Comparing the detection of HIV antigen P24 by human and mouse monoclonal antibodies

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Abstract

Previous studies have shown that antigen p24 appears in the primary stages of the infection before there is any evidence of antibodies. Therefore, the evaluation of antigen P24 can be an appropriate index for diagnosing the infection in its first stages.

Three hundred negative blood samples were tested with the third-generation kit for determining the antibody against HIV. Thirty positive samples were collected from the AIDS Research Center, and it was confirmed that the patients were HIV positive using Immunoblot and nucleic acid amplification test (NAT) methods. All the samples were investigated with the kit designed for determining antigen p24 in the ELISA method.

The average of optical density of the positive samples in the antigen test was 1.6, whereas the average optical density for the negative samples was 0.08. The difference between these two average values of optical density was statistically significant ($p<0.005$). By using human monoclonal antibodies, one unit per milliliter of WHO (World Health Organization) antigen, and two picograms per milliliter of recombinant antigen, the analytic sensitivity of the measurements was obtained. According to results obtained for the negative samples, the measurement specificity is 100%. In pretreatment, the samples of serum positive and the samples of BBI (Boston Biomedica Inc.) panels for which the antigen, antibody, and polymerase chain reaction (PCR) tests were positive; the 1.5 M glycine buffer with a pH of 2 increased the diagnostic sensitivity from 70% to 93%.

This test has high sensitivity and specificity in diagnosing HIV and is simpler, faster, more accurate, and more economical than other diagnostic methods.

Key words: ELISA test; Antigen P24; Immunity measurement; HIV

Introduction

HIV is a virus with a single-string RNA genome that has about 10000 nucleotides. HIV is the cause of AIDS, and its initial origin was injecting contaminated blood and blood products (1). The method currently proposed for diagnosing individuals with HIV is to determine the antibody that works against the virus. However, this test has false positive results. The Western Blot test (1) was used for examining the false positive cases (2, 3). The diagnosis of the infection in the early stages is very important for preventing secondary infections in blood transfusions. Therefore, a method is required for diagnosing HIV in blood

samples, especially during the early stages of infection (4). Monoclonal and polyclonal antibodies are being used increasingly for detecting virus antigens, especially antigen P24 of the virus. Two of the objectives of the present research were to use the ELISA test for detecting this antigen with high sensitivity and specificity and to compare its effectiveness to other methods for diagnosing the infection in its early stages. Based on the results of our study of the ELISA test, the diagnostic problems may have been solved.

Materials and methods

Sample collection

Three hundred samples of serum that were negative for HIV were selected randomly from individuals who had no symptoms. Thirty positive samples were collected from the AIDS Research Center, and it was confirmed that the patients were HIV positive by the use of Immunoblot and NAT methods.

Testing antibodies against HIV

By using the kit produced by Pishtaz Teb Zaman Co., all 300 collected samples were tested in compliance with the method specified in the brochure of the kit. The optical densities of these samples were less than the cutoff determined for the kit (i.e., an average optical density of negative control + 0.2). In this kit, gp120, gp41, and P24 were used for detecting antibodies.

Designing the diagnostic kit for antigen P24 in the ELISA Method

Plate Preparation: We acquired human monoclonal antibodies produced by Biomaric Co. (catalog Nos. 200.004 and 200.008) and mouse monoclonal antibodies (catalog Nos. 200.005 and 201.004) with concentrations of 1 and 2.5 mg/ml. Each of these products was dissolved in coating buffer. One hundred microliters of the solution were placed in a suitable vessel and incubated at a temperature of 4 oC for one night. The wells were maintain at room temperature for one hour after washing the ELISA plate with a phosphate buffer containing Tween 20 and a blocker solution that contained 1% bovine serum albumin and carbohydrate. Then, the contents of the wells were discharged and kept at room temperature for six hours in order to dry them. The plates were kept in foil containing damp absorbent in the range of 2-8 oC until the tests were performed.

Conjugated Solution Preparation

Conjugated solution 1. Biotinated monoclonal antibodies produced by Biomaric Co. were used. Considering the initial and final volumes of the antibodies, a concentration of 0.1 microgram in per milliliter was calculated for the biotinated antibodies. Conjugated solution 2. Different concentrations of the conjugated Strepto Avidine HRP (Hydrogen Proxidas) produced by Sigma Co. were used for identifying the antigen-antibody complex. This conjugated solution was diluted in a stabilizing solution produced by Pishtaz Teb, which is used especially for HRP preservation.

The Testing Procedure

One hundred microliters of the serum sample and 50 microliters of conjugated solution 1 were added to each well. The antigen P24 of HIV produced by the Russian RPC Co. (catalog no.90/636) with a concentration of 100 picograms in per milliliter was used for positive control. The wells were incubated at a temperature of 37 ^{o}C for one hour.

After the incubation, the wells were washed five times with a solution containing the phosphate buffer and Tween; then, the washing solution was completely discharged and 100 microliters of conjugated solution 2 were added to each well and incubated at a temperature of 37 ^{o}C for 30 minutes. After the incubation was completed, the wells were washed five times with washing buffer and 100 microliters. Then, substrate chromogen solution was added to each well after the washing solution (the substrate chromogen solution containing tetramethylbenzidine and hydrogen peroxi-

de produced by Pishtaz Teb) had been completely discharged. After 15 minutes, the solution became blue in color, indicating that the reactions had taken place. One hundred microliters of blocker (1N hydrochloric acid) were added to stop the reaction, and color changed to yellow. At a wavelength of 450 nm, the optical densities of the contents of the wells were read against reference filter 630.

Cutoff

The average and standard deviation of the optical densities of the negative samples were calculated using the +3SD formula in order to determine the cutoff and the mean cutoff for the kit.

Dissociation of the antigen-antibody complex

For preventing the probable interaction of the antibodies with antigen P24 in the samples, serum samples, the antibodies, antigen, and PCR tests of the positive samples were adjoined with various solutions including glycin buffer with a pH of 2, 0.5 N hydrochloric acid, Triton X100 with a concentration of 0.1%, alkaline buffer no.1 containing 2 M amine ethanol, 2.5% Triton X100, 0.15 M NaCl with a pH of 10, and alkaline buffer no.2 containing 4 M urea, 1% Supanin, 0.02% ethanol, and 0.15 M NaCl, and 0.01 M Na3PO4 with a pH of 10. One hundred microliters of the sample with 100 microliters of the abovementioned solutions were mixed and were kept in the incubator in a temperature of 37 oC for periods of 30, 60, 90, and 120 minutes. Concerning the last two solutions, 20 microliters of the solution were mixed with 100 microliters of the serum sample. The pH of the sample with the Tris buffer with a pH pf 10.6 was adjusted to approximately 7. Several negative samples were examined along with the positive samples in order to investigate the probable effect of these solutions on background optical density.

In addition, the antibody test was repeated, and the results were compared with a titer of the antibodies in the serum sample without pretreatment with the solution in order to be sure of the reduction of the effect of the antibodies in the serum on samples adjoined with acidic solutions and Triton.

Diagnostic Sensitivity

Seroconversion panels no. PRB926 (Mixed titer) and PRB108 (M) were chosen and were investigated with the designed kit for studying the diagnostic sensitivity by using BBI international panels.

Analytic Sensitivity

In this stage, the analytic sensitivity was determined by two methods:

1. The measurement of analytic sensitivity designed with human monoclonal antibodies and mouse monoclonal antibodies was investigated by diluting the series of P24 antigens recombinant.
2. The measurement of analytic sensitivity was investigated by using the dilutions of P24 antigen series proposed by the World Health Organization (WHO).

Imprecision Test

Three samples with different concentrations of antigen were used for investigating the imprecision of measurement in which ten working series (duplicate for any series) were studied during five different days.

Results

The average optical density of the samples that tested negative for antibodies was 0.07, and the standard deviation was 0.023.

From the average optical density of the negative samples, the cut-off optical density for distinguishing negative from positive samples by using the +3SD approach was determined to be 0.15. Samples that had optical density values greater than 0.15 were considered to be positive. The range of the optical densities of the positive samples was 0.35-2.91, and the average was 1.6.

The effect of the pretreatment of serum samples with acidic, alkaline solutions and X-100 Triton

The titer of the antibodies available in serums adjoined with acid was reduced significantly compared to the serum sample without adjoining. But, in samples that were adjoined with X-100, no titer reduction was found. In samples that had negative antigen test results and in which the existence of antibodies was confirmed, the titer of the antibodies was reduced significantly after being adjoined with acidic solutions.

Considering the comparison of the optical density of the samples with positive antibody test and negative antigen test, pretreatment with glycine buffer (pH = 2) at a temperature of 37 °C for one hour caused an increase in the optical density compared to the hydrochloric acid at 37 °C. Pretreatment with Triton X100 and solutions 1 and 2 had no effect in enhancing optical density. No increase in optical density was found in the samples with negative antibody tests, and they were used as control samples. This indicates that the enhancement of optical density in samples with positive antibodies after being adjoined with acidic solution for the antigen test is a specific phenomenon that results from the separation of antibodies from the antigen and the detection of antigen P24 by the measurement methods.

Considering the results obtained, the diagnostic sensitivity and specificity were increased by 93% and 100%, respectively.

The values of different markers of HIV in samples of commercially-available seroconversion panels were studied. These panels contain a group of serial samples that has been sampled at various times, and they indicate the time required to detect antibodies in the samples.

The international panels specified in the following tables were used for investigating diagnostic sensitivity.

Analytic sensitivity of the measurement

In this stage, the analytic sensitivity was determined by two methods:

1. The measurement of analytic sensitivity designed with human monoclonal antibodies and mouse monoclonal antibodies was investigated by diluting the series of P24 antigens recombinant.
2. The measurement of analytic sensitivity was investigated by using the dilutions of P24 antigen series proposed by the World Health Organization (WHO).

Considering the dilution of the serial of recombinant antigen P24 in the sample of negative serum for evaluation of the analytic sensitivity of measurement and with regard to the cutoff defined for negative samples, the minimum detectable value by this measurement technique was two picograms in per milliliter.

Specificity test

For assessing of specificity, we observed the cross reaction between antibodies and other viral structural antigens. In this method, we prepared the antigens gp41 and gp120 at concentrations of 200 pg/ml. We also examined 300 negative cases in the same way. Before treatment by glycin buffer, we found antigens in 21 of the 30 positive cases when we designed the kit using human monoclonal antibodies, and we found the antigen in 18 cases when using the mouse monoclonal antibodies. After treatment with glycin buffer, the sensitivities were 93% (28 positive cases) and 90% (27 positive cases), respectively. We performed the same test on random negative cases, and the specifity of the kit was 100% for both methods, and no false positive cases were observed. Our findings showed that the diagnostic sensitivity and specificity of the human monoclonal antibody tests before treatment by glycin were 70% and 100%, respectively. However, after treatment with 1.5 M glycin buffer, the sensitivity and specificity were calculated as 93% and 100%, respectively.

Imprecision Test

The results of the imprecision of the intra-assay measurement obtained from three samples with different concentrations of antigen showed that the de-

signed kit had appropriate imprecision for various limits of antigen. The range of the imprecision of the measurements was calculated on the basis of the coefficient of variables (CV%) as 4.2 to 12.8.

Discussion and conclusions

The ELISA method is applicable for detecting antigen p24 of HIV through using the specific antibodies that trap antigen. The studies showed that antigen p24 of the virus are detectable within one day after the appearance of RNA (5, 6).

Mouse monoclonal or polyclonal antibodies have been used for the detection of antigen p24 (7, 8. In our research, the comparison of two mouse and human antibodies showed that the analytic sensitivity of measurement, which is an important parameter in determining diagnostic sensitivity, is improved when human antibodies are used.

Separation of the complex of antigens and antibodies has been done using different methods in various research studies (9). In a study done by M. A. Rodrigues et al. in 2003, antigen p24 was detected in 554 serum samples (509 samples from individuals who were HIV positive and 45 control samples from people who were HIV negative). They used the acid treatment method for separating the complex of antigen p24 and its antibody. In individuals with no symptoms, a significant increase in antigen P24 detection was found (48.2% versus 8.4%), and the same results were obtained for the patients (85.7% versus 37.1%). This indicates that acid treatment increases the sensitivity of the technique for detecting antigen p24 (10).

In this study, it was found that samples that were treated with glycine buffer before measurements were taken had an increase of diagnostic sensitivity in both measurements (70% before pretreatment versus 93% after adjoining).

The increase in the percentage of measurement sensitivity after treating the samples with 1.5 M glycine buffer indicated the removal of the effect of antibody intervention of antigen P24 existing in the serum.

These antibodies prevent the connection of antigen to trapping antibodies in measurement through the formation of a complex with antigen and covering antigenic epitope (11, 12).

Pretreatment with a special buffer has also been used in Perkin Elmer, Abbott, and Coulter kits. In a study done by Fedyuk et al. in1993, The ELISA Test was used for detecting antigen p24. They investigated 97 serum samples from HIV positive patients. They studied the effect of the formation of the complex of antigen p24 and its antibody in detecting antigen P24. Their results showed that this complex causes confusion in antigen detection. After acid treatment, 50% of the serum samples were positive, while, before treatment, only 5% was positive (13).

The sensitivity and specificity of the measurement techniques we studied were 93% and 100%, respectively, after pretreatment with 1.5 M glycine solution, which shows optimal sensitivity and specificity that compare favorably with results obtained from other studies. Analytic sensitivity of measurement in using human monoclonal antibody was one unit per milliliter with consideration to the standard proposed by the World Health Organization; while using mouse monoclonal antibody, this value was four units per milliliter. This difference indicates that the analytical sensitivity of human monoclonal antibodies is improved, which is due to the high affinity and avidity of the human antibodies for the antigen. The analytic sensitivity of this measurement and the comparison of the results with the results of other detection kits of antigen P24 are illustrated in Table 4.

Considering the results obtained in this study using the seroconversion panels produced by BBI Co., employing P24 antigen detection is accompanied with significant reduction of serologic window period (14).

The ELISA method for determining antigen p24 is a direct method for diagnosing HIV infection. This method is economical and easily feasible in most laboratories, and no specialized personnel are required to conduct the tests. There is no cross-sectional contamination in PCR, and the preservation of the sample is easier than RNA because of the greater durability of the antigen.

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