Evaluation of a new dot blot assay for confirmation of human immunodeficiency virus type 1 and 2 infections using recombinant p24, gp41, gp120 and gp36 antigens

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ABSTRACT

Objectives: A sensitive and accurate dot blot assay using recombinant p24 (gag), gp41 and gp120 (env) proteins of HIV-1 and also recombinant gp36, the specific HIV-2 antigen was developed to confirm the presence of antibodies in sera reactive in screening enzyme-linked immunosorbent assays.

Methods: We collected sera from Iranian 125 confirmed HIV positive Iranian samples (seropositive group) from AIDS patients, asymptomatic HIV-infected subjects, HIV-infected intravenous drug users and also hemophilic infected subjects. The samples were obtained from the AIDS Specimen Bank, Pasture Institute, Iran during 2002 to 2003. We also obtained 180 samples (seronegative group) from healthy blood donors. Recombinant antigens were expressed in *Escherichia coli*. By use of highly purified antigens, the dot blot procedure was developed. Analysis of the results was accomplished by capturing the dot blot images.

Results: We established and interpreted the results using Centers for Disease Control criteria. We defined the positive test result as the presence of antibody against at least 2 different HIV gene products, one of which had to be an env gene product while a negative test result was defined as no antibodies against any of the HIV gene products and an indeterminate result was defined as antibodies reacting with only one HIV env gene product or against gag gene product only.

Conclusions: The recombinant HIV dot blotting assay identified seropositive individuals with a high degree of accuracy; none of the HIV-seropositive subjects had a negative test result. Reactivity with these antigens, demonstrated 100% sensitivity and specificity in distinguishing seronegative from seropositive sera. The different sets of Western blot interpretative accepted criteria did not make a difference in interpretation of the seronegative and seropositive samples.

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The human immunodeficiency virus (HIV) is the etiologic agent of acquired immunodeficiency syndrome,[1-3] and the demonstration of an antibody response specifically directed against HIV proteins is accepted as evidence of infection.[4-6] There are many different methods available for testing for antibodies to HIV, including screening tests, which use enzyme-linked immunoassays (ELISAs), and confirmatory (supplementary) tests using immunoblot (Western Blot Assay [WBA] or Dot Blot Assay [DBA]), radioimmuno

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precipitation, and immunofluorescence assays.[7-10] Nearly all the currently licensed primary blood screening and supplemental tests use recombinant HIV antigens or HIV-infected cells as the antigen source. Approximately 0.3% of all donor blood units screened by ELISA had positive test results, but only 10% of these (namely 0.03% overall) can be verified as true-positive results after supplementary testing.[10,11] This means that 90% of initial positive test results in a low-prevalence population are false-positive results. False positive results are often due to nonspecific reactions. Recombinant HIV proteins have been generated in an attempt to provide a pure source of HIV antigen(s) free from cellular protein contamination for serological testing.[12-16] The developed in-house DBA is one of the confirmatory tests that use a panel of recombinant proteins derived from the 2 major HIV structural genes (gag and env) and HIV-2 specific antigen (gp36).[17] We have used this assay to test sera from HIV seropositive and seronegative individuals and to assess its utility as a confirmatory assay for the presence of HIV-specific antibodies. A commercial WBA (Organon Teknika Co, Belgium) was also used for comparison and interpretation of the results.

Methods. The blood serum was collected from 125 confirmed HIV positive Iranian samples from AIDS patients, asymptomatic HIV-infected subjects, HIV-infected intravenous drug users and hemophilic infected subjects (seropositive sample group). The samples were obtained from the AIDS Specimen Bank, Pasture Institute, Iran between 2002 and 2003. All samples containing HIV antibodies were verified by in-house dot blot (in which a positive was defined as antibody reactivity with env [gpl20, or gp4l] and gag [p24] and specific HIV-2 antigen [gp36] gene products) as well as by separate commercial WBA (Organon Teknika Co., Belgium). A total of 180 samples from healthy blood donors (seronegative sample group) were obtained from Iran Blood Transfusion Organization, Iran. From healthy blood donor samples, which were tested, 4 serum specimens had a positive reaction on one of the HIV screening ELISA (Vironostika, Italy) but did not react on the developed DBA. These 4 samples were classified as sera, which present rise to false-positive ELISA results. A total of 3 serum samples from patients with *Escherichia coli (E. coli)* sepsis were also provided.

Recombinant HIV antigens. Recombinant antigens were expressed in *E. coli*. Briefly, genes were cloned in pET32a+ expression vector, a derivative of the pET expression system (Novagen Co., USA).[18] Using this vector, the antigens were expressed with 6 histidines incorporated at the carboxyl terminus of the antigens for affinity purification procedures. The recombinant gp41 contains the N-terminal segment (amino acids 510–684) of the transmembrane HIV-1 glycoprotein. The *E. coli* k12 BL21 (DE3) (Novagen Co., USA) strains were transformed with the pET32a+ vector and were cultured in LB medium supplemented with ampicillin (5 μg/ml). When cells reached an optical density of 0.8 (610 nm), the expression was induced by isopropyl-beta-D-thio galactopyranoside (Fermentas, France) and incubated at 37°C for 2 hours. The cells were harvested by centrifugation. Ten grams of biomass was resuspended and homogenized in 100ml of 10 mM Tris, 1 mM ethylenediaminetetr aacetic acid (EDTA). The suspension was sonicated for 3 min and centrifugated. The recombinant gp41 protein was extracted with 100 ml of 4 M urea, 0.5 M NaCl pH 8 (buffer A), clearing the solution by centrifugation. A total of 100 ml of clear supernatant were loaded at a flow rate of 0.25 ml/min onto a fast flow Chelating Sepharose column (Amersham-Pharmacia, Sweden) equilibrated in buffer A. The absorbed proteins were eluted with an imidazole-step gradient (20–500 mM) at a flow-rate of 1 ml:min. The recombinant p24, gp21 and gp36 proteins comprise the whole sequence of the natural antigens, and were produced using procedures similar to those described for gp41.

Dot blot procedure. Recombinant antigens p24, gp41, gp120 and gp36 were diluted to optimal concentrations, in a 20mM Tris and 500mM NaCl, pH 7.2, buffer (coating buffer). A 5-μg sample of an antigen was pipetted into each dot in a vertical row of a Nitrocellulose strip (Hybond C; Amersham Pharmacia Inc., Sweden). Horizontal rows, A through D, contained antigens p24, gp41, gp120, and gp36, while row G was coated with 50 ng per dot of human immunoglobulin G (Sigma-Aldrich Inc., Germany) and row H was dotted with coating buffer only. The slots were washed once with 1 ml of 20mM Tris containing 500 mM NaCl (TBS), pH 7.2, containing 0.1% (vol/vol) Tween 20 (TBS-Tween 20) and aspirated again, after incubation overnight at 4°C. Blocking (1 g of Bovine Serum Albumin, and 0.1 ml of Tween 20 in 100 ml of TBS) was added, followed by one hour incubation at room temperature when the plates were rotated on a platform. Slot contents were aspirated, washed once with TBS-Tween 20, and aspirated again. At this point, strips could be used immediately or stored for several weeks at -20°C after being dried and sealed in plastic-lined aluminum bags containing a desiccant.

Each serum sample to be tested was diluted to 1:50 TBS-Tween 20 with sample diluent and allowed

to stand for 30 min at 20°C. Ten serum samples could be tested per plate, with the first 2 columns of the plate reserved for positive and negative control samples. Diluted sera were pipetted into each slot and incubated for 45 min at 37°C. Slots were aspirated and rinsed twice with TBS-Tween 20, and incubated with 800-μl of a 1:2,000 dilution of alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma-Aldrich, Inc., Germany). Slots were aspirated and rinsed twice with TBS-Tween 20 and incubated with 5-bromo 4-choloro 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma-Aldrich, Inc., Germany) for 10 min at 37°C in the dark chamber. Membranes were washed in water and air-dried.

Analysis of the results was accomplished by capturing the strip images, measuring the reflectance density (DR) of anti-p24, -gp41, -gp120 and –gp36 antigen bands, and calculating the relative intensity (RI) by a video densitometer (Amersham-Pharmacia, Sweden). The DR was calculated with the formula:

$$DR = \frac{DR\ of\ Test\ Sample - DR\ of\ Negative\ Control}{DR\ of\ Positive\ Control - DR\ of\ Negative\ Control}$$

The negative control sera were used to determine cut-off values for the RI of each antigen band. The cut-off values were set to minimize false-positive results and were at least 3 standard deviations above the means of the readings obtained with the negative control sera. The absorbance of each of the antigens for the positive as well as negative control sera had to be 2 standard deviations of the mean before the run was considered valid. To assess reproducibility, intra-assay (Positive control sera with a strong reacting for all HIV proteins in eight replicates) and inter-assay (Ten serum samples examined eight times) analysis were performed. Mean RI of each band and corresponding coefficient of variability (CV) was calculated.[19]

A positive test result was defined as the presence of antibody against at least 2 different HIV gene products, one of which had to be an env gene product. A negative test result was defined as no antibodies against any of the HIV gene products. An indeterminate result was defined as antibodies reacting with only one HIV env gene product or against gag gene product only.[19,20] Positive results were established by 3 different criteria: (i) the criteria established by Centers for Disease Control (CDC), (ii) the criteria articulated by the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) Third Consensus Conference on HIV Testing, and (iii) the criteria suggested by the Consortium for Retrovirus Serology Standardization (CRSS). A negative result had no reactivity with any HIV protein, and an indeterminate result had bands observed which did not meet the criteria necessary for a positive interpretation.[21]

Results. A total of 180 seronegative samples were analyzed. These samples were obtained from healthy blood donors including 4 persons whose sera were presented as false-positive HIV screening from ELISA results; overall, none of these seronegative samples were positive; however 97.8% have negative results, and 2.2% have indeterminate results (Table 1). The indeterminate group primarily had reactivity against p24 gag and none of these samples reacted with gp4l or gp120 env gene product. Eighteen sera with other abnormalities (4 with elevated bilirubin, 10 hemolyzed specimens, and 4 containing hepatitis B surface antigen) have negative results when tested. Sera from 3 patients with *E. coli* sepsis were also tested. None of the 21 sera from patients with the above conditions reacted with any of the recombinant antigens. Sera from 125 HIV-infected subjects at different clinical stages of disease were tested (**Table 1**). Overall, 97.6% positive, 0% negative, and 2.4% indeterminate results. All of these samples reacted with the p24 gag gene product. The samples giving indeterminate result (3 patients) failed to react with an env gene product (gp41 or gp120). The distribution of reactivity against the HIV recombinant gene products as compared with those of the WBA is shown in **Table 2**. The majority of the seronegative samples had no reactivity in the recombinant HIV DBA and therefore would be classified as negative. None of the seronegative samples reacted with 2 or more HIV gene products to be interpreted as positive. Virtually, all samples from asymptomatic HIV-infected subjects were positive in the recombinant HIV DBA, and were samples from patients with AIDS (**Table 2**). There were no negative test results among the HIV-infected subjects. Indeterminate results correlated directly with the severity of clinical disease. A commercial WBA was performed (Organon Teknika, Belgium). Proteins corresponding to the different gene products are as described above. All reactivities against 2 or more HIV-gene products included reactivity against at least one env gene product. A direct comparison of HIV reactivity with the subset of sera tested by both commercial WBA and in-house developed DBA is shown in **Table 3**. Although the data from a smaller number of samples are presented, the overall performance of DBA versus WBA was not significantly different than that presented in **Table 2**.

Statistical analysis. A comparison of sensitivity and specificity between the recombinant HIV DBA

Table 1 - Sensitivity and specificity of the recombinant in-house HIV dot blot assay.

Sample Group	Number of Specimens with indicated results		
	Positive	**Negative**	**Indeterminate**
Seronegative Subjects (n=180)			
Healthy blood donors (n=176)	0	176	0
False Positive by ELISA† (n=4)	0	0	4
Other Clinical Conditions† (n=21)	0	21	0
Total (%)	**0 (0)**	**176 (97.8)**	**4 (2.2)**
Seropositive Subjects† (n=125)			
AIDS Patients (n=32)	32	0	0
Asymptomatic HIV Infected Subjects (n=28)	28	0	0
HIV-Infected Intravenous drug users (n=48)	47	0	1
Hemophilic infected subjects (n=17)	15	0	2
Total (%)	**122 (97.6)**	**0 (0)**	**3 (2.4)**

†Confirmed by reference western blot assay (Abbot Laboratories, USA) and Commercial RT-polymerase chain reaction assay (Roche Molecular Diag, France).
ELISA - enzyme-linked immunosorbent assay, AIDS - acquired immune deficiency syndrome

Table 2 - Sera reacting with different HIV recombinant proteins: Comparison of the recombinant in-house dot blot assay with commercial western blot assay.

Sample group	Number of specimens reacting with the indicated no. of HIV recombinant proteins by in-house DBA with Commercial WBA					
	1 of 3		**2 of 3**		**3 of 3**	
	DBA	**WBA**	**DBA**	**WBA**	**DBA**	**WBA**
Seronegative subjects (n=180)	4	6	0	1	0	0
Seropositive subjects† (n=125)						
AIDS Patients	30	28	32	32	32	32
Asymptomatic HIV infected subjects	27	26	28	28	28	28
HIV-infected intravenous drug users	41	40	48	43	48	45
Hemophilic infected subjects	14	15	16	15	17	15

† Western blot assay (WBA) was performed using manufacturer criteria and in-house dot blot assay (DBA) were performed using Centers for Disease Control criteria. Reactivity was determined as described in the Methods sections.

Table 3 - Comparison of the recombinant in-house dot blot assay results with results of commercial western blot assay as interpreted by various criteria.

Sample group	Results (%) of tests as interpreted by indicated criteria†			
	Dot blot	**Western Blot**		
		WBA	**DBA**	**WBA**
Seronegative	0 pos 2.2 ind 97.8 neg	0 pos 2.8 ind 97.2 neg	0 pos 2.8 ind 97.2 neg	0 pos 2.8 ind 97.2 neg
Seropositive	97.6 pos 2.4 ind 0 neg	96.6 pos 3.4 ind 0 neg	96.8 pos 3.2 ind 0 neg	96.8 pos 3.2 ind 0 neg

†DBA - dot blot assay, pos - positive, ind - indeterminate, neg - negative.

and commercially WBA as interpreted by criteria established by CDC, ASTPHLD, and CRSS is shown in **Table 3**. The results of the recombinant HIV DBA and commercial WBA had statistical correlation and significance when compared and calculated by the SPSS version 13 software. The recombinant HIV DBA had fewer indeterminate results and more negative results than WBA. The 3 different sets of WBA interpretative criteria did not make a difference in interpretation of the seronegative samples. None of the seronegative samples were positive when interpreted by any set of criteria.

None of the seropositive samples was negative in either the recombinant HIV Dot blot or the commercial WBA. The recombinant HIV DBA had more positive results and fewer indeterminate results compared with the WBA, as interpreted by CDC criteria (**Table 3**). When the WBA data were interpreted by the ASTPHLD or the CRSS criteria, the seropositive samples presented results comparable with the results of the recombinant HIV DBA (**Table 3**).

Discussion. Confirmatory assays for HIV antibodies (WBA, radioimmunoprecipitation, and immunofluorescence assay), in contrast to screening assays, are technically difficult to perform, require subjective interpretation, are impossible to automate for large-volume screening, and are not sufficiently standardized to yield reproducible results. Desirable features in a confirmatory assay include a high degree of sensitivity, specificity, reproducibility, and the potential for automation. The recombinant HIV DBA tested in this study fulfills all the above criteria. It was highly sensitive and specific for detecting antibodies to HIV, it yielded objective and reproducible results, and it had fewer indeterminate results than did commercial WBA (Organon Teknika Corp, Belgium). One recombinant antigen in particular, gp41, was 100% accurate in distinguishing between seropositive and seronegative individuals. In theory, reactivity with this antigen alone could discriminate an affirmative positive from a false-positive reaction in screening ELISA for HIV antibodies. A similar observation was made by Burke et al,[22] who used a single molecularly cloned and expressed HIV env gene product to test sera, which have positive results in the screening ELISA. However, our criteria requiring reactivity against more than one HIV gene product (of which one must be against an env gene product) would virtually eliminate false-positive results. The other HIV env gene products, gpl20, were not as sensitive in detecting seropositive samples. However, since completion of this study, we tested one serum sample, which reacted with gpl20 and not with gp4l (this pattern of reactivity was verified by Reference WBA, Abbot Laboratories), warranting the continued inclusion of this antigen in the panel. An increasing number of indeterminate results were observed with increasing severity of HIV-related disease. These results were called indeterminate in view of lack of reactivity with more than one HIV gene product; all samples reacted with at least one HIV env gene product (gp4l), but a significant proportion did not react with an additional gag-derived protein (p24). This increased the frequency of indeterminate results concomitant with the severity of clinical disease is most likely attributable to the well-described phenomenon of declining titers of antibodies to HIV gag antigens with progression of disease.[7,24-26]

In this study, the recombinant HIV DBA showed fewer indeterminate results when compared with the commercial WBA, which was interpreted by CDC criteria. This is most likely explained by the stringent criteria necessary to interpret a WBA test as positive as required by the manufacturer (Organon Teknika Corp, Belgium). When less stringent criteria (those stated by ASTPHLD or the CRSS) were used for interpretation, the recombinant HIV DBA was comparable in sensitivity to commercial WBA. Less than 2.2% of samples from healthy blood donors reacted with any individual HIV antigen; however, none of them reacted with 2 or more HIV gene products. Most of these reactions were against gag gene products; only samples from one healthy blood donors reacted with gpl20, and none reacted with gp4l. Isolated reactivity with gag proteins alone, as determined by WBA, has been observed in healthy blood donors.[14,20,27-29] These gag-reactive antibodies are often a source of false-positive screening ELISA results and the indeterminate commercial WBA, and their significance is unclear. Alternatively, antibodies reactive to gag only could be the first evidence of seroconversion after an individual have been exposed to and infected with HIV.[15,16,30-32] Only repeated serological testing of these patients will determine whether the observed reactivity with individual HIV antigens is due to background cross-reactivity to unrelated antigens or represents an early antibody response to HIV infection.

In summary, we found the recombinant HIV DBA to be highly accurate in distinguishing HIV seropositive from seronegative individuals. This assay, when used as a confirmatory (supplemental) test, had fewer indeterminate results, resolved a significant proportion of the indeterminate results obtained by conventional WBA, and was able to verify more positive HIV screening ELISA results than conventional WBA (interpreted by CDC criteria). Other potential uses for this test include longitudinal

studies of seropositive individuals to quantitative and correlative levels of antibodies to HIV in relation to disease progression or antiretroviral therapy.

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