Assessment of a New Immunoassay for Serological Confirmation and Discrimination of Human T-Cell Lymphotropic Virus Infections

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The present study evaluated a new confirmatory assay for antibodies to human T-cell lymphotropic virus type 1 and 2 (HTLV-1 and HTLV-2) proteins performed with serum samples from various commercial sources. The new test is a line immunoassay (LIA) with a nylon membrane sensitized with the most relevant antigens of HTLVs: the envelope gp46 and gp21 as well as the gag p24 and p19 antigens, represented by either recombinant proteins or synthetic peptides. A total of 176 serum or plasma samples were tested, of which 66 were HTLV-1 positive, 72 were HTLV-2 positive, and 38 were HTLV negative; of the 38 HTLV-negative samples 23 were indeterminate by Western blotting (WB). Serially diluted samples (n = 33) from HTLV-1- and HTLV-2-infected patients were also analyzed to determine the sensitivity of the new assay. The new confirmatory assay (INNO-LIA HTLV) performed markedly better than WB assays for those samples reactive by screening. Accurate confirmation of the presence of HTLV-1 and HTLV-2 antibodies and accurate discrimination of HTLV-1 and HTLV-2 antibodies were obtained for all the HTLV-seropositive samples. Due to its enhanced specificity and sensitivity, the new assay not only improves the ability to confirm and discriminate HTLV infections but also eliminates the vast majority of WB-indeterminate and false-positive specimens.

Human T-cell lymphotropic viruses type 1 (HTLV-1) and HTLV-2 are the only known human Oncoviridae. Since their discovery in the early 1980s, these retroviruses have been found to be endemic in many parts of the world including Japan, the Caribbean, Melanesia, and equatorial Africa (2). HTLV-1 has been etiologically linked to adult T-cell leukemia, tropical spastic paraparesis, and several other conditions (12, 31). Although HTLV-2 was initially isolated from two patients with hairy-cell leukemia, it has not been possible to conclusively associate the virus with any type of leukemia or other disease. However, HTLV-2 infections are frequently found in intravenous drug users and seem to be endemic among certain Amerindian tribes.

In order to prevent the spread of HTLV infections to areas where such infections are not endemic, various public health authorities have recommended the routine screening of blood donations for the presence of serological markers to these viruses (20). However, various strategies have been adopted in different countries to screen blood donors. For instance, screening is performed for every donation in France and the United States and only for first-time donors in Sweden. Those countries with very low prevalence rates (<0.01%) tend to remain hesitant to introduce routine screening for HTLV antibodies (e.g., Germany, The Netherlands, and the United Kingdom).

Unfortunately, the prevailing method used for routine screening for HTLV is hampered by relatively poor specificity, thereby giving rise to the need for a large number of confirmatory assays. For this purpose, the Western blotting (WB) technology is most frequently used. This technique is based on the use of HTLV antigens extracted from HTLV-infected cells. In some cases, recombinant HTLV antigens are added to improve the envelope sensitivity of WB. However, the complexity of WB reactivity patterns often makes interpretation of the results quite difficult since many inconclusive results are generated because of the presence of nonspecific bands (8, 18, 27). Blood units classified as indeterminate are usually subjected to further investigation by PCR or are simply discarded from the transfusional circuit. However, in actual practice, WB-reactive, PCR-negative results may not necessarily qualify the blood sample for use in transfusions (13, 16, 24).

HTLVs have been extensively studied since their discovery. Independent groups have precisely mapped the B-cell epitopes (5, 10, 11, 14, 17, 21). Although synthetic peptides and recombinant proteins of the most immunogenic HTLV proteins have been successfully used for the detection (11, 15, 32) and the discrimination (5, 10, 30) of HTLV-1 and HTLV-2 antibodies, antibodies cross-reactive with HTLV proteins are frequently encountered in various autoimmune disorders (1, 3, 28) as well as in multiple sclerosis (7, 22). Additionally, antibodies cross-reactive with HTLV proteins have been reported in different infections such as those caused by varicella-zoster virus (26) and Plasmodium falciparum (23) or those following influenza vaccination (4).

In this study, we evaluated a newly developed line immunoassay (LIA: INNO-LIA HTLV) for the confirmation and the differentiation of HTLV-1 and HTLV-2 infections. Commercially available panels of samples, including well-documented samples, were tested. The results obtained for each individual antigen line were statistically analyzed to define an interpretation algorithm. The newly defined algorithm for interpretation shows high sensitivity and specificity compared to those of the classical WB techniques. The improved specificity was further demonstrated with 279 serum samples repeatedly reactive by
an enzyme-linked immunosorbent assay (ELISA) screening (25).

**MATERIALS AND METHODS**

**Tested samples.** Commercially available panels of different origins containing HTLV-infected samples were tested. Boston Biomedica Inc. (BBI; Rockville, Mass.) supplied four HTLV panels that included mainly HTLV-2-infected samples: panels BBI-AO2 (n = 25), PRP-203 (n = 25), PRP-204 (n = 25), and PRP-205 (n = 25) were tested. Two French HTLV panels included mainly HTLV-1-infected samples as well as those that were either HTLV-2 positive, WB indeterminate, or HTLV-2 positive, WB indeterminate, or HTLV positive but diluted. These panels, SFTS-93 (n = 45) and SFTS-94 (n = 59), supplied by the Société Française de Transfusion Sanguine (SFTS; Montpellier, France), were tested. In addition to these proficiency panels, we investigated samples from European blood donations that tested negative by registered, routinely used screening assays. We also tested some serum samples initially reactive by enzyme immunoassay which then showed negative or indeterminate results by two different WB techniques.

**WB kits.** The two kits available for HTLV serology confirmation were manufactured by Genelabs (DBL versions 2.3 and 2.4; Genelabs, Geneva, Switzerland) and by Cambridge Biotech Corporation (CBC; Worcester, Mass.). These kits are based on viral lysates of HTLV-1-infected cells to which HTLV-1 and HTLV-2 envelope recombinant antigens have been added. The test procedures and interpretation of the results were performed according to the corresponding manufacturer’s instructions.

**INNO-LIA HTLV.** The INNO-LIA HTLV kit uses recombinant antigens and synthetic peptides derived from both HTLV-1 and HTLV-2 protein sequences. The antigens used in this technique are presented in Table 1. In addition to these HTLV antigens, control lines are used for a semiquantitative evaluation of the results as well as for the verification of sample addition and reagents. A schematic layout of the strips is shown in Fig. 1.

The assay procedure can be summarized as follows. Serum or plasma samples were diluted 1:100 and were incubated in the troughs containing LIA strips at a room temperature (RT) of 25°C overnight for 16 h. This was followed by three washing steps with washing buffer before the addition of an alkaline phosphatase anti-human immunoglobulin conjugate and incubation for 30 min at RT. Three washing steps were again performed, followed by the addition of a chromogen for 30 min at RT. Color development was then stopped with an appropriate stop solution.

Following the visual interpretation protocol, after color development each lane was compared to the control lines, and the intensity was scored as follows: 0 (−), absent or less than the cutoff line; 0.5 (+), intensity equal to that of the cutoff line; 1 (+), intensity between that of the cutoff line and that of the 1+ control line; 2 (++), intensity between that of the 1+ control line and that of the 3+ control line; 3 (+++), intensity equal to that of the 3+ control line; 4 (++++) intensity higher than that of the 3+ control line. The use of control lines with different staining intensities allows for semiquantitative interpretation and diminishes the subjectivity of visual reading. Figure 2 shows INNO-LIA HTLV tests with recombinant antibodies and the presence of type-specific antibodies and the presence of type-specific antibodies were calculated with a high probability for correct interpretation (79%), while the alternative interpretations, having a very low probability (<1%), were statistically nonsignificant (data not shown). The samples whose results were difficult to interpret had no typical pattern, and the patterns were considered according to the highest probability of occurrence.

**RESULTS**

**Panels of HTLV-infected samples.** The results obtained for the different panels of HTLV-infected samples are summarized in Table 3. Reactivities with the antigens in the INNO-LIA HTLV are expressed as relative intensities by visual reading. Depending on the panel, the status for each member of a panel is a consensus diagnostic result by different techniques such as enzyme immunoassays, WB, PCR, radioimmunoprecipitation, or agglutination assays used in different kits registered in the United States or Europe. These consensus results were obtained from the panel suppliers (BBI or SFTS).

**BBI panels.** The older BBI panel (AO2; no longer commercially available) is less well documented, while the newer one (PRP-205) has been tested by a wide variety of techniques. For ease of comparison, we considered only the overall interpretation and summarized the results found by the INNO-LIA HTLV. Two of three indeterminate specimens in panels AO2 and PRP-203 were resolved as negative by INNO-LIA HTLV. Those samples were not fully characterized, and the infections could not be efficiently diagnosed by any other serological method. Moreover, INNO-LIA HTLV correctly diagnosed all members of the new, fully characterized panels, PRP-204 and PRP-205. A summary of the results obtained with these panels is presented in Table 3, and the results are compared with those obtained by the WB techniques available when the panels were formed.

**SFTS-93 panel.** The SFTS panel (n = 45) included samples which were HTLV-1 positive (n = 14), HTLV-2 positive (n = 2), and HTLV negative (n = 13). For sensitivity assessment, serially diluted samples positive for HTLV-1 (n = 3) and HTLV-2 (n = 2) were also included in this panel. All HTLV-1- and HTLV-2-positive samples were found to be positive by INNO-LIA HTLV. Two of the HTLV-1-positive samples could not be typed by INNO-LIA HTLV. The WB techniques used for this panel have no typing ability. In general, INNO-LIA HTLV showed higher sensitivity than WB techniques in

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**TABLE 1. Antigens used in INNO-LIA HTLV**

| Line no. | Protein/sequence | Test material | Immunoreactivity |
|----------|------------------|---------------|------------------|
| 1        | gag p19/HTLV-1+  | Recombinant protein + SP* | HTLV-1 and HTLV-2 |
| 2        | gag p24/HTLV-1   | Recombinant protein | HTLV-1 and HTLV-2 |
| 3        | Envelope gp46/HTLV-1 + HTLV-2 | SP | HTLV-1 and HTLV-2 |
| 4        | Envelope gp21/HTLV-1 | Recombinant protein | HTLV-1 and HTLV-2 |
| 5        | gag p19/HTLV-1   | SP | HTLV-1 |
| 6        | Envelope gp46/HTLV-1 | SP | HTLV-1 |
| 7        | Envelope gp46/HTLV-2 | SP | HTLV-2 |

* SP, synthetic peptides.
identifying diluted samples. Some of the diluted HTLV-1 and HTLV-2-positive samples became indeterminate or negative on WB, while INNO-LIA HTLV remained fully sensitive and discriminatory. For the seronegative members of this panel, only one was found to be indeterminate by INNO-LIA HTLV, whereas eight were indeterminate by WB.

SFTS-94 HTLV panel. The SFTS-94 HTLV panel (n = 59) included samples positive for HTLV-1 (n = 26) and HTLV-2 (n = 6) and HTLV-negative samples (n = 14). For sensitivity assessment, serially diluted samples positive for HTLV-1 (n = 3) and HTLV-2 (n = 2) were also included in this panel. INNO-LIA HTLV was able to detect virus in all HTLV-1-positive samples (26 of 26) and typed the virus in 25 of 26 samples. Sample 221 lacked specific antibodies for HTLV-1 and therefore was scored as positive but untypeable. Similarly, antibodies in all HTLV-2-positive samples were detected and typed efficiently by the INNO-LIA HTLV technique. Among the diluted series, depending on the original titers of HTLV antibodies, the detection and discrimination of HTLV-1 and HTLV-2 antibodies performed well for sample 220 diluted 1:200, sample 201 diluted 1:10, sample 207 diluted 1:200, sample 240 diluted 1:5, and sample 241 diluted 1:5. Further dilutions of these samples resulted in a loss of sensitivity for antibodies to HTLV. Negative samples in this panel were classified as negative (13 of 14) or indeterminate (1 of 14), while WB indicated indeterminate results for 13 of 14 negative samples.

Sensitivity. The overall sensitivity of the INNO-LIA HTLV for the detection of HTLV antibodies in samples confirmed to be positive for HTLV was 100% (138 of 138), while the sensitivity for discrimination, expressed as the capacity to differentiate between HTLV-1 and HTLV-2 antibodies, was 96.4% (133 of 138). Because the WB results provided by the panel suppliers were obtained by different commercially available tests, the overall sensitivity and the ability to discriminate the two virus types cannot be assessed. However, these parameters can be determined separately for each panel from Table 3.

DISCUSSION

As diagnostic tests for HTLV become more reliable, the worldwide patterns of distribution of HTLV infections are becoming more clear. Many samples initially thought to be HTLV positive because of some WB reactivities were subsequently found to be negative by other techniques such as PCR (13, 29).

WB techniques commonly use HTLV-1 viral lysate spiked with recombinant antigens derived from HTLV-1 and HTLV-2 protein sequences. To detect the humoral immune response, which varies among infected individuals, an optimal concentration of each HTLV antigen is required. However, detection and typing of HTLV antibodies are not always feasible by WB due to inherent difficulties for optimization of the technique. In addition to the variability of the immune response to HTLV infections, the parameters measured by serological assays for HTLV seem to widely occur in other infectious and noninfectious diseases. Thus, the results for many ELISA-reactive samples remain unresolved by WB techniques. Other tests, such as the immunofluorescence or the radioimmunoprecipitation assay as well as follow-up testing may be used to rule out HTLV infection. PCR techniques are sometimes required to confirm HTLV infections, but PCR remains inconvenient for large-scale screening such as in blood banks (29). Additionally, PCR primers are not fully standardized, thereby resulting in laboratory-to-laboratory variations.

The HTLV WB kit from CBC does not allow for serotyping, while that from Genelabs has typing ability. However, the criteria currently defined for the latest WB technique do not allow for the accurate typing of all positive samples (27). Many WB-positive but untypeable samples are, in fact, found to be negative when further investigations are possible. This finding has been supported previously by other studies (6, 19, 24). More recently, Vrielink et al. (29) have studied WB-indeterminate samples (n = 228) by PCR; all were reported to be negative. Repeated attempts to isolate HTLVs from blood donors with isolated gag antibodies have failed (13, 16). Therefore, many users have amended these criteria to meet their own needs, leading to inconsistency in reports from different studies. The well-known false reactivity with recombinant gp21 by WB was only partially resolved in a more recent version that uses a truncated recombinant gp21 (9). However, this truncation did not significantly decrease the numbers of indeterminate samples, because false reactivities to other proteins (usually gag) are still unsolved. Clearly, this shows the difficulty in defining the detection and discrimination criteria for HTLV-1 and HTLV-2 antibody patterns by WB (19).

The interpretation criteria for HTLV serology have heretofore been technique dependent and need to be adapted according to each set of parameters related to a specific technique. Unlike WB tests, the newly developed INNO-LIA HTLV was optimized by using HTLV-specific antigens. The

![FIG. 2. Representative INNO-LIA HTLV patterns for negative, indeterminate (Ind.), and HTLV-1, HTLV-2, and HTLV (untypeable)-positive (Pos.) serum samples.](image)

**TABLE 2. Guidelines for interpretation of INNO-LIA HTLV results.**

| Test and reading | Results$^a$ |
|------------------|------------|
| **Confirmation**  |            |
| 1. No band        | Negative   |
| 2. Isolated band  | Negative   |
| 3. Multiple bands | Indeterminate |
| **Sum of intensities, <2** | Indeterminate |
| | | |
| a. Only gag bands (p19 + p24) | Indeterminate |
| b. Only env bands (gp46 + gp21) | Positive |
| c. gag and env bands | Positive |

| **Discrimination of positive samples** | |
| 1. env gp46-1 $>$ env gp46-2 | HTLV-1 |
| Alternatively, if gag p19-1 $>$ 0 | HTLV-1 |
| 2. env gp46-2 $>$ env gp46-1 | HTLV-2 |
| Alternatively, if gag p19-1 = 0 | HTLV-2 |
| 3. Other combinations | Positive (untypeable) |

$^a$ Each sample is first determined to be negative, positive, or indeterminate. Positive samples are then classified either as HTLV-1, HTLV-2, or HTLV positive but untypeable according to the relative intensities of type-specific env gp46 antibodies. Alternatively, the presence or absence of gag p19 antibodies can allow for serotyping.
use of such antigens dramatically reduced the numbers of indeterminate results found by WB. This was shown in a recent study involving 279 ELISA-reactive Brazilian samples, of which the majority were better resolved by INNO-LIA HTLV than by classical WB (25). The few remaining samples indeterminate by INNO-LIA HTLV usually react with gag proteins (p19 and p24) and may be due to cross-reactivities with autoantibodies or antibodies of unknown origin. Nevertheless, the significance of antibodies cross-reactive with HTLV remains highly controversial; many HTLV proteins have been reported to be potential targets for antibodies with distinct specificities. Although the pattern of seroconversion against HTLV antigens has not yet been elucidated, the long incubation time, generally observed after an HTLV infection, should allow the immune system to fully develop the B-cell response. Thus, antibody reactivity to a single HTLV antigen is unlikely to represent seroconversion. This is supported by a recent trend toward the omission of the most cross-reactive antigens, more specifically, the gag antigens, from newly developed HTLV screening assays. However, it is more advisable not to exclude cross-reactive antibodies at the screening stage but rather to use an improved algorithm at the confirmation level such as that proposed for the INNO-LIA HTLV technique. An epidemiological survey of cross-reactive antibodies, at least with samples from high-risk populations, will contribute to the elucidation of their significance.

In conclusion, the INNO-LIA HTLV technique appears to be at least as sensitive as WB and allows for the more accurate confirmation and discrimination of HTLV by serology than are possible by WB techniques. None of the samples confirmed to be HTLV positive was missed by using the proposed algorithm for the INNO-LIA HTLV technique. Furthermore, among the 279 samples from Brazilian donors that were studied, none of the WB-indeterminate or WB-positive (untypeable) samples showed any evidence of HTLV infection by additional investigations (25). Therefore, these samples were considered false positive by WB. Most of such samples were found to be negative by the new technique, supporting the very low probability of HTLV infection. The use of the INNO-LIA HTLV technique will improve the ability to confirm the presence of HTLV infection and discriminate the HTLV type causing the infection and should also eliminate most WB-indeterminate and false-positive samples.

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