NEW SUPPLEMENTAL ASSAY FOR THE CONFIRMATION OF HIV-1/2 POSITIVE SAMPLES

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SUMMARY

A new membrane assay for the detection of antibodies against HIV-1 and HIV-2 has been developed. Recombinant proteins from HIV-1 (p41r, p120r, p24r) and HIV-2 (p36r) are spotted onto nitrocellulose membranes, and constitute the basis of the assay.

The sensitivity and specificity of the test for the detection of anti HIV antibodies were analyzed against 60 HIV-1 positive sera, 30 HIV-2 positive sera and 100 blood donor sera.

All the HIV-1 positive samples recognized the combination p41r/p120r or p41r/p24r. The 100% of the HIV-2 positive sera were p36r reactive, and 50% were p36r/p24r positive. One of the normal sera (1%) recognized only p24r and was classified as indeterminate. The new membrane assay, based on the use of recombinant proteins is a useful test for the confirmation of the presence of anti-HIV-1 and anti-HIV-2 antibodies.

RESUMEN

Un nuevo ensayo de membrana para la detección de anticuerpos contra el VIH-1 y el VIH-2 ha sido desarrollado. Proteínas recombinantes del VIH-1 (p41r, p120r, p24r) y del VIH-2 (p36r) son aplicadas sobre membranas de nitrocelulosa y constituyen la base del ensayo. La sensibilidad y especificidad para la detección de anticuerpos anti-VIH fue analizada empleando 60 sueros VIH-1 positivos, 30 sueros VIH-2 positivos y 100 sueros de donantes de sangre.

Todas las muestras VIH-1 positivas reconocieron la combinación p41r/p120r o p41r/p24r. El 100% de los sueros VIH-2 positivos resultaron reactivos a la p36r, y el 50% de ellos fue positivo a la combinación p36r/p24r. Uno de los sueros normales (1%) reconoció solamente la p24 y fue clasificado como indeterminado. El nuevo ensayo de membrana, basado en el uso de proteínas recombinantes, es útil para la confirmación de la presencia de anticuerpos anti-VIH-1 y anti-VIH-2.

INTRODUCTION

The most widely used test for the detection of antibodies against HIV-1 and HIV-2 is the enzyme-linked immunosorbent assay (ELISA). All the samples that are reactive in the primary screening test are usually retested in duplicate to ensure reproducibility. Repeatedly reactive sera are then confirmed by supplementary assays.

The Western immunoblot (WB) is the most widely test used for the confirmation of HIV seropositivity for ELISA reactive samples. WB assays have difficulties with the reproducibility of the quantitative ratios between viral antigens. In addition, env-gene derived products, relevant in the detection of early antibodies are often not available in sufficient quantities. Furthermore, using WB assays it is impossible to discriminate between an actually negative result, and a strip to which one of the reagents (sample, conjugate, substrate) was not added or was inactivated. With the advent of HIV-2 infections the use of confirmatory assays able to detect antibodies against both of the viruses became a necessity.

A new generation of supplemental tests for the diagnosis of HIV-1 and HIV-2 infection is under development. It has been described the use of recombinant protein (RP) based ELISAs for the confirmation of anti-HIV positive samples with results highly concordant with the WB (Lepine *et al*, 1990: van der Groen *et al.*, 1991). Synthetic peptides are also used in the detection of anti-HIV antibodies employing strip formats. These assays are simple to perform and do not require the use of highly sophisticated equipment. We report the development of a nitrocellulose assay based on recombinant antigens for the detection of antibodies against different HIV proteins.

MATERIALS AND METHODS

Recombinant Antigens

Two HIV-1 envelope (transmembrane gp41 and external gp120) proteins are used, as well as the major core protein p24. In addition, the transmembrane protein (gp36) of the HIV-2 was included. All the antigens were cloned and expressed as inclusion bodies in *E.coli*. The proteins were purified by washed pellet cell techniques and finally by the use of HPLC chromatography (Novoa *et al.*, 1992).

Samples

Sixty WB confirmed HIV-1 positive, 30 HIV-2 positive and 100 HIV negative individual were tested. Serum samples were stored at -20°C and thawed no more than three times.

Strips

All the antigens were separately spotted onto nitrocellulose strips (0.45 μm, S & S) in 1 μl of 0.1 M sodium carbonate buffer pH = 9.6 at 1 mg/ml. The membranes were rinsed with double distilled water by shaking 5 min at room temperature (RT). After that, free sites on the membrane were blocked with 5% solution of skim milk in phosphate buffered saline (PBS), by 1 hour incubation at 37°C. Blocked membranes were air-dried, sealed and stored at 4°C.

Immunoenzymatic reaction

Each strip was incubated at RT for 30 min with 50 μl of the sample and 1 ml of PBS + 0.05% Tween-20 (PBS-T). Strips were washed with 1 ml of PBS-T by shaking 5 min, and 1 ml of protein A - peroxidase conjugate was added to each strip. After a 30 min incubation at RT, the excess of conjugate was eliminated by shaking 5 min with 1 ml of PBS-T. Reaction was developed by a 5 min incubation with 0.05% diaminobenzidine (Kanto Chemical), 0.015% H_2O_2 in 1 ml of PBS-T. Finally the strips were rinsed and dried prior to the interpretation of the results.

RESULTS AND DISCUSSION

Figure 1 shows typical results obtained for HIV positive and negative samples. All the 60 HIV-1 (+) sera were reactive for p41r, 75% for p24r, and 79% for p120r (figure 2). Furthermore, 83% were positive for p36r.

All the HIV-2 positive samples were p36r reactive, while the reactivity rates of these sera for HIV-1 proteins were 50% for p24r, 45% for p41r, and 5% for p120r (figure 3). Cross reaction for p24 has been reported by other authors (Pollet *et al.*, 1991). The patterns for HIV-1 sera showed 100% reactivity for the combination of p41r/p24r or p41r/p120r. Twenty two percent of the samples were positive for p24r/p41r, but negative for p120r; 25% were positive for p120r/p41r, but negative for p24r; and 53% were positive for p120r/p24r/p41r (figure 4). Fifty percent of the HIV-2 sera were positive for p36r/p24r. One negative sera (1%) was p24 reactive, significantly lower than the frequency of WB indeterminate results (usually as high as 20% in persons with negative HIV ELISA, Davey and Lane, 1990), and similar to the results obtained for RP based assays (Pollet *et al.*, 1991).

The occurrence of p24 or p55 bands may correspond to known HIV-1 proteins or to other proteins of cellular origin and currently represents the presence of antibodies with low specificity. Nevertheless, our results confirmed the fact that RP based assays are comparatively superior to the WB test for the confirmation of HIV seropositivity, concerning the quantity of indeterminate results.

It has been found that some WB conventional tests detected only 68-95% of core antibodies (Pohlod and Saravolats, 1991). In our study, 25% of the HIV-1 positive samples were p24r negative. This phenomenon could cause false negative results in case of including the presence of core bands as a criterium of HIV positivity. On this basis, the correct criterium must be the detection of anti-env (p41r, p36r) antibodies which are present in all the HIV(+) individuals, and are generated preceding the response to gag gene products.

For economical reasons, the use of WB in primary evaluations is prohibitive. Immunoblot tests which use recombinant engineered proteins blotted on nitrocellulose filters are compared favorably with WB in terms of sensitivity and specificity (Hofbaur *et al*, 1988).

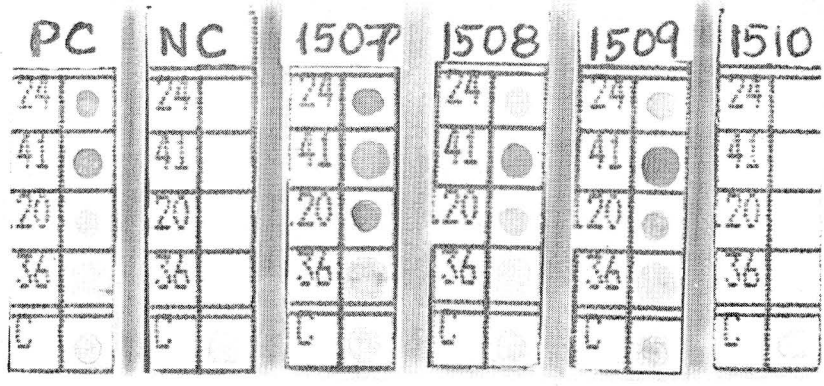

Fig. 1 Antigen profile on the membrane. PC- HIV positive control, NC- negative control, 1507' 1509- HIV-1 positive samples, 1510- blood donor sample. 24, 41, 120, 36 recombinant antigens, C- assay control.

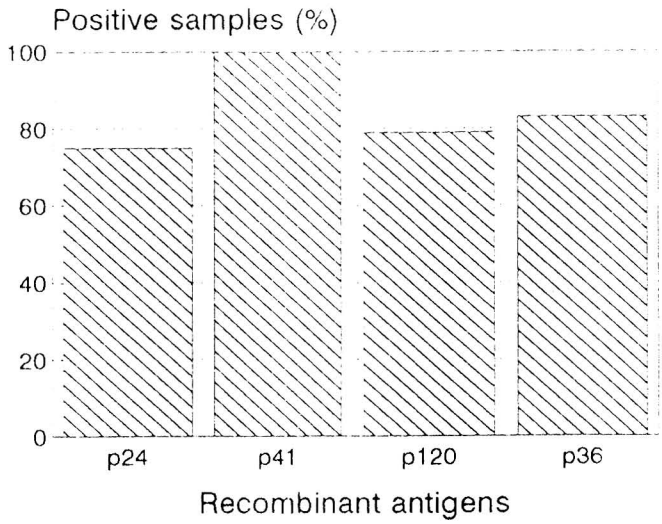

Fig. 2 Positivity rates obtained for HIV-1 positive samples.

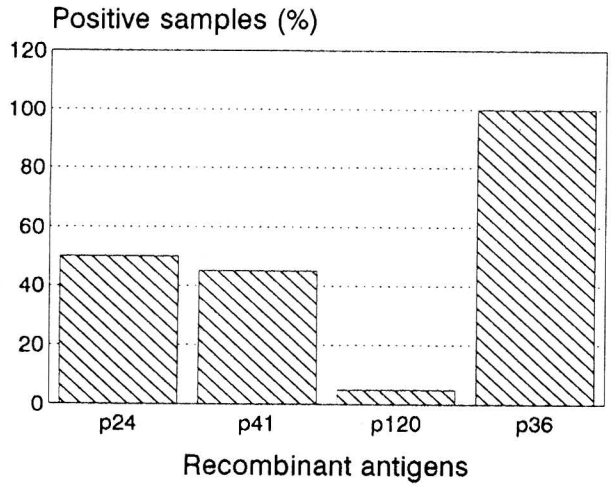

Fig. 3 Positivity rates obtained for HIV-2 positive samples.

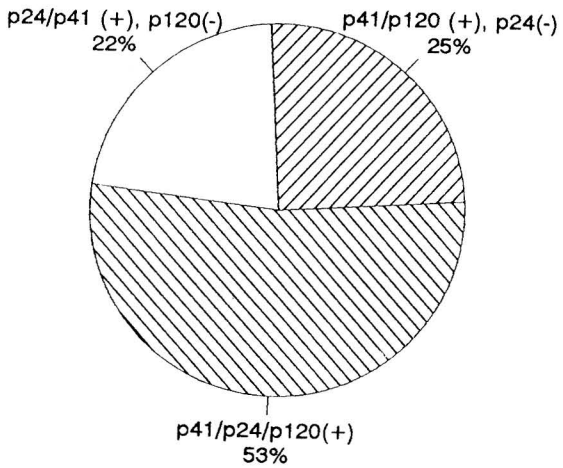

Fig. 4 Combined reactivity for HIV-1 samples.

They could be used not only for the confirmation of HIV ELISA positive samples, but also as primary assays for testing high risk individuals.

According to the standards adopted by most of the researchers a positive blot must contain at least two of the three major bands of diagnostic importance: anti-gp120, anti-gp41 and anti-p24 (Anonymous, 1989; Hausler, 1988). Nevertheless, in some conventional WB systems, quantitative ratios between viral proteins are difficult to reproduce from one preparation to the other (Döpel *et al*, 1991). Moreover, env-derived proteins (gp41, gp120 and gp160) are often not available in sufficient quantities, which makes difficult the detection of early antibodies (Saah *et al.*, 1987).

In the RP based assays the relative quantities of the antigens can be correctly determined and reproduced from one preparation to others. Furthermore, the most relevant regions of the viral antigens can be included, increasing in this way the amount of epitopes fixed to the solid phase.

According to our results, the criteria for positivity was established as the combined reactivity to p24r/p41r or to p120r/p41r, as well as all HIV-1 positive samples are correctly identified in this way. This is in agreement with the most widely used criterium for HIV positivity (Anonymous, 1989). For HIV-2, the criterium of positivity is the reactivity to p36r, recognized by all the HIV-2 positive sera.

It has been reported that in WB assays the 160 kDa band represents a tetramer of the gp41 transmembrane protein. A similar event has been found in some cases for the gp120 protein. This fact makes difficult to apply the WHO criteria for HIV positivity, as well as, antibodies recognizing gp41 would cause the occurrence of both 120 and 160 kDa WB bands. This paradox can be avoided by the use of RP based tests, which are able to discriminate between antibodies against the gp41 and gp120 proteins. In our assay 21% of the samples were p41r positive and p120r negative, which could be partially explained by an unequal recognition of the p120r by antibodies against different HIV strains.

Finally, it is impossible to distinguish between a WB strip corresponding to a negative sample, and a WB strip to which one of the reagents was not added by error or was inactivated. In the RP based assays this event can be easily detected by the inclusion of an IgG(h) control spot, which must always appear in an anti correct assay and will not be present in the case of error.

The new membrane assay described could be used as an economical and simple supplemental test for the confirmation of HIV-1/2 positive samples.

REFERENCES

ANONYMOUS. (1989). Centers for Disease Control. Interpretation and use of the western blot assay for the serodiagnosis of human immunodeficiency virus type 1 infection. *MMWR* **38**: 1,7

DAVEY, R. and H. LANE (1990). Laboratory methods in the diagnosis and prognostic staging of infection with human immunodeficiency virus type 1. *Review of Infectious Diseases*, **12**(5): 912-930

DÖPEL S.H., U. SCHUBERT, R. GRUNOW, P. PAS, W. RÖNSPECK, G. PAULI and T. PORSTMAN (1991). Comparison of four anti-HIV screening assays which belong to different test generations. *Eur. J. Clin. Chem. Clin. Bioche.* **29**: 331-337

HAUSLER, W. J. (1988). Report of the third consensus conference on HIV testing sponsored by the association of state and territorial public health laboratory directors. *Infect. Control Hosp. Epidemiol.*, **9**: 345-349

HOFBAUR, J. M.; T. F. SCHULZ, P. HEUGSTER, C. LANDER, R. ZANGERTE, H. KOFLER, P. FRITSCH, H. WACHTER, and M.P. DIERICH (1988). Comparison of WB (immunoblot) based on recombinant derived p41 with conventional test for serodiagnosis of HIV infections. *J. Clin. Microbiol.* **26**: 116-120

LEPINE, D. G., P. W. NEWMANN, S. L. FRENETTE and M. V. O'SHAUGHNESSY (1990). Evaluation of human immunodeficiency virus test algorithm utilizing a recombinant proteins enzyme immunoassay. *J. Clin. Microb.* **28**(6): 1169-1171

NOVOA, L.; J. MACHADO, J. BENITEZ, J. GARCIA, R. NARCIANDI, G. PADRON, E. PENTON y L. HERRERA (1992) Desarrollo de un ensayo de segunda generación para la detección de anticuerpos contra componentes del HIV-1. *Biotecnología Aplicada* **9** (2):140-147

POHLOD, D. and L. SARAVOLATS (1991). Comparison of five commercial western blot kits for detection of HIV-1. *Eur. J. Clin. Microbiol. Infect. Dis.* **10**: 453-457

POLLET, D. E., C. J. WOUNTERS, G. BEELAERT, G. van der GROEN and H. VAN HEUVERSWYN (1991). Confirmation of antibodies to HIV-1 and HIV-2 with a strip based assay including recombinant antigens and synthetic peptides. *Clin. Chem.* **37**(10) :1700-07

SAAH, A. J., H. FARZADEGAN, R. FOX, P. NISHANIAN, CH. RINALDO, J. P. PHAIR, J. L. FAHEY, T. LEE and B.F. POLK (1987). Detection of early antibodies in HIV infection by ELISA, western blot and radioimmunoprecipitation. *J. Clin. Microbiol.* **26:** 1005-1610

VAN DER GROEN, G., I. VAN KERCKHOVEN, G. VERCAUTEREN AND P. PIOT (1991). *Bulletin of the World Health Organization* **69**(6): 747-752