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Research paper

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An Accurate Confirmation of Human Immunodeficiency Virus Type 1 (HIV-1) and 2 (HIV-2) Infections with a Dot blot assay Using Recombinant p24, gp41, gp120 and gp36 Antigens

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Abstract

An immunoblot assay using four recombinant proteins corresponding to human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) gene products was developed to confirm the presence of antibodies to HIV-1 and 2 in sera reactive in screening ELISAs. Serum samples for testing were obtained from healthy seronegative blood donors and from different categories of HIV-infected individuals (asymptomatic HIV-infected, and AIDS). A positive reaction was defined as reactivity against gag (p24) and at least one other env (either gp41 or gp120) HIV gene products; negative result was defined as no reaction with any antigen; and indeterminate result was defined as reactivity with gag (p24) or with env (gp41 or gp120) alone. None of the 180 serum samples from healthy seronegative blood donors gave a positive result, and only 4 of these samples (2.2%) gave indeterminate results. The recombinant HIV Dot blotting assay identified seropositive individuals with a high degree of accuracy; none of the 125 HIV-seropositive subjects had a negative test result. Reactivity with these antigens, demonstrated 100% sensitivity and specificity in distinguishing seronegative from seropositive sera. All seronegative and seropositive samples were tested both with the commercially available ELISA and by Western blot. The recombinant in-house HIV Dot blot assay accurately identified more seropositive and seronegative samples and had fewer indeterminate results than did commercial Western blot (as interpreted by CDC criteria).

Key words

Human Immunodeficiency Virus, Recombinant p24, gp41, gp120 and gp36 Proteins, Dot Blot assay

Author biography

Mehrdad Ravanshad: The research presented in this article is performed for fulfilling part of the requirement for degree of PhD in virology. His main specialty is protein expression and purification and large scale production of proteins used in Elisa and western blots, as well as setting up and optimization of Elisa, Dot and western blots for HSV and HIV viruses.
Farzaneh Sabahi is an associate professor of virology. Her research interest involves development of new or molecular techniques for rapid viral diagnosis and viral immunology. She has worked on measles vaccine immunology and at present is working on blood born viruses such as HIV, CMV and a number of hepatitis viruses such as HBV, HCV and hepatitis delta virus. She obtained M.Sc and PhD in Microbiology/Immunology from Rutgers University and the University of Medicine and Dentistry of New Jersey (UMDNJ) USA, where she was a research associate and faculty member for several years.

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1. INTRODUCTION

The human immunodeficiency virus (HIV) is the etiologic agent of acquired immunodeficiency syndrome (AIDS) [3, 21, 29, 34], and demonstration of an antibody response specifically directed against HIV proteins is accepted as evidence of infection [9, 34]. There are many different methods available for testing for antibodies to HIV, including screening tests, which use enzyme-linked immunoassays (ELISAs), confirmatory (supplementary) tests using immunoblot (Western or Dot blot), radioimmunoprecipitation, and immunofluorescence assays [6, 13, 16, 20, 28, 32, 43]. Nearly all of the currently licensed primary blood screening and supplemental tests use recombinant HIV antigens or HIV-infected cells as the antigen source. About 0.3% of all donor blood units screened by ELISA give positive test results, but only 10% of these (i.e., 0.03% overall) can be verified as true-positive results after supplementary testing [17]. 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. Synthetic peptides and 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 [5, 11, 18, 37-39, 42]. However, these studies used recombinant antigens representative of only one HIV gene products, and in all but one study [5], only small numbers of patient sera were analyzed [11, 18, 37-39, 42]. The developed in-house dot blot assay is one of the first confirmatory tests to use a panel of recombinant proteins derived from the two major HIV structural genes (gag and env) and HIV-2 specific antigen (gp36). We have used this assay to test sera from HIV-seropositive and –seronegative individuals to assess its utility as a supplementary test for the presence of HIV-Specific antibodies. A commercial western blot assay (Organon Teknika co, Belgium) was also used for comparison and interpretation of the results.

2. MATERIALS AND METHODS

Serum specimens. Serum specimens from AIDS patients and asymptomatic HIV-infected subjects, and HIV-infected intravenous drug users and also hemophilic infected subjects were obtained from Pasture Institute of Iran, AIDS specimen bank. All samples containing HIV antibodies were verified by two separate EIAs (HIV Rec, Abbot Laborotories; and Vironostika, Belgium) as well as by in-house dot blot (in which a positive was defined as antibody reactivity with env [gpl20, or gp4l] and gag [p24] and/or specific HIV-2 antigen [gp36] gene products). A total of 180 serum samples from healthy blood donors were obtained from Iran Blood Transfusion Organization. From healthy blood donor samples which being tested, 4 serum specimens gave a positive reaction on one of the HIV screening ELISA but did not react on developed dot blot assay. These 4 samples were classified as sera, which gave, rise to false-positive ELISA results. A total of 18 specimens with other abnormalities (four with elevated bilirubin, ten hemolyzed specimens, four containing hepatitis B surface antigen and three from patients with E.cloi sepsis) were also provided.

Recombinant HIV antigens. HIV gag, and env coding regions were inserted in the Escherichia coli pET32a+ expression vector, a derivative of the pET plasmids [35]. In each construct, a translational fusion was created between the HIV coding segment and the 5' end of the galactokinase sequence present on the expression vector. The construction, production, and purification of these antigens are described in detail elsewhere [M.Ravanashd, F.Mahboodi, F.Sabahi and M.H.Roostaee, Journal of biotechnology, submitted for publication].

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-µl 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 the antigens p24, gp41, gp120, and gp36, respectively, 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. After incubation over night at 4°C, the slots were washed once with 1 ml of 20mM Tris, 500 mM NaCl (TBS), pH 7.2, containing 0.1% (vol/vol) Tween 20 (TBS-Tween 20) and aspirated again. Blocking (1 g of Bovine Serum Albumin, and 0.1 ml of Tween 20 in 100 ml of TBS) was added, followed by a 1-h incubation at room temperature during which 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 1:50 with sample diluent (TBS-Tween 20) and allowed to stand for 30 min at 20°C. Ten serum samples could be tested per plate, with the first two 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. The reaction was stopped with washing the nitrocellulose strips with water. The results were determined with densitometer (Amersham-Pharmacia, Sweden).

Net absorbance of antigens for a given sample were calculated by subtracting absorbance of the individual blank (Dot H) from absorbance of each antigen (Dot A to G). Net absorbance of each of the antigen-coated dots was proportional to the amount of antibody bound to the dot. All results and calculations are based on net absorbance.

Reference ranges were determined by testing 180 healthy blood donor sera, which were non reactive by standard HIV ELISA, in the recombinant HIV Dot blotting. Antibody against any antigen was defined as being present if the dots were visible.

Interpretation, A positive test result was defined as the presence of antibody against at least two 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.

Positive results were established by three different criteria: (i) the criteria established by 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)[8]. 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.

3. RESULTS

A total of 180 seronegative samples were analyzed. These samples were obtained from healthy blood donors, 4 persons whose sera gave false-positive HIV screening ELISA results. Overall, none of these Seronegative samples gave a positive result; 97.8% gave a negative result, and 2.2% gave an indeterminate result (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 (four with elevated bilirubin, ten hemolyzed specimens, and four containing hepatitis B surface antigen) gave negative results when tested. Sera from three patients with E. coli sepsis were also tested. None of the sera from patients with E. coli sepsis 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% gave positive results, 0% gave a negative result, and 2.4% gave indeterminate results. All of these samples reacted with the p24 gag gene product. The samples giving indeterminate result, from 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 Western blot is shown in Table 2. The majority of the seronegative samples had no reactivity in the recombinant HIV dot blot assay and therefore would have been classified as negative. None of the seronegative samples reacted with two or more HIV gene products to give a positive test result.

Virtually all samples from asymptomatic HIV-infected subjects were positive in the recombinant HIV Dot blot assay, as were of samples from patients with AIDS (Table 2).

There were no negative test results among the HIV-infected subjects.

A commercial Western blot assay (WBA) was performed (Organon Teknika, Belgium). Proteins corresponding to the different gene products are as described in Materials and Methods. All reactivity against two 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 Western blot and in-house developed Dot blot assay is shown in Table 3. Although the data from a smaller number of samples are presented, the overall performance of Dot blot assay versus Western blot was not significantly different than that presented in Table 2.

Statistical Analysis. A comparison of sensitivity and specificity between the recombinant HIV Dot blot assay and commercial Western blot as interpreted by criteria established by CDC, ASTPHLD [2], and CRSS [8] is shown in Table 3. The recombinant HIV Dot blot assay had fewer indeterminate results and more negative results than Western blot. The three different sets of Western blot interpretative criteria did not make a difference in interpretation of the seronegative samples. None of the seronegative samples was 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 Western blot assay. The recombinant HIV Dot blot assay had more positive results and fewer indeterminate results than Western blot, as interpreted by CDC criteria (Table 3). When the Western blot data were interpreted by the ASTPHLD or the CRSS criteria, the seropositive samples gave results comparable with the results of the recombinant HIV DOT blot assay (Table 3).

4. DISCUSSION

Confirmatory assays for HIV antibodies (Western blot, 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 dot blot assay tested in this study fulfills all of the above criteria. It was found to be highly sensitive and specific for detecting antibodies to HIV, it yielded objective and reproducible results, and it had fewer indeterminate results than did commercial Western blot (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 a true positive from a false-positive reaction in a screening ELISA for HIV antibodies. In fact, a similar observation was made by Burke and co-workers [5], who used a single molecularly cloned and expressed HIV env gene product to test sera, which gave positive results in the screening ELISAs.

However, the criteria used in this study, which is based on CDC and WHO 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 have tested one serum sample, which reacted with gpl20 and not with gp4l (this pattern of reactivity was verified by Reference Western blot assay, 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 because of a lack of reactivity with more than one HIV gene product; in other words, 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 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 [1, 12, 15, 23, 25, 37, 38].

In this study, the recombinant HIV Dot blot assay showed fewer indeterminate results when compared with commercial Western blot, which was interpreted by CDC criteria. This is most likely explained by the stringent criteria necessary to interpret a Western blot 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 dot blot assay was comparable in sensitivity to commercial Western blot.

Less than 2.2% of samples from healthy blood donors reacted with any individual HIV antigen; however, none of them reacted with two 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 Western blot, has been

observed in healthy blood donors [4, 10, 19, 31, 40, 41]. These gag-reactive antibodies are often a source of false-positive screening ELISA results and indeterminate commercial Western blot results, and their significance is unclear. Alternatively, antibodies reactive to gag only could be the first evidence of seroconversion after an individual has been exposed to and infected with HIV [9, 30]. 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 Dot blot assay 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 Western blot, and was able to verify more positive HIV screening ELISA results than conventional Western blot (interpreted by CDC criteria). Other potential uses for this test include longitudinal studies of seropositive individuals to quantitate and correlate levels of antibodies to HIV in relation to disease progression or antiretroviral therapy.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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Tables

Table 1. Sensitivity and Specificity of the Recombinant in-house HIV Dot Blot assay.

Sample Group	No of Specimens with Indicated Results		
	Positive	**Negative**	**Indeterminate**
Seronegative Subjects (180)			
Healthy blood donors (176)	0	176	0
False Positive by ELISA[†] (4)	0	0	4
Other Clinical Conditions[†] (21)	0	21	0
Total	**0 (0%)**	**176 (97.8%)**	**4 (2.2%)**
Seropositive Subjects[†] (125)			
AIDS Patients (32)	32	0	0
Asymptomatic HIV Infected Subjects (28)	28	0	0
HIV-Infected Intravenous drug users (48)	47	0	1
Hemophilic infected subjects (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-PCR Assay (Roche Molecular Diag., France).

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	No of Specimens reacting with the indicated HIV recombinant proteins by in-house Dot Blot or Commercial Western Blot[†]					
	P24/gp41/gp120		**P24 and gp41/120**		**P24 and gp41 and gp120**	
	DBA	**WBA**	**DBA**	**WBA**	**DBA**	**WBA**
Seronegative Subjects (180)	4	6	0	1	0	0
Seropositive Subjects (125)						
AIDS Patients (32)	30	28	32	32	32	32
Asymptomatic HIV Infected (28)	27	26	28	28	28	28
Infected Intravenous drug users (48)	41	40	48	43	48	45
Hemophilic infected subjects (17)	14	15	16	15	17	15

[†] Western Blot Assay (WBA) was performed using manufacturer criteria and in-house Dot Blot Assay (DBA) was performed using CDC Criteria. Proteins are as described in Material and Methods.

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 (No. Of Specimens)	Results (%) of test as interpreted by indicated criteria†			
	Dot Blot	Western Blot		
		CDC	ASTPHLD	CRSS
Seronegative	0 pos.	0 pos.	0 pos.	0 pos.
	2.2 ind.	2.8 ind.	2.8 ind.	2.8 ind.
	97.8 neg.	97.2 neg.	97.2 neg.	97.2 neg.
Seropositive	97.6 pos.	96.6 pos.	96.8 pos.	96.8 pos.
	2.4 ind.	3.4 ind.	3.2 ind.	3.2 ind.
	0 neg.	0 neg.	0 neg.	0 neg.

† DBA, Dot Blot Assay; pos, Positive; ind, Indeterminate; neg, Negative.

Author biography (continued from front page)

Mohammad Hassan Roustai is a full professor of virology with main area of interest in DNA vaccine of HSV1 and 2. He has expertise in both classical as well as new molecular techniques and worked with a number of viruses in humans or animals. At present, he is spending his Post-doctorate training in Sherbrook University. He is a graduate of Virology Department of Tehran Medical University.

Fereidoun Mahboudi is a biotechnologist and the head of Iranian network of Biotechnology and Biotechnology Research Center at Pasteur Institute of Iran. He is an associate professor and his main area of interest is HIV and AIDs. He is a graduate of Pathology Department of University of Medicine and Dentistry of New Jersey.

Ramin Sarrami Forooshani is the supervisor of HIV laboratory at Pasteur institute of Iran and is working with Dr Mahboudi. He has MS degree and has had a number of long term training courses in both Pasteur institute of Iran and International center for genetic engineering and biotechnology in India. He is accepted for continuing his PhD degree.

Anooshirvan Kazemnejad is an associate professor in Biostatistics cooperating with the projects in the virology department of Tarbiat Modarres