV-2 positivity in samples that showed p19, GD21 and rgp46-II bands. In addition, when gag bands were undetectable, but both envelope bands (GD21 and rgp46-I or rgp46-II) were present, it was impossible to rule out true HTLV-1 or HTLV-2 infection, since seroconversion could
be taking place. Indeed, when all bands showed reactivity to HTLV-1 viral lysate antigens, even in the absence of rgp46-I, it was possible to confirm HTLV-1 infection.

In conclusion, LIA was shown to be the best serological test for confirming HTLV-1 and HTLV-2 infections, regardless of whether HTLV-monoinfected or -coinfected individuals. We further highlight the need to review some WB criteria based on our results and those published by others. It remains to be determined whether the superior performance of LIA was due to the less stringent criteria employed in relation to the WB. Further studies are necessary to confirm these results in a variety of risk populations from Brazil and elsewhere.

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LEGENDS OF TABLES AND FIGURES

**TABLE 1** Summary of the characteristics (number, age and sex) of the study-groups whose serum samples were analyzed for the presence of HTLV-1/2 antibodies and results of HTLV-1/2 screening and Western Blot confirmatory assay

| Study Group | Number of Individuals | Age | Results of HTLV-1/2 Screening and Western Blot Confirmatory Assay |
|-------------|-----------------------|-----|---------------------------------------------------------------|
| G1          |                       |     |                                                               |
| G2          |                       |     |                                                               |
| G3          |                       |     |                                                               |

\( g \) Screening assays according to described in Material and Methods; \( b \) Western Blot (results according to HTLV Blot 2.4, MP Biomedicals manufacturer’s criteria); IND: indeterminate; \( c \) Age data obtained only from patients with WB-inconclusive results; \( d \) Five samples positive for both, HTLV-1 and HTLV-2.

**TABLE 2** Characteristics (sex and age) and number of individuals whose serum samples yielded HTLV inconclusive WB results and were available for LIA analysis in each study-group

| Study Group | Number of Individuals |
|-------------|-----------------------|
| G1          |                       |
| G2          |                       |
| G3          |                       |

G1, group of HIV/AIDS patients from São Paulo-SP, Brazil; G2, group of patients with hepatitis B (HBV) and hepatitis C (HCV) from São Paulo-SP; G3, group of individuals from HTLV outpatients clinic in Salvador-BA, Brazil; N, number of individuals
aWB-indeterminate and WB-HTLV according to HTLV Blot 2.4, MP Biomedicals manufacturer's criteria; b*p-value statistically significant using Chi-square test

TABLE 3 WB-indeterminate profiles detected in serum samples of each study-group, and number and percentage of serum samples that confirmed HTLV-1/2 infection after LIA analysis

aAccording to the criteria established by HTLV Blot 2.4, MP Biomedicals; 
bAccording to the criteria established by INNO-LIA HTLV-I/II, Fujirebio

TABLE 4 Final results and profiles obtained with confirmatory serologic assays for detecting HTLV-1/2 antibodies in 111 serum samples that yielded WB-inconclusive results and were tested by LIA

aWB, Western Blot (HTLV Blot 2.4, MP Biomedicals); bLIA, Line ImmunoAssay (INNO-LIA HTLV-I/II, Fujirebio); cfaint bands

FIG 1 Number of serum samples in each study-group who scored WB-indeterminate or HTLV untypeable in previous analysis and confirmed or excluded HTLV infections by LIA

WB, Western Blot (HTLV Blot 2.4, MP Biomedicals); LIA, Line ImmunoAssay (INNO-LIA HTLV-I/II, Fujirebio)
Table 1. Summary of the characteristics (number, age and sex) of the study-groups whose serum samples were analyzed for the presence of HTLV-1/2 antibodies and results of HTLV-1/2 screening and Western Blot confirmatory assay.

| Groups   | Study population | Number of individuals | Sex | Nº | %  | Age (years) Mean | Min-Max | Screening positive* | WB-Positive HTLV-1 | HTLV-2 | WB-Inconclusive HTLV | IND | WB-HTLV a [%] | WB-IND b [%] |
|----------|------------------|-----------------------|-----|----|----|------------------|---------|---------------------|-------------------|--------|---------------------|------|--------------|--------------|
| G1       | HIV              | 4,395                 | Male | 2,935 | 66.78 | 39.12 | (16 - 84) | 311 | 97 | 65 | 16 | 66 | 5.14 | 21.22 |
|          |                  |                       | Female | 1,459 | 33.20 | 40.36 | (16 - 83) | 221 | 45 | 29 | 3 | 21 | 3.03 | 21.21 |
| G2       | HBV/HCV          | 3,228                 | Male | 1,749 | 54.18 | 48.40 | (14 - 88) | 99  | 45 | 29 | 3  | 21 | 3.03 | 21.21 |
|          |                  |                       | Female | 1,479 | 45.82 | 48.50 | (13 - 94) | 122 | 45 | 29 | 3  | 21 | 3.03 | 21.21 |
| G3       | Monoinfection    | 362                   | Male | 105  | 29.01 | 39.00 | (24 - 60) | 207 | 127 | 36 | 11 | 27 | 5.31 | 13.04 |
|          |                  |                       | Female | 257  | 70.99 | 42.20 | (16 - 73) | 155 | 127 | 36 | 11 | 27 | 5.31 | 13.04 |

G1, group of HIV/AIDS patients from São Paulo-SP, Brazil; G2, group of patients with hepatitis B (HBV) and hepatitis C (HCV) from São Paulo-SP; G3, group of individuals from HTLV out-patients clinic in Salvador-BA, Brazil; N, number of individuals.

*Screening assays according to described in Material and Methods; "WB, Western Blot (results according to HTLV Blot 2.4, MP Biomedicals manufacturer' criteria); IND: indeterminate; "Age data obtained only from patients with WB-inconclusive results; "Five samples positive for both, HTLV-1 and HTLV-2.
Table 2. Characteristics (sex and age) and number of individuals whose serum samples yielded HTLV inconclusive WB results and were available for LIA analysis in each study group.

|               | Groups                             |       |       | p-value          |
|---------------|------------------------------------|-------|-------|------------------|
|               | G1 (N= 62)                         | G2 (N= 24) | G3 (N= 25) |                |
| Sex           | Male                               | 31 (50.00) | 20 (83.30) | 10 (40.00) | 0.0048<sup>b</sup> |
|               | Female                             | 31 (50.00) | 4 (16.70)  | 15 (60.00) |                |
| Age - years   | means (min - max)                  | 44.06 (18 - 68) | 49.50 (35 - 76) | 41.08 (16 - 73) |    |
| WB-Indeterminate<sup>a</sup> |                                   | 48 | 21 | 16 |                |
| WB-HTLV untypeable<sup>a</sup> |                                   | 14 | 3 | 9 |                |

G1, group of HIV/AIDS patients from São Paulo-SP, Brazil; G2, group of patients with hepatitis B (HBV) and hepatitis C (HCV) from São Paulo-SP; G3, group of individuals from HTLV outpatients clinic in Salvador-BA, Brazil; N, number of individuals.

<sup>a</sup>WB-indeterminate and WB-HTLV, according to HTLV Blot 2.4, MP Biomedicals manufacturer’ criteria; <sup>b</sup>p-value statistically significant using Chi-square test.
Table 3. WB-indeterminate profiles detected in serum samples of each study-group, and number and percentage of serum samples that confirmed HTLV-1/2 infection after LIA analysis.

| WB-Indeterminate Profile | G1 (N=48) | G2 (N=21) | G3 (N=16) | Total | LIA-Positiveb |
|--------------------------|-----------|-----------|-----------|-------|---------------|
|                          |           |           |           |       | N (%)         |
| GD21, p24                | 10        | 3         | -         | 13    | 12 (92.30)    |
| rgp46 only (-I or -II)   | -         | 5         | 1         | 6     | 5 (83.33)     |
| GD21, p19                | 3         | 3         | 3         | 9     | 8 (88.89)     |
| p24, rgp46-II            | 4         | 3         | -         | 7     | 7 (100.00)    |
| GD21, rgp46 (-I and/or -II) | 7    | 1         | 4         | 12    | 9 (75.00)     |
| GD21                      | 4         | 4         | 6         | 14    | 3 (21.43)     |
| p19                      | 5         | -         | -         | 5     | 1 (20.00)     |
| p24                      | 4         | 1         | 1         | 6     | 1 (16.67)     |
| GD21, p24, rgp46-I       | 3         | -         | -         | 3     | 1 (33.33)     |
| p19, rgp46-II            | 1         | -         | 1         | 2     | 2 (100.00)    |
| GD21, gp21, rgp46-II     | 1         | -         | -         | 1     | 1 (100.00)    |
| GD21, p19, p26           | 1         | -         | -         | 1     | 1 (100.00)    |
| GD21, p24, p32, p36      | 1         | -         | -         | 1     | 1 (100.00)    |
| p19, p24, rgp46-II       | -         | 1         | -         | 1     | 1 (100.00)    |
| p24, p36, rgp46-II       | 1         | -         | -         | 1     | 1 (100.00)    |
| p19, p24                 | 1         | -         | -         | 1     | -             |
| p19, p26, p28, p53       | 2         | -         | -         | 2     | -             |

aAccording to the criteria established by HTLV Blot 2.4, MP Biomedicals; bAccording to the requirements established by INNO-LIA HTLV-I/II, Fujirebio.
Table 4. Final results and profiles obtained with confirmatory serologic assays for detecting HTLV-1/2 antibodies in 111 serum samples that yielded WB-inconclusive results and were tested by LIA.

| Group | WB-Result | WB Profile | LIA-Result | LIA Profile | Final Result |
|-------|-----------|------------|------------|-------------|--------------|
| G1    | Indeterminate | GD21, p24  | HTLV      | p19/I, II, gp21/II | Indeterminate |
| HTLV  | GD21, p19, p24 | HTLV      | p19/I, II, gp21/II | HTLV        | Indeterminate |
| HTLV  | GD21, p19, p24, p26, p28, p32, p36, p53 | HTLV      | p19/I, II, gp21/II | HTLV        | Indeterminate |
| Indeterminate | p19, p26, p28, p53 | Negative | p19/II | Negative | Indeterminate |
| Indeterminate | GD21, p24 | HTLV | p19/I, II, gp21/II, gp21/II | HTLV | Indeterminate |
| Indeterminate | GD21, p24 | HTLV | p19/I, II, gp21/II | HTLV | Indeterminate |
| Indeterminate | p24, gp46-II | HTLV-2 | p19/I, II, gp21/II, gp46/II, gp46-II | HTLV-2 | Indeterminate |
| Indeterminate | GD21 | Indeterminate | gp21/II | Indeterminate | Indeterminate |
| Indeterminate | p24 | Indeterminate | p19/I, II, gp21/II | Indeterminate | Indeterminate |
| Indeterminate | GD21, rgp46-II | HTLV-2 | gp46/II, gp21/II, gp46-II | HTLV-2 | Indeterminate |
| Indeterminate | GD21, p19 | HTLV-1 | p19/I, II, gp21/II, p19-I | HTLV-1 | Indeterminate |
| Indeterminate | p24, p36, rgp46-II | HTLV-2 | p19/I, II, gp21/II, gp46/II, gp21/II, gp46-II | HTLV-2 | Indeterminate |
| Indeterminate | GD21 | Indeterminate | gp21/II | Indeterminate | Indeterminate |
| Indeterminate | p19, p26, p28, p53 | Negative | p19/II | Negative | Indeterminate |
| Indeterminate | p24 | Indeterminate | gp21/II | Indeterminate | Indeterminate |
| HTLV  | GD21, p19, p24 | HTLV-1 | p19/I, II, gp21/II, p19-I | HTLV-1 | Indeterminate |
| Indeterminate | p19 | Negative | p19/I, II, p19-I | Negative | Indeterminate |
| Indeterminate | p19, p24 | Negative | p19/II | Negative | Indeterminate |
| Indeterminate | GD21, p24 | Indeterminate | gp21/II | Indeterminate | Indeterminate |
| HTLV  | GD21, p19, p24 | HTLV-2 | p19/I, II, gp21/II, gp46/II, gp21/II, gp46-II | HTLV-2 | Indeterminate |
| Indeterminate | GD21, gp46-II, rgp46-II | HTLV-2 | p19/I, II, gp21/II, gp46/II, gp21/II, gp46-II | HTLV-2 | Indeterminate |
| Indeterminate | GD21, p24, rgp46-I | HTLV-1 | p19/I, II, gp21/II, gp46-I | HTLV-1 | Indeterminate |
| Indeterminate | GD21 | Negative | p19/II | Negative | Indeterminate |
| HTLV | GD21, p19, p24, p26, p28, p32 | HTLV | p19/II, p24/II, gp21/II | HTLV |
| HTLV | GD21, p19, p24 | HTLV | p19/II, gp21/II | HTLV |
| Indeterminate | p19, rpp46-1 | HTLV | p19/II, p24/II, gp46/II, gp21/II, gp46-I | HTLV-1 |
| Indeterminate | p24, rpp46-II | HTLV | gp46/II, gp21/II, gp46-II | HTLV-2 |
| HTLV | GD21, p19, p24, p26, p32, p53 | HTLV | p19/II, p24/II, gp46/II, gp21/II, gp46-II | HTLV |
| HTLV | GD21, p19, p24 | HTLV | p19/II, p24/II, gp46/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | p24 | HTLV | gp46/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | p19 | HTLV | p19/II, gp46/II, gp21/II | HTLV |
| HTLV | GD21, p19, p24 | HTLV | p19/II, p24/II, gp21/II | HTLV |
| Indeterminate | GD21', p24 | HTLV | gp46/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | GD21, GD21, rpp46-II | HTLV-2 | gp46/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | p24', rpp46-II | HTLV-2 | gp46/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | GD21, p24 | HTLV | gp46/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | p24 | HTLV | p19/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | p19 | Negative | p19/II | Negative |
| Indeterminate | GD21, rpp46-F | Indeterminate | gp21/II | Indeterminate |
| Indeterminate | GD21, GD21, rpp46-H | Indeterminate | gp21/II | Indeterminate |
| Indeterminate | GD21, p24, rpp46-F | Indeterminate | gp21/II | Indeterminate |
| Indeterminate | GD21, rpp46-I | Indeterminate | gp21/II | Indeterminate |
| HTLV | GD21, p19, p24 | HTLV | p19/II, p24/II, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | GD21, rpp46-I, rpp46-F | HTLV-2 | gp46/I, gp21/II, gp46-II | HTLV-2 |
| Indeterminate | GD21, p24 | HTLV-2 | p19/II, p24/II, gp46/II, gp46-II | HTLV-2 |
| Indeterminate | p24 | Indeterminate | p19/II, p24/II | Indeterminate |
| Indeterminate | p19 | Negative | p19/II | Negative |
| Sample Type | Unspecific Antibodies | Specific Antibodies |
|-------------|----------------------|---------------------|
| Indeterminate p19 | HTLV | p19 l/I, gp21 l/I, gp46 I/II |
| Indeterminate GD21, rgp46-II | HTLV-2 | gp46 l/I, gp21 l/I, gp46 l/I, gp46 l/I, gp46 l/I, gp46 l/I, gp46 l/I |
| Indeterminate GD21, p19 | HTLV-2 | p19 l/I, gp21 l/I, gp46 l/I, gp46 l/I, gp46 l/I, gp46 l/I, gp46 l/I |
| HTLV | GD21, p19, p24, p26, p32, p36 | HTLV-1 | p19 l/I, p24 l/I, p21 l/I, gp21 l/I, gp19 l/I, gp46 l/I |
| Indeterminate GD21, p24 | HTLV-2 | p19 l/I, gp46 l/I, gp21 l/I, gp46 l/I, gp46 l/I, gp46 l/I, gp46 l/I |
| HTLV | GD21, p19, p24 | HTLV-1 | p19 l/I, p24 l/I, gp21 l/I, p19 l/I, gp46 l/I |
| Indeterminate p19 | Negative | p19 l/I |
| HTLV | GD21, p19, p24 | HTLV-1 | p19 l/I, p24 l/I, gp21 l/I, p19 l/I |
| Indeterminate rgp46-I | HTLV-1 | p24 l/I, gp46 l/I, gp21 l/I, gp46 l/I, gp46 l/I |
| Indeterminate p24, rgp46-II | HTLV-2 | gp46 l/I, gp21 l/I, gp46 l/I, gp46 l/I, gp46 l/I |
| HTLV | GD21, p19, p24, p26, p28, p32, p36 | HTLV-1 | p19 l/I, p24 l/I, gp46 l/I, gp21 l/I, p19 l/I, gp46 l/I |
| Indeterminate GD21 | HTLV-1 | Negative, no bands |
| Indeterminate rgp46-I | HTLV-1 | gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate GD21, p19 | HTLV-1 | gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate GD21, rgp46-I | HTLV-1 | gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate GD21, rgp46-I | HTLV-1 | gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate GD21, p19 | HTLV-2 | gp46 l/I, gp21 l/I, gp46 l/I, gp46 l/I, gp46 l/I |
| Indeterminate GD21, p24, rgp46-II | HTLV-2 | p19 l/I, gp24 l/I, gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate GD21 | HTLV | p19 l/I, p24 l/I, gp21 l/I, gp46 l/I |
| Indeterminate GD21, p24, rgp46-II | HTLV-2 | p19 l/I, p24 l/I, gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate GD21, p24 | HTLV-2 | p19 l/I, p24 l/I, gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate rgp46-II | HTLV-2 | p19 l/I, p24 l/I, gp46 l/I, gp21 l/I, gp46 l/I |
| Indeterminate rgp46-II | HTLV-2 | p19 l/I, p24 l/I, gp46 l/I, gp21 l/I, gp46 l/I |
WB, Western Blot (HTLV Blot 2.4, MP Biomedicals); LIA, Line ImmunoAssay (INNO-LIA HTLV-I/II, Fujirebio); faint bands.
WB-indeterminate or HTLV untypeable
(n = 111)