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Quantification Analysis of Dot Blot Assays for Human Immunodeficiency Virus Type 1 and 2 Antibodies

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Abstract

Objective

Dot Blot (DB) assay provides highly specific results, but usually is not reliable for quantification of antibody production. The need for a more objective DB assay to provide a better definition of the immune status, against HIV antigens, promoted this study to develop a quantitative DB assay.

Materials and Methods

Dot blot (DB) strips for antibodies, directed to human immunodeficiency virus (HIV) type 1 and 2, were analyzed by a video densitometer. This method was used to quantify the antibody response to different HIV proteins in infected patients. In order to increase reproducibility, reagents and protocols were accurately standardized and internal controls were added. In the first format, an internal control band consisting of Human IgG was added to each dot to minimize the effects of band intensity variation. In the second format, antibody concentrations were calculated from the ratio of the densities produced by test sera and by positive and negative standard sera.

Results

The sera under scrutiny were also examined by standard enzyme-linked immunosorbent assay (ELISA) and the obtained results were compared with those of the corresponding DB. A statistically significant positive correlation was found between the results obtained with the two methods, and this was especially evident when ELISA titers were compared to corrected DB values ($p = 0.001$).

Conclusion

Densitometric analysis of DB assays led to quantify the antibodies against HIV-1 and 2 Gag and Env proteins and might be useful to investigate possible humoral immune correlates of production in HIV vaccine studies and antibody production in the early phase of infection.

Keywords: Densitometric Analysis, Dot Blot assay, Human Immunodeficiency Virus.

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Introduction

The human immunodeficiency virus (HIV) type 1 and 2 are the etiologic agents of acquired immunodeficiency syndrome, and demonstration of an antibody response specifically directed against HIV proteins is accepted as the evidence of infection (1, 2). Serological studies have shown that, following infection, patients develop antibodies to both nucleocapsid proteins and the envelope glycoproteins, but the qualitative and quantitative differences in the immune response occur between individuals. The humoral immune response of HIV-infected patients had been extensively investigated (3, 4).

Antibodies to individual HIV-1 and 2 proteins can be detected by dot blot (DB) and radioimmunoprecipitation assay (RIPA) using native HIV-1 and 2 proteins and enzyme-linked immunosorbent assays (ELISAs) using recombinant HIV Gag and Env proteins (3, 5, 6). The development of these sensitive and specific ELISA methods based on recombinant proteins allows the investigation of the production to individual HIV-1 and 2 antigens (7), but such studies are very time- and labor-demanding. DB and RIPA provide highly specific results, but they are usually not reliable for quantification of antibody production (8, 9).

The need for a more objective DB assay to provide a better definition of the immune status against HIV antigens promoted us to develop a quantitative DB assay (8). This report describes two DB assays that enable us to determine the antibody response against single HIV antigens by using a computer assisted densitometer for image analysis. The obtained results using these quantitative DB assays were also compared with the results obtained by a standard ELISA.

Materials and Methods

Human sera: The study was conducted on sera from Iranian collection of 110 confirmed HIV-1 positive samples from AIDS patients; asymptomatic HIV-infected subjects, HIV-infected intravenous drug users and also hemophilic infected subjects collected in 2002 - 2003, as well as 15 HIV-2 positive samples were obtained from Pasture Institute of Iran, AIDS specimen bank. Local Ethics Committee approval was taken in the session held on 27 Oct. 2007. A total of 180 samples from healthy blood donors were obtained from Iran Blood Transfusion Organization. A total of three serum samples from patients with *Escherichia. coli* sepsis were also provided (Prof. Alborzi Research Center, Namazee Hospital, Shiraz, Iran).

Recombinant HIV antigens: Recombinant antigens were expressed in *E.coli* (10). Briefly, genes were cloned in pET32a+ expression vector, a derivative of the pET expression system (Novagen Co, USA). Using this vector, the antigens were expressed with six 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 transmembranic HIV-1 glycoprotein. The *E.coli* k12 BL21 (DE3) (Novagen Co, USA) strains were transformed with the pET32a+ vector and cultured in LB medium supplemented with ampicillin (5 µg/ml). When cells reached an optical density of 0.8 (610 nm), the expression were induced by IPTG (Fermentas, France) and incubated at 37°C for 2 h. The cells were harvested by centrifugation. 10 grams of biomass was resuspended and homogenized in 100 ml of 10 mM Tris, 1 mM 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 was 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, gp120 and gp36 proteins comprise the whole sequence of the respective 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 20 mM Tris and 500 mM NaCl, pH 7.2, buffer (coating buffer).

A 5-µg sample of an antigen was pipetted into each dot in a Horizontal row of a nitrocellulose strip (hybond C; Amersham Pharmacia Inc., Sweden).

Vertical rows, A to 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 strips were washed once with 1 ml of 20 mM Tris containing 500 mM NaCl (TBS), pH 7.2, containing 0.1% (vol/vol) Tween 20 (TBS-Tween 20).

Blocking (1 g of Bovine Serum Albumin and 0.1 ml of Tween 20 in 100 ml of TBS) was added, followed by 1-h incubation at room temperature during which the plates were rotated on a platform.

The strips were washed once with TBS-Tween 20. 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 before being tested was diluted 1:100 with sample diluent (TBS-Tween 20) and allowed to stand for 30 min at 20°C. In each plate ten serum samples could be tested, with the first two columns of the plate reserved for positive and negative control samples. Diluted sera were pipetted onto each strip and incubated for 45 min at 37°C.

Strips were 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). Strips were 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 dots, and calculating the relative intensity (RI) by a video desitometer (Amersham-Pharmacia, Sweden).

To increase reproducibility of measurements and score dot intensity two different assays with internal controls were developed and performed every time the sera were tested.

In the first format, DB A, purified human IgG (Sigma-Aldrich, Inc.,Germany) were inserted in each strip and used as internal control to account for inter-assay variability. A serial dilution on a scale 1:2 was made from concentrated solution of human IgG (250 µg/1ml Tris buffer). From each dilution, 10 µl were withdrawn and dotted on the membrane.

The RI of the single antigen dots was hence quantitated by comparing the DR of each dot with the DR of the internal IgG control on each strip after substraction of the RI detected using negative control human serum. In the second format, DB B, the reproducibility of measurement was increased by using the ratio of antibody concentrations as determined by including positive and negative controls in each

experiment. The DR was calculated with the formula:
(*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 dot. The cut-off values were quantified by adding three times the standard deviation to the means of the readings obtained with the negative control sera.

Standard ELISA for anti-HIV

The antibody response to HIV was also determined by Biotest ELISA based on HIV1 p24, HIV1 gp41, HIV1 gp120 and HIV2 gp36 recombinant proteins (Germany).

The antibody titer was expressed as the reciprocal of the highest dilution of serum that gave optical density readings higher than the average values obtained with 110 Control HIV-1 positive serum samples and 15 HIV-2 positive plus three times the standard deviation (11, 12).

Statistical analysis

To access reproducibility, intra-assay (one serum with strong reactivity for all HIV-1 and HIV-2 proteins in eight replicate) and inter-assay (three serum samples examined eight times) analysis were performed. Mean RI of each dot and corresponding coefficient of variability (CV) were calculated. The correlation between data obtained using the standard ELISA and the different DB assays was analyzed by regression ANOVA and the Pearson correlation coefficient using SPSS 10.0 (SPSS, Bologna, Italy).

Results

To determine optimal DB conditions, different amounts of antigen and different dilutions of serum samples and of the conjugate were tested to obtain the highest signal-to-noise ratios for positive and negative sera, respectively (results not shown). The optimal amount of antigen for preparing the strips was found to be 5 ng per dot. Optimal signal-to-noise were found with sera diluted 1:100 and conjugate diluted 1:2000. In the examination of the different dilutions of Human IgG used as internal control, a linear relationship between DR of bands and human IgG dilution was observed ($r= 0.869$; $p= 0.05$). The optimal amount of human IgG for quantifying the single dots of anti-HIV antibodies was found to be 0.04 µg per strip.

Serum samples of infected patients were examined with both DB formats. DB A used human IgG as internal control, DB B was developed together with positive and negative control sera to determine ratio of antibody concentrations. With both formats, the highest intensities were detected against the gag product p24. Other HIV proteins consistently detected were, although with a lower RI, the *env* products gp41 and gp120 and the HIV-2 specific antigen gp36.

Cut-off values of RI obtained for each dot corresponding to HIV antigens are presented in Table 1. Using DB A, the cut-off values for each HIV protein were lower than those observed with DB B. Table 2 shows the relationship between the RI obtained with the two DB assays for the different dots corresponding to individual HIV protein and the results of a standard ELISA. A strong correlation was found between the results obtained with the two different DB assays for the HIV antigens.

Table 1. Cut-off values[1] of relative intensity obtained for each antigen dot using the two dot blot formats developed[2].

HIV Proteins	DB A	DB B
p24	0.06	0.20
gp41	0.07	0.40
gp120	0.05	0.31
gp36	0.05	0.50

[1]Mean of negative control sera plus three standard deviations.
[2]DB A: Dot blot A with internal human IgG standard; DB B in which the relative intensity (RI) of bands was calculated using the ratio of the RIs produced by test sera and by positive and negative standard sera.

The measure of antibody production against individual HIV antigens obtained with both DB formats was also statistically correlated with the antibody titers determined using a standard ELISA.

Table 2. Correlation between the results obtained with the two Dot Blot (DB) formats and between the relative intensities obtained with the two DB and anti-HIV titer determined with a standard ELISA.

HIV Proteins	DB A vs. DB B		DB A vs. ELISA		DB B vs. ELISA	
	r	*p*	*r*	*p*	*r*	*P*
p24	0.856	< 0.00001	0.464	0.001	0.778	< 0.0001
gp41	0.969	< 0.00001	0.714	<0.0001	0.738	< 0.0001
gp120	0.924	< 0.00001	0.759	< 0.0001	0.707	< 0.0001
gp36	0.874	< 0.00001	0.734	< 0.0001	0.685	< 0.0001

To further evaluate the reproducibility of quantitation of HIV antibodies, the RI obtained using both strips from the same blot or antigen bound to different blots were compared in intra- and inter-assay tests. The results are presented in Table 3. Intra-assay CV ranged from 2.4% for gp41 and gp120 to 3.4% for p24, while inter-assay CV ranged from 2.3% for p24 to 7.8% for gp36.

Table 3. Intra- and inter-assay reproducibility of dot blot tests developed.

HIV	Intra-assay[1]		Inter-assay[2]	
Proteins	Mean relative intensity[3]	CV[3] (%)	Mean relative intensity[3]	CV[3] (%)
p24	169.6	2.7	170.8	2.3
gp41	58.3	2.4	65.4	5.2
gp120	60.9	3.4	65.0	7.8
gp36	40.8	2.4	44.4	5.1
Human IgG	123.9	3.2	127.0	4.0

[1] Intra-assay reproducibility was determined using one serum with a strong reactivity for all HIV proteins in eight replicate.
[2] Inter-assay reproducibility was calculated with three serum samples examined eight times.
[3] Mean relative intensities of each band and corresponding coefficient of variability (CV) were calculated.

Discussion

Two quantitative DB assays, which were read using a video camera based densitometer connected to a personal computer for quantitative image analysis, are described. Taking into account that automated quantification of band intensities gives objective results, the presence of a low cut-off allowed avoiding the misclassification of dots.

A statistically significant concordance was found when the results obtained with the two different DB assays were compared: the Pearson correlation coefficient estimated for each pair of variable ranged from 0.969 for gp41 to 0.856 for p24 ($p<0.0001$). The highest correlation was found for the RI of the bands gp120 and gp36. The RIs obtained with both DB assays for the individual HIV proteins were also correlated with total anti-HIV antibody titers as determined by a standard ELISA.

This demonstrated that the quantification of antibodies against single HIV antigens by quantitative DB assays is comparable with the titers obtained using a standard ELISA. With regard to reproducibility of the results, both intra- and inter-assay precision tests gave acceptable results. It must be noted that the inter-assay CV of the RIs in the DB was lower than 10% for all the dots. In intra-assay tests, the CV was always below 5% for each band. Of the two different formats of quantitative DB developed, DB A, with internal human IgG standard, was found to be preferable due to good correlation with the results obtained with a standard ELISA and to low cut-off values. The results of our study demonstrated that the quantitative DB assay with internal human IgG standard (DB A) proved to be convenient for characterization of specific antibody production against single HIV antigens. DB A has the advantage that

specific anti-HIV antibody can be quantified using a single dilution of test sera, thus saving time and labor.

Furthermore, the use of a computer assisted video camera offered the advantage of a permanent storage of image data, thus allowing comparison between samples analyzed at different times, while membranes stained with chromogenic substrates can lose their color differentiation overtime. Although the technical equipment employed in this study is still mainly a research tool, its potential for a clinical application could be explained. DB is the standard confirmatory test for detecting antibodies to HIV-1 and HIV-2 (9, 13, 14).

The pattern of antibody response in HIV-infected patients, anti-p24 antibodies received considerable attention as a potential clinical marker early in the epidemic (6, 8, 14). Subsequent studies in which antibody to specific HIV antigen were quantitated showed a strong relationship between baseline values of p24 antibodies and subsequent disease progression (9, 12, 13), demonstrating that low levels of these antibodies herald disease aggravation.

Further investigation demonstrated that the ratio of p24 and gp41 antibodies was highly predictive of the risk of developing AIDS (15, 17, 18).

The use of an internally-controlled DB assay for measuring anti-HIV antibodies could be applied to study whether the changes in production of antibodies to Gag or Env proteins might give further information on the course of HIV infection (17, 19, 20).

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