Immunofluorescence assay in India for confirmation of HIV-1 infection using a T-cell line infected with defective HIV-1

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1. Introduction

The human immunodeficiency virus (HIV) epidemic continues to be a burden worldwide, especially in developing countries. Though there has been dramatic progress in the diagnostic methodologies, the detection of antibodies continues to be the mainstay of diagnosis in most of these countries. Serologic assays for the detection of anti-HIV antibodies, rapid HIV tests, and enzyme immunoassays (EIAs) are universally used for initial antibody testing of HIV infection, and all HIV reactive samples are further confirmed by two other rapid tests working on different principles; however, Western blotting (WB) and immunofluorescence (IF) assays are not routinely performed in this country.

Methods: A total of 2104 sera from Indian subjects were tested for the presence of HIV-1 antibody using EIA/rapid tests, according to the guidelines of the National AIDS Control Organization of India, and were also subjected to IF test using L-2 cells persistently infected with defective HIV-1. WB and a nested reverse transcriptase polymerase chain reaction (RT-PCR) were performed on discrepant samples.

Results: IF results were 100% concordant with EIA/rapid tests for 212 HIV-1-positive samples and 1889 HIV-1-negative samples. Interestingly, three (0.14%) samples negative by EIA/rapid tests were weakly or moderately positive (1+/2+) by IF test. All three of these samples were confirmed to be negative by WB (reactive with Gag/Pol, but not with Env), but positive by RT-PCR with primers targeting the C2–V5 fragment of the env gene. These three samples were from individuals who voluntarily reported for HIV testing because of high-risk practices, and they may have been at an early stage of HIV infection.

Conclusions: These results confirm that the IF test using L-2 cells is a sensitive and specific alternative method for confirmation of HIV-1 infection and could be included in the diagnostic algorithm in reference laboratories in developing countries.

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In contrast to WB, the IF assay is a well-accepted and widely used confirmatory test in many laboratories. The primary advantages of the IF assay are that it is technically simple, more convenient, less expensive, and more rapid than WB. Commercial WB tests usually require overnight incubation for optimal results, while commercial IF assays can be performed within 2 h. Additionally, cells are maintained by dilution passage every 4 days, which takes less than 5 min. Moreover, the use of an uninfected cell control ensures high assay specificity by easily differentiating non-specific staining from specific staining.

The source of viral antigen in HIV IF assays has commonly been continuous lymphoid cell lines such as H9 or HUT78 and non-lymphoid cell lines such as the T4 HeLa cell line. In this study, a previously developed human T-cell line persistently infected with defective HIV-1 (L-2 cell clone) was used in an IF assay system along with MT-4 cells as control cells. The advantage of using this cell clone is that L-2 cells show exceptionally strong HIV-1 antigen expression compared with other persistently HIV-1 infected human T-cell lines and continuously produce non-infectious, reverse transcriptase-negative doughnut-shaped particles. The goal of the present study was to explore the suitability of L-2 cells as a potentially useful source of viral antigens in IF assays for HIV confirmatory testing.

2. Methods

2.1. Serum samples

From October 2006 to May 2008, 2104 serum samples were tested. Sera were obtained from 954 consecutive diagnostic specimens received for HIV antibody testing from individuals attending the Integrated Counseling and Testing Centre (ICTC) of a tertiary care hospital in India and 1150 consecutive samples collected during a sentinel surveillance program. Out of the 1150 samples, 400 were from antenatal clinic attendees and 250 each were from female sex workers, intravenous drug users, and men having sex with men (MSM). Each patient gave written informed consent for study participation and received pretest counseling prior to sample collection, in accordance with the guidelines of the National AIDS Control Organization (NACO), India.

2.2. Routine HIV serology

In this study, screening and confirmatory antibody testing for HIV infection was performed as recommended by NACO, India, following a serial testing strategy (Figure 1). Briefly, the samples were initially screened for HIV antibody by EIA/rapid test, i.e., CombAids-RS (Span Diagnostics Ltd, India; Dot Immunoassay)/Microlisa-HIV (J. Mitra & Co. Pvt. Ltd, India; Indirect ELISA principle). All samples found negative by the first screening test were reported negative. To reduce the costs of HIV testing as recommended by the World Health Organization, all the HIV reactive samples were further confirmed by two more rapid tests working on different principles, such as ACON-HIV (Rapid Diagnostic Pvt Ltd, India; Tri-line rapid chromatographic immun assay), Capillus test (Trinity Biotech plc, Bray, Co. Wicklow, Ireland; agglutination test), or Retroquic-HIV (Qualpro Diagnostics, India; rapid immunoconcentration test). Samples reactive in all three assays were reported as positive for HIV antibody. For
sentinel surveillance samples, the NACHO testing strategy is screening and then confirmation of the positive samples by one test. All the kits used for the detection of HIV antibody were approved and supplied by the Delhi State AIDS Control Society, India. In India, WB testing and IF assays are not routinely performed and are restricted to specimens with atypical serological profiles (discordant results by three EIA/rapid tests).

2.3. Cell lines

The uninfected MT-4 human T-cell line and the infected L-2 cell line were used. MT-4 was the parental cell line used for the preparation of the L-2 cell clone that was persistently infected with the defective HIV-1 subtype B LAI strain. These cell lines were maintained at the Department of Virology, Research Institute of Microbial Diseases, Osaka University, Japan. L-2 and MT-4 cells were propagated by incubation in RPMI-1640 supplemented with 5–10% heat-inactivated fetal bovine serum at 37 °C in a CO₂ incubator. Cells were cultured at 5 × 10⁵/ml until the cell density reached 2–3 × 10⁶/ml on the 4th day post-incubation. Cells were maintained by subdividing 1:4 (v/v) in fresh medium every 4 days.

2.4. Slide preparation

The cells were harvested, washed with phosphate-buffered saline (PBS), and suspended in PBS at 1 × 10⁶ cells/ml. One part L-2 cell suspension was mixed with three parts uninfected MT-4 cell suspension. The MT-4 and L-2 cells were smeared on a 12-well glass slide (Flow Laboratories). Approximately 5 × 10³ cells (5 µl of a 1 × 10⁶ cells/ml suspension) were placed in each well. The slides were air-dried and fixed in cold acetone for 10 min at 4 °C. The slides were packed in heat-sealed plastic bags and stored at −20 °C until use.

2.5. IF assay procedure

All patient sera were heat-inactivated at 56 °C for 30 min and diluted 1:100 in PBS. Ten microliters of each diluted serum were applied to wells and incubated in a humid chamber at 37 °C for 30 min. PBS and HIV-positive and HIV-negative sera were used as internal controls each time. After washing three times with PBS at room temperature, 10 µl of the fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc.) was applied to the wells; these were again incubated at 37 °C for 30 min. The slides were rinsed three times with PBS and mounted in 90% glycerol in PBS and read independently by two trained persons, who were blinded to serological results, under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). This test was performed as a single assay (without replicates). Any ambiguous or discordant samples were retested in duplicate.

2.6. Interpretation of IF assay results

The typical cytoplasmic staining patterns of L-2 cells were graded from 0 to +4 according to the intensity of FITC in approximately 25% of L-2 cells present in a monolayer of the HIV-infected wells (approximately 1.25 × 10⁶ L-2 cells/well), as described elsewhere. A false-positive reaction was detected when both the L-2 and MT-4 cells were stained.

2.7. Western blot

WB was performed on the samples with discordant rapid test and IF assay results. WB testing was performed with the HIV WB kit from J. Mitra & Co. Pvt Ltd, India, following the manufacturer's instructions. Results were interpreted according to the criteria suggested by the manufacturer; interpretation criteria for a positive test were 2 Env + 1 Gag ± 1 Pol band and for an indeterminate test were 1 Env ± Gag ± Pol or Gag ± Pol or only Gag or only Pol.

2.8. RNA extraction and envelope PCR amplification

Viral RNA was extracted from the patient serum samples using the QiAamp blood kit (Qiagen, Chatsworth, CA, USA). A reverse transcriptase polymerase chain reaction (RT-PCR) was performed to amplify a 708-base pair C2–V5 fragment of the env gene, using previously described primer sets. We used an infectious molecular clone of Indian subtype C HIV-1 – Indie-C1 – as a positive control. Briefly, viral RNA was initially reverse-transcribed at 50 °C for 50 min using the Thermoscript kit (Invitrogen, Carlsbad, CA, USA). Subsequently, for the cDNA amplification, the EX Taq PCR kit (Takara, Kyoto, Japan) was used as per the manufacturer’s instructions. A nested PCR reaction protocol was used. In the first round reaction, the forward primers were ED31 (CCTGCGCATTACAAGCCTGTCAAG) and BH2 (CCTTGCGCGCTCTCTTTAAGGTCA). Five microliters of the first round reaction product was subjected to a second round of amplification, with the primers DR7 (TCACCTCAGGCTGCCTGCTCAGGCCTGCTAATGACGACTCTACG) and DR8 (CACTCCTCAATTGTGCTCCTACATCTCTCCTC). The cycling conditions for both amplifications consisted of initial activation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 7 min. Amplified PCR products were visualized by electrophoresis in 1% agarose gel.

3. Results

Out of the 2104 samples, 1892 tested HIV-negative and 212 HIV-positive by EIA/rapid test (Table 1). Specific IF patterns of reactivity to HIV antigens were clearly demonstrated in all 212 sera positive by EIA/rapid test, as well as the positive control serum from an HIV-1-infected individual (Figure 2, C). Of the 212 serum samples, 184 were IF-strongly positive (3+ or 4+), 21 were moderately positive (2+), and seven were weakly positive (1+). These seven samples weakly positive (1+) at a dilution of 1:100, were strongly positive (3+ or 4+) at a dilution of 1:20, which gave negative staining in MT-4. In addition, three sera (termed X, Y, and Z) from the ICTC that were negative by EIA/rapid test, were also weakly to moderately positive (one sample 2+, two samples 1+) by IF assay (Figure 2, A, C, and E). The positive reactions of the three sera by IF were confirmed on repeat testing. We also observed that another nine (seven from the ICTC and two from MSM) of the sera negative by EIA/rapid test were also weakly positive by IF. However, all the sera showed negative reactions on repeat testing with the IF test. Hence we concluded that the results were negative in these nine serum samples. The other 1880 sera negative by EIA/rapid test were also negative by IF.

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Number tested</th>
<th>Number reactive by EIA/rapid test</th>
<th>Number reactive by IF assay</th>
</tr>
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<tbody>
<tr>
<td>Integrated counseling and testing centre</td>
<td>954</td>
<td>202</td>
<td>205</td>
</tr>
<tr>
<td>Antenatal clinic</td>
<td>400</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Female sex workers</td>
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<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Intravenous drug users</td>
<td>250</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Men having sex with men</td>
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<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>2104</td>
<td>212</td>
<td>215</td>
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</table>
The above-described X, Y, and Z sera were determined to be negative by WB because reactions with Gag and Pol proteins were observed, however there were no reactions with Env proteins in any of the three serum samples (Figure 3, A). When we subjected these three plasma samples to RT-PCR analysis for the detection of HIV-1 RNA in plasma, it was found that all the samples were positive (Figure 3, B).

4. Discussion

The IF assay has been used successfully in diagnostic laboratories for three decades and has proven to be an acceptable test for the detection of anti-HIV-1 antibodies.14–20 We have described here the performance of one such IF assay using L-2 cells as an alternative antigen source, within the context of a state reference...
laboratory that is accustomed to screening large numbers of suspected cases of HIV infection. The IF assay was compared with EIA/rapid tests for the ability to detect antibodies to HIV. A total of 2104 specimens were tested by both systems. When both assays were used, 212 serum samples were positive and 1889 samples were negative (99.86% concordance). Only three (0.14%) specimens gave discrepant results, i.e., IF produced weakly to moderately positive results, which required retesting for resolution. The three discrepant samples were also negative on WB but were positive on PCR. These samples were from direct walk-in, asymptomatic individuals attending the ICTC for HIV testing, who were involved in high-risk practices. The IF assay and PCR positivity in these cases denotes an early stage of infection, indicating the high sensitivity of the IF assay in comparison to WB. No false-negative results were observed with the IF assay on single testing of 212 HIV-positive sera. The IF assay was found to be almost as sensitive and specific as ELISA as reported in earlier studies. However, in this study it was observed to be slightly more sensitive and equally specific to the EIA/rapid test. Lennette et al. tested 181 sera by both IF assay and ELISA and there were 91 positive and 85 negative sera. They reported that an additional five sera were positive by ELISA only. All five were shown to be negative on retesting and were considered as false-ELISA positives. Gallo et al. observed 100% agreement between EIA and IF on 142 serum specimens from homosexual men and on 88 sera from frank AIDS cases. It has been reported that if the IF assay is performed in a diagnostic laboratory with experience in IF, the false-positive rate should be less than 1% on initial testing and should approach zero on repeat testing. In the present study no false positivity was observed. Additionally, the cost–performance ratio must be considered when deciding which confirmatory test to use for the evaluation of sera that show discrepant results in two screening assays. Previous studies have suggested both WB and IF to have comparable performance characteristics, but confirmation by IF assay can be implemented at a cost that is on average five times less than WB. Therefore IF constitutes a better cost–performance alternative to WB, especially in the case of healthcare centers in India where immunofluorescent microscopy is routinely done to evaluate other infectious disease, and thus can be implemented at no additional cost.

To conclude, the evaluation of IF using HIV-1–infected L-2 cells as a source of antigen shows it to be a rapid, reliable, and less expensive test. As it was observed to be slightly more sensitive than WB after evaluating the results of discordant samples with PCR, we feel that it is well suited for use in reference laboratories for confirmation of HIV infection, replacing or complementing WB. However, its sensitivity for the diagnosis of early infection needs to be evaluated further. The IF assay reagents are simple to prepare within the capacity of most reference laboratories. The reagents are also quite stable with a shelf life of many months.

Ethical approval

Study samples were obtained from HIV-1–infected patients attending the Integrated Counseling and Testing Centre at Safdarjang Hospital, New Delhi, India. The study was approved by the local ethics committee, and all patients provided their written informed consent to participate.

Conflicts of interest

No conflict of interest to declare.

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References


Figure 3. Confirmation of the three discordant serum samples by WB and RT-PCR. The three serum samples (X, Y, and Z) were subjected to WB (A) and nested RT-PCR (B). For WB: lane 1, X serum; lane 2, Y serum; lane 3, Z serum; lane 4, reagent blank as a negative control; lane 5, Indie-C1 as a positive control.


