

# Agreement Study between Two Laboratories of Immunofluorescence as a Confirmatory Test for Human Immunodeficiency Virus Type 1 Antibody Screening

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Received 27 October 1988/Accepted 1 March 1989

**A total of 114 serum specimens from 76 blood donors, 21 patients with acquired immune deficiency syndrome or acquired immune deficiency syndrome-related complex, 7 multiply transfused patients, 3 hemophiliacs, and 7 others were tested for anti-human immunodeficiency virus type 1 (HIV-1) antibody by enzyme immunoassay (EIA) and Western blot (WB) and then blindly tested by immunofluorescence (IF), independently, in two separate laboratories. The IF technique used acetone-fixed HIV-1-infected E cells and uninfected HUT-78 cells mixed at a 1:3 ratio in one spot on a glass slide and uninfected HUT-78 cells (to assess nonspecific fluorescence) alone in a second spot. Of 114 serum specimens, 85 were repeat EIA positive, and 21 of these were WB positive. A total of 129 of 134 of the IF results (included were 20 duplicates) were identical between laboratories, for a Kappa agreement statistic of 0.93. All five IF results discordant between laboratories were EIA repeat positive and WB negative. Included in the study were eight WB-indeterminate sera, of which five blood donor serum specimens and one hemophiliac serum specimen were IF negative and two acquired immune deficiency syndrome serum specimens were IF positive. As a confirmatory test for HIV-1 antibodies, IF provided a faster alternative or supplementary test for confirming EIA results.**

Serologic screening for antibody to human immunodeficiency virus type 1 (HIV-1) has been a major responsibility of blood banks and more recently of transplantation centers in order to ensure that HIV-1 infection is not transmitted iatrogenically. Antibody to HIV has been detected by various assays, including enzyme immunoassay (EIA) with whole virus antigen (4, 28) or recombinant antigens (15, 29), Western blot (WB) (30, 32), indirect immunofluorescence (IF) (11, 13, 16, 27, 33, 34), time-resolved fluoroimmunoassay (1), radioimmunoprecipitation (26), immunoperoxidase slide test (14), neutralization test (18, 20, 22, 23), latex agglutination (19, 21), and passive particle agglutination (PPA) (35). Although second-generation EIAs have better performance characteristics (sensitivity and specificity) than earlier screening tests, the continuing problem of false-positive results necessitates the use of a highly specific confirmatory test. WB has been a very useful test for detecting EIA false-positive sera, but it is not without serious disadvantages, including a specificity problem (2, 3, 5, 6, 9, 17, 24, 25) which yields indeterminate results in most laboratories. It can also be expensive, cumbersome, and time-consuming and requires technical expertise usually found only in reference laboratories. By performing a blinded study of agreement between two laboratories, we have evaluated IF on a variety of sera with different WB patterns as a confirmatory test for detecting HIV-1 antibody. Our results show good agreement and suggest that the IF test could easily be incorporated into most diagnostic virology laboratories for use as an HIV-1 confirmatory test, replacing or complementing WB.

## MATERIALS AND METHODS

**Sera.** Sera were collected from various populations in Hamilton, Ontario, Canada, including blood donors (tested by the Hamilton Centre of the Canadian Red Cross Blood Transfusion Service during 1986); acquired immune deficiency syndrome (AIDS) and AIDS-related complex (ARC) patients, as defined by the case definition criteria of the Centers for Disease Control (7); hemophiliacs; multiply transfused patients; and seven others who were undergoing other viral serology testing. Blood donor sera which were positive by a screening EIA without repeat testing or WB confirmation were selected for the IF observer agreement study. One set of 20 repeat serum specimens selected part way through the study and consisting of 15 IF assay-WB positives and 5 IF assay-WB negatives was included with the sera to provide a sample with approximately 50% positivity for the interlaboratory observer agreement study.

**Cell lines.** The uninfected human T-cell line HUT-78 and the infected E-cell line were obtained from Jay Levy, School of Medicine, University of California, San Francisco. The E-cell line is chronically infected with HIV-1, and 95% of the cells express viral antigens detectable by IF (13). Both HUT-78 and E cells were propagated in RPMI 1640 medium containing 10% fetal bovine serum, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1% penicillin-streptomycin, 1% L-glutamine, and 0.1%  $\beta$ -mercaptoethanol. Cultures were split biweekly by diluting them 1:4 in fresh medium to maintain cells in logarithmic growth phase.

**IF.** IF was performed essentially as described by Lennette et al. (16). HUT-78 and E-cell cultures were centrifuged for 10 min at 500  $\times$  *g*, and pelleted cells were suspended in phosphate-buffered saline containing 0.25% bovine serum albumin at a concentration of 10<sup>7</sup>/ml. HUT-78 cells were

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mixed with E cells in a 3:1 ratio so that each cell smear contained uninfected negative control cells as well as positive cells. One could distinguish an autoimmune serum giving a weak 1+ fluorescence with all cells from an HIV-positive serum giving fluorescence with only 25% of the cells. Smears were prepared by placing approximately 2 µl of the mixed-cell suspension onto microscope slides and then air drying them. The dried smears were fixed in acetone at room temperature for 5 min and then stored at -20°C. Sera were diluted 1:10 in phosphate-buffered saline and heated at 56°C for 30 min to inactivate HIV-1. Diluted serum samples were incubated with cell smears for 30 min, and slides were rinsed twice with phosphate-buffered saline and stained with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (Organon Teknika, Malvern, Pa.) diluted 1:20 in phosphate-buffered saline containing 0.1% (wt/vol) amido black for 30 min. Cover slips were mounted with 10% glycerol in phosphate-buffered saline, pH 9.0.

**Interlaboratory comparison of IF testing.** Two separate laboratories, one a regional diagnostic virology laboratory (laboratory A) and the other a university research laboratory (laboratory B), were used to conduct the interlaboratory agreement study of IF for the detection of anti-HIV antibody. In the initial pilot study, a small panel of sera was tested by IF in each laboratory, with each using its own staining protocol. Laboratory B used separate smears of infected and uninfected cells, while laboratory A used combined uninfected and infected cell smears with 0.1% amido black to quench nonspecific fluorescence. After a meeting to compare staining results, it was decided that the combined use of amido black and mixed-cell smears gave clearer staining reactions, with better discrimination between a positive and a negative result. Both laboratories then used the same protocol (amido black plus mixed-cell spots) and the same commercial source of fluorescein isothiocyanate-anti-human immunoglobulin G conjugate. The criterion for a positive IF test was established as one of the following: (i) a diffuse cytoplasmic staining pattern, (ii) a focal cytoplasmic pattern, or (iii) a membrane or capping staining without nuclear staining (13). Both laboratories were cognizant of the occurrence of antilymphocyte antibodies in sera, but no specific discussion of criteria and no observer training regarding antilymphocyte staining took place. During the 5-week period of testing, seven groups of coded serum specimens were transported between laboratories and tested blindly for HIV-1 antibody by IF in each laboratory.

**EIA.** EIA screening for HIV-1 antibody was performed with the Abbott HTLV-III EIA kit (Abbott Laboratories, North Chicago, Ill.) according to the instructions of the manufacturer.

**WB.** We used the recommended Centers for Disease Control procedure for WB as described by Tsang et al. (32), and the test was performed at one center, the Provincial Public Health Laboratory. At this laboratory, WB can detect an HIV-1-positive serum diluted 1:10<sup>-6</sup> and will normally show up to 10 discrete bands. Purified HIV-1 was purchased from Organon Teknika, and 180 µg of protein was electrophoresed in a 12% polyacrylamide gel (2-mm thickness by 15 cm) by using a modified comb which makes one continuous well the full width of the gel plus a second well at one edge of the gel for molecular weight markers. Proteins were transferred to nitrocellulose, and the paper was cut into 3-mm strips. Sera were tested at a single dilution of 1:100, and anti-HIV-1 antibody was detected with biotin-conjugated goat anti-human immunoglobulin, horseradish peroxidase-labeled avidin D (Dupont-Biotech Research Laborato-

TABLE 1. Comparison of IF results from two laboratories<sup>a</sup>

Laboratory B result	No. of laboratory A results		
	Positive	Negative	Total
Positive	38	4	42
Negative	1	91	92
Total	39	95	134

<sup>a</sup> Blind testing by IF in separate laboratories as described in Materials and Methods.

ries, Rockville, Md.), and 4-chloro-1-naphthol substrate (Sigma Chemical Co., St. Louis, Mo.).

**Statistical analysis.** The Kappa agreement statistic which corrects for agreement due to chance alone was calculated by the method of Fleiss (10).

RESULTS

A total of 134 samples were tested in both laboratories, including 114 unique serum samples and 20 blinded repeat serum samples. Both laboratories agreed on the IF results for 129 of 134 serum samples, for a 96.3% Kappa agreement (Table 1). A total of 38 positives were in agreement. The 38 positives were made up of 23 original specimens and 15 specimens put in as blinded duplicate repeats, along with 5 true-negative repeats. Both laboratories identified these 38 positives correctly, yielding 100% repeatability. All five discordant serum specimens were repeatedly positive by EIA but negative by WB and were from blood donors. One was read as falsely positive by laboratory A, and four were read as falsely positive by laboratory B. All five were scored negative in both laboratories when they were repeated.

Table 2 shows the sources of the sera and the HIV-1 antibody results for the 76 blood donors, 21 AIDS-ARC patients, 3 hemophiliacs, 7 multiply transfused patients, and

TABLE 2. Source and HIV antibody results for study sera tested by EIA, WB, and IF

Clinical status	Result in:		No. in WB			
	EIA (no.)	IF	Positive	Negative	Indeterminate <sup>a</sup>	
					p24	gp41
Blood donor	+ (62)	+	1	0	0	0
		-	0	56	2	3
		- (14)	+	0	0	0
AIDS-ARC	+ (21)	+	19	0	0	2
		-	0	0	0	0
		- (0)	+	0	0	0
Hemophiliac	+ (1)	+	0	0	0	0
		-	0	0	0	1
		- (2)	+	0	0	0
Transfused	+ (0)	+	0	0	0	0
		-	0	0	0	0
		- (7)	+	0	0	0
Other	+ (1)	+	1	0	0	0
		-	0	0	0	0
		- (6)	+	0	0	0
		-	0	6	0	0

<sup>a</sup> Only a single band, either anti-p24 or anti-gp41.

TABLE 3. Comparison of WB, EIA, and IF results for eight WB-indeterminate serum specimens

Clinical status (serum specimen no.)	HIV antibody present by:			
	WB <sup>a</sup>		EIA	IF <sup>b</sup>
	anti-p24	anti-gp41		
Blood donor (1)	+	-	+	-
Blood donor (2)	+	-	+	-
Blood donor (3)	-	+	+	-
Blood donor (4)	-	+	+	-
Blood donor (5)	-	+	+	-
AIDS-ARC (6)	-	+	+	+
Hemophiliac (7)	-	+	+	-
AIDS-ARC (8)	-	+	+	+

<sup>a</sup> Indeterminate WB result with only a single band, either anti-p24 or anti-gp41.

<sup>b</sup> All IF results were the same in both laboratories.

7 other individuals undergoing other viral testing (total, 114). Of 76 blood donor serum specimens, 62 were repeat EIA positive, and one of these was positive by both WB and IF. Of the 62, 5 were indeterminate by WB, 3 had anti-gp41, and 2 others had anti-p24 only. All five of these WB-indeterminate blood donor serum specimens were negative by IF. All 21 AIDS-ARC serum specimens were positive by EIA and IF; 19 were WB positive, and 2 were indeterminate. One of three serum specimens from hemophiliacs was positive by EIA, indeterminate by WB (anti-gp41 only), and negative by IF. The other two hemophiliacs were negative by all three tests. All seven sera from multiply transfused patients with idiopathic thrombocytopenia were HIV-1 antibody negative by EIA, WB, and IF. Three of these seven serum specimens reacted with HUT-78 cells in the IF test but lacked the characteristic pattern of staining on E cells and were clearly negative.

Of special interest were eight serum specimens (five blood donors, two AIDS-ARC patients, and one hemophiliac) which were repeat EIA positive and WB indeterminate, having only anti-p24 or anti-gp41 bands (Table 3). Both laboratories agreed on the IF results for all eight serum specimens; six were negative and two were positive. Both IF-positive sera were from AIDS patients, while the IF-negative sera were from five blood donors and one hemophiliac.

## DISCUSSION

Two separate laboratories agreed on the IF results for 129 of 134 serum specimens. The Kappa agreement statistic which corrects for agreement due to chance alone was 0.93, indicating that the IF test was performed with reproducible results in the two laboratories. In a previous study in which this IF technique was used, Lennette et al. (16) reported a sensitivity and specificity of 100% compared with WB. Insertion of blinded positive and negative sera in our study demonstrated 100% repeatability, whereas repeating the five false-positive IF samples reduced the false-positive IF rate to zero.

The good agreement obtained in our separate laboratories may be attributable to two factors. Our preliminary comparison indicated that the use of separate smears of infected and uninfected cells did not easily distinguish between sera containing anti-HIV-1 antibody and sera containing antilymphocyte antibody, which led to some IF false-positive results in the latter case. The use of mixed-cell smears containing

25% infected cells greatly facilitated reading, eliminated the need for removal of antilymphocyte antibody by absorbing serum with uninfected cells, and readily indicated the presence of antilymphocyte antibody in serum. Similarly, the use of amido black counterstain seemed to quench nonspecific IF, making reading easier.

If the IF test is performed in a diagnostic laboratory with experience in IF, the false-positive rate should be less than 1% (1 of 134, or 0.74%, reduced from 4 of 134, or 2.99%, in a nondiagnostic laboratory; Table 1) on initial testing and should approach zero on repeat testing.

The IF test was rapid and inexpensive to perform and could be adapted by most diagnostic virology laboratories with experience in cell culture and IF. If a laboratory uses preprepared slides, the IF test should only take 2 h to perform, making it significantly faster than the WB test, which is usually an overnight test. If a laboratory prepares its own HIV slides, then the cost of the test will be minimal, since large numbers of slides can be prepared in bulk and stored at -20°C for later use. The actual cost per test in our laboratory, including reagents and technologist time (excluding laboratory overhead), was approximately \$2.25, which is much less than that for most commercially available WB confirmatory tests. The simplicity, low cost, quick turnaround time, and good reproducibility make the IF test an attractive alternative or supplement to WB as a confirmatory test for EIA-positive sera.

Longitudinal follow-up of HIV-1-infected individuals has revealed that anti-p24 antibody may be the first antibody detectable by WB early after infection (2, 9, 12, 26, 31) and that anti-p24 antibody may be lost from serum late in the course of infection (5, 15). Two serum specimens from AIDS patients, which normally show 10 bands by WB, showed only anti-gp41 antibody discretely. These sera were clearly positive by IF, radioimmunoprecipitation, and a synthetic-peptide EIA, demonstrating that the IF test may be useful for confirming AIDS in patients who lack anti-p24 antibody. Another six WB-indeterminate serum specimens from asymptomatic individuals (five blood donors and one hemophiliac) with only anti-p24 or only anti-gp41 antibody were negative by IF, suggesting that the IF test may be helpful in the analysis of sera which give a WB-indeterminate pattern. We have not tested any sera for AIDS patients that contained only anti-p24 antibody, but Cooper et al. have shown that immunoglobulin M class anti-p24 antibody can be detected by IF shortly after the onset of symptoms and prior to detection by either WB or EIA (8).

Recently, rapid agglutination tests for the detection of HIV-1 antibody have appeared, including a latex agglutination test which uses recombinant envelope polypeptide-coated latex beads and a PPA test which uses detergent-disrupted virion antigen-coated gelatin particles (19, 21, 35). Yoshida et al. reported excellent agreement between the PPA test and IF, with the former being slightly more sensitive (35). PPA detected immunoglobulin M antibody to HIV-1 and failed to yield any false-positive results when over 400 problem serum specimens were tested. Since latex agglutination and PPA, like IF, can be sensitive and specific, all three tests may confirm EIA results or act as supplemental tests to WB. Future well-designed comparisons are needed, since speed and cost are important factors in HIV testing.

## ACKNOWLEDGMENTS

We thank J. Levy, University of California, San Francisco, for supplying us with the HUT-78 and E-cell lines. We are indebted to

Anna Pietruszkiewicz and Peter Farrell for excellent technical assistance and to Karen Warren for secretarial assistance.

D. G. Harnish is a research scholar of the Ontario Ministry of Health. This work was supported in part by National Health and Welfare, Canada.

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