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Full Length Research Paper

Development and evaluation of a line immunoassay as a confirmatory test for human immunodeficiency virus (HIV)-1/2 infections

Xihong Zhao[1], Xiaowei He[1]*, Wenmei Li[1,2], Liansheng Yang[1] and Jihua Wang[2]

[1]College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, China.
[2]Guangzhou Wondfo Biotech. CO., LTD, 510530, Guangzhou, China.

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Serological methods are the main methods in diagnosis of human immunodeficiency virus (HIV)-1 and HIV-2 infection. The western Blot (WB) is considered as a gold standard test for the confirmation of enzyme-linked immunosorbent assay (ELISA) and/or other rapid screened reactive results samples in the diagnosis of HIV infection. However, problems of WB, particularly in regard to the significance of indeterminate results, still remain. To develop and evaluate a recombinant HIV-1/2 antigens-based line immunoassay as confirmatory assay for HIV-1 and HIV-2 infections is the objective of this study. HIV-1 envelope proteins (gp160, gp120, gp41), HIV-1 integrase protein (p31), HIV-1 gag proteins (p24, p17) and HIV-2 envelope protein (gp 36) were cloned and expressed in *Escherichia coli*. All recombinant HIV antigens were purified and immobilized on nitrocellulose membranes and applied in a line immunoassay, which supplement detected anti-HIV antibodies in a single strip. Specific antibodies to these antigens in human sera were revealed using an alkaline phosphatase labeled anti-human immunoglobulin G (IgG) as second antibody. Two anti-human IgG lines were used as a control for the addition of serum. Positive or negative results were determined by visual comparison of the antigen lines intensity with the two control lines. The recombinant HIV antigens-based line immunoassay identified seropositive individuals with a high degree of accuracy; none of the HIV-seropositive subjects yielded a negative result. Reactivity with these antigens, demonstrated 100% sensitivity and specificity in distinguishing seronegative from seropositive sera. This line immunoassay was much more sensitive and specific than WB. In particular, the line immunoassay allowed for a significant reduction in the number of indeterminate results and for more accurate distinction between HIV-1 and/or HIV-2 infections.

Key words: HIV antibodies, line immunoassay, recombinant antigens, confirmatory test.

INTRODUCTION

Human immunodeficiency virus (HIV) is the etiological agent for acquired immunodeficiency syndrome (AIDS) which remains one of the most important global public health problems. The HIV infection causes a gradual depletion and weakening of the immune system. This results in an increased susceptibility of the body to infections, such as pneumonia and tuberculosis and can lead to the development of AIDS. It was estimated that 33 million people worldwide are living with HIV, with 2.7million newly infected; and 2 million died from HIV-relaxd causes (UNAIDS, 2008). HIV type 1(HIV-1) is the predominant virus worldwide, while HIV-2 differs from HIV-1 in its lower pathogenicity and higher lever of intra-subtype strain diversity (Wang et al., 2000). Prevention and treatment of AIDS depends on the prompt and accurate detection of HIV infected patients (Organization, 2009).

The serological methods, such as enzyme-linked immunosorbent assay (ELISA), western blot (WB) and colloidal gold immunochromatgraphic strip, are the main methods in the diagnosis of HIV-1 and HIV-2 infection.

*Corresponding author. E-mail: biosci18@163.com. Tel: +86-20-87058470.

The positive results after serologic screening should be supplementary tested of HIV confirmatory assays such as WB or the indirect fluorescent assay. WB is the most widely used confirmatory tests for detection of HIV antibodies worldwide (Luft et al., 2004). However, interpretation of WB assays requires considerable expertise as the WB-reaction bands are sometimes difficult to identify. Furthermore, 15–20% of samples from HIV-negative blood donors are indeterminate by using current WB assays (Arens, 2000), which are inconclusive and needed to be further examined by alternative means such as p24 antigen tests, polymerase chain reaction (PCR) and/or virus isolation, which can incur further cost (Ma et al., 2004). Insufficient accuracy, laboratory requirement and high expense of current WB assay proved to be an obstacle for their broad application as confirmatory method; so development of a sensitive, specific and reliable confirmatory HIV1/2 detected method was of urgent necessity and utmost importance.

Recombinant DNA-derived HIV-1, HIV-1 main structural proteins and synthetic peptides as antigens have been used in anti-HIV antibodies assays (Mas et al., 1997; Ishikawa et al., 1999; Schuepbach et al., 2007). Because these antigens contain the immunodominant regions of selected viral antigens and are not contaminated with human cellular proteins, these antigens theoretically should provide less variable, more sensitive and, in particular, more specific tests than those based on native virus (Mas et al., 1997). Furthermore, these antigens are less dangerous than native HIV virus.

In this report, we described the development and preliminary evaluation of a recombinant HIV-1/2 antigens-based line immunoassay as confirmatory assay testing clinical samples.

MATERIALS AND METHODS

Samples and kits

A total of 153 serum specimens from healthy blood donors were collected. A total of 35 seropositive serum specimens was obtained, among these, 18 confirmed HIV positive samples from AIDS patients, 10 asymptomatic HIV-infection subjects, 6 HIV-infected intravenous drug users (as well as HIV-2 positive samples) and 1 hemophilic infected subjects, were collected in our laboratory.

ELISA 4th HIV test kit (Vironostika® HIV Uni-Form II Ag/Ab, bioMérieux, France) was used as a pre-screening assay and HIV Blot 2.2 Western Blot kit (Genelabs, Singapore) was included as comparative confirmatory test with line immunoassay. The authors have no financial interest in either of the kits.

Expression and purification of HIV antigens

HIV-1 envelope proteins (gp160, gp120, gp41), HIV-1 integrase protein (p31), HIV-1 gag proteins (p24, p17) and HIV-2 envelope protein (gp36) were cloned and expressed in *Escherichia coli*. Briefly, genes were cloned in pET-His expression vector, a derivative of the pET expression system (Cxbio Co, USA). Antigens were expressed with six histidines incorporated at the carboxyl terminus of the antigens for affinity purification procedures.

The recombinant gp41 (amino acids 511-659) contains the N-terminal segment of the transmembranic HIV-1 glycoprotein. The *E. coli* BL21 (DE3) (Novagen Co, USA) strains were transformed with the pET-His vector and cultured in lysogeny broth (LB) medium supplemented with ampicillin (5 µg/ml) at 37°C.

When cells reached an optical density of approximately 0.6 at 600 nm, the expression vector were induced by isopropylthio-β-D-galactoside (IPTG, final concentration 1 mmol/L) and incubated at 37°C for 4 h. The cells were harvested by centrifugation (10,800 × g, 10 min, 4°C). 1 g of biomass was resuspended and homogenized in 10 ml of 20 mM Tris-HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0. The suspension was sonicated for 10 min under 400 W then centrifuged (10,800 × g, 10 min, 4°C). The recombinant gp41 protein was extracted with solution buffer (100 ml of 3 M urea, 0.5 M NaCl pH 8.0), 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 solution buffer.

The bound proteins were eluted by conduction in loading buffer (50 mmol/L sodium phosphate, pH 8.0) with an imidazole-step gradient (20 – 500 mM) at a flow-rate of 1 ml/min.

The recombinant gp160, gp120, p31, p24, p17 and gp36 proteins comprise the main epitope sequence of the respective natural antigens and were produced using procedures similar to those described for gp41.

Preparation of line immunoassay strips

Recombinant antigens (gp160, gp120, gp41, p31, p24, gp36 and p17) were diluted to optimal concentrations in coating buffer (20 mM Tris and 500 mM NaCl, pH 7.2), then coated onto nitrocellulose sheet (0.45 µm; Sartorortius stedim, Germany) in parallel lines 50 mm long as shown in Figure 1. For use in the line immunoassay procedure, coated nitrocellulose sheets were cut into 3 mm wide strips perpendicular to the antigen lines.

Horizontal rows, C to I, contained the antigens gp160, gp120, gp41, p31, p24, gp36 and p17, respectively, while rows A and B were coated with different concentrations of goat anti-human immunoglobulin G (Sigma-Aldrich Inc., Germany) as internal control for the quality of the conjugates used in the test and as reference control for positive test results.

After incubation over night at 4°C, each strip was blocked with 1 mL blocking buffer (1 g of Bovine Serum Albumin and 0.1 ml of Tween 20 in 100 ml of tris buffered saline (TBS)), followed by 2 h incubation at room temperature during which the plates were rotated on a platform. The strips were incubated for 2 h at 37°C and then strips could be used immediately or stored for several weeks at -20°C after air-dried and sealed in plastic-lined aluminum bags containing a desiccant.

Procedure of line immunoassay strips

Each serum sample before being tested was diluted 1:100 with sample dilution buffer (TBS-Tween 20) and allowed to stand for 30 min at 25°C. In each plate, eight serum samples could be tested, with two columns of the plate reserved for positive and negative control samples. Nitrocellulose strips were incubated for 1 h at room temperature with 1 mL diluted serum samples. Each strip was washed twice with TBS-Tween 20 and incubated with 1000 µl of a 1:8,000 dilution of alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma-Aldrich, Inc., Germany) for 30 min at 20°C.

Strips were washed three times with TBS-Tween 20 and then incubated with 5-bromo 4-choloro 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma-Aldrich, Inc., Germany) for 10 min at

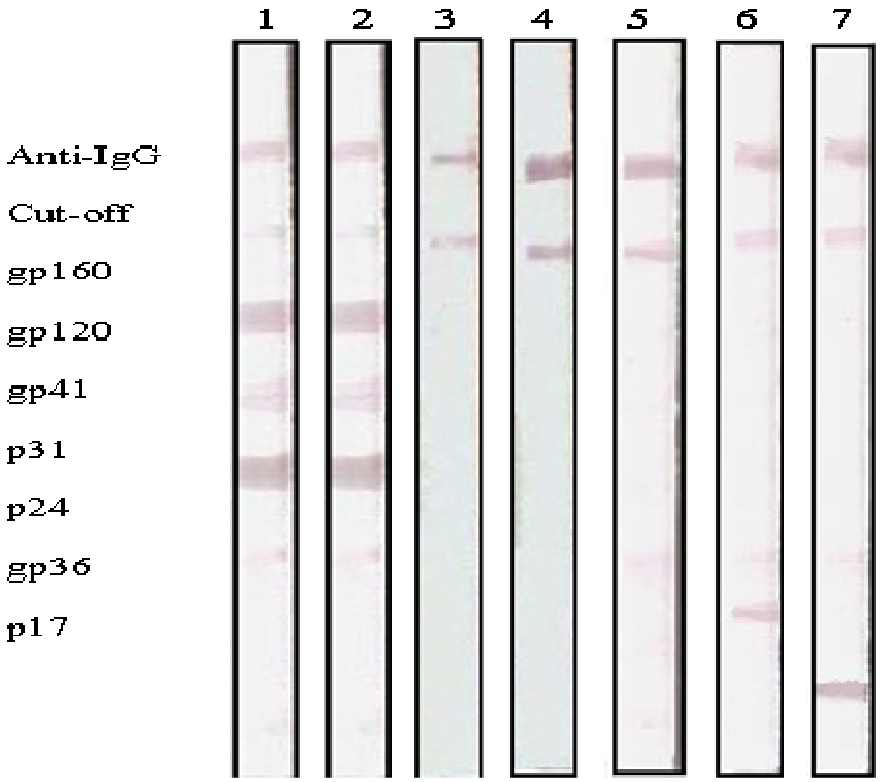

Figure 1. Evaluation of line immunoassay with clinical samples. Samples 1 and 2 were HIV-1 positive result, samples 3, 4 and 5 were HIV negative result, sample 6 was indeterminate result and sample 7 was HIV-2 positive result.

37℃ in the dark chamber. Strips were washed in deionized water to end the reaction and air-dried.

Mechanism of colorization development and interpretation of the results

An ideal system for western blotting is the combination of BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) and NBT (Nitro-Blue Tetrazolium Chloride). BCIP is hydrolyzed by alkaline phosphatase to form an intermediate that undergoes dimerization to produce an indigo dye. The NBT is reduced to the NBT-formazan by the two reducing equivalents generated by dimerization.

A confirmatory positive result was defined as the presence of antibodies against at least two different HIV gene products, one of which must be HIV env gene products. A confirmatory negative result was defined as no antibody against any of the HIV gene products. An indeterminate result was defined as antibodies against only one of HIV gene products.

RESULTS

Production of recombinant antigens

HIV-1 (gp160, gp120, gp41, p31, p24) and HIV-2 (gp36, p17) structural proteins were heterologously expressed in *E. coli* and purified to homogeneity. Lack of any interfering protein contamination originating from the *E. coli* host was demonstrated by western blotting. In purified protein fractions, no bacterial proteins were detected using mouse anti-*E. coli* antibodies. Recombinant antigens could be identified during the purification process by the use of antibodies recognizing the C-terminal histidine tag (data not shown).

Development and evaluation of line immunoassay strips

Recombinant antigens were immobilized as horizontal lines on membrane strips. To determine optimal line immunoassay conditions, different amounts of antigens and different dilutions of serum samples and of the conjugate were tested to obtain the best signal-to-noise ratios for positive and negative sera, respectively (results not shown). The optimal concentration of antigens for preparing the strips was found to be 100ng/μL. Optimal signal-to-noise was found with sera diluted 1:100 and conjugate diluted 1:8,000. Reliability of the testing system was improved by including a strong positive (goat anti-human IgG; 50 ng/μL) and a weak positive control (goat anti-human IgG; 10 ng/μL) on each strip. The weak positive control line represented a cutoff control. Only signals of the same intensity as or stronger than the

Table 1. Sensitivity and specificity of line immunoassay.

Samples	No. of samples with indicated results		
	Positive	Negative	Indeterminate
Seronegative subjects (153)			
Healthy blood donors (151)	0	151	0
False-positive by ELISA (2)*	0	0	2
Total	0	151 (98.7%)	2 (1.3%)
Seropositive subjects (35)			
AIDS patients (18)	18	0	0
asymptomatic HIV-infection (10)	9	0	1
HIV-infected intravenous drug users(6)	6	0	0
hemophilic infected subjects (1)	1	0	0
Total	34 (97.1%)	0	1 (2.9%)

*Confirmed by reference HIV Blot 2.2 Western Blot kit (Genelabs, Singapore) and Commercial RT-polymerase chain reaction assay (Roche Molecular Diag, France).

Table 2. Comparison of line immunoassay results with results of commercial WB assay as interpreted by CDC criteria.

Sample group	Results(%) of test as interpreted by CDC criteria	
	Line immunoassay	Western blot
Seronegative (153)		
Positive	0	0
Negative	98.7	97.4
Indeterminate	1.3	2.6
Seropositive (35)		
Positive	97.1	94.3
Negative	0	0
Indeterminate	2.9	5.7

defined weak positive control were considered positive.

A total of 153 seronegative samples from healthy blood donors were tested. Among the samples, two samples gave false-weak positive by ELISA results. None of these seronegative samples gave a positive result; 98.7% gave a negative result, and 1.3% gave an indeterminate result (Table 1). The indeterminate sample primarily had reactivity against p24 gag and none of these samples reacted with envelop proteins.

Sera from 35 HIV-infected subjects at different clinical stages of disease were analyzed (Table 1). From Table 1, 97.1% (34/35) gave positive results, 0% gave a negative result and 2.9% (1/35) gave indeterminate results. All of these samples reacted with the p24 gag gene product. The samples giving indeterminate result, from one young pregnant, failed to react with an env gene product. Clinical sample showed specific reactions with their respective antigens (Figure 1).

A comparison of sensitivity and specificity between the line immunoassay and commercial Western blot as interpreted by the Centers for Disease Control (CDC) criteria is shown in Table 2. The line immunoassay had fewer indeterminate and more negative results than western blot in seronegative group samples. None of the seropositive group samples was negative in either line immunoassay or WB. The line immunoassay had more positive and fewer indeterminate results than WB, as interpreted by CDC criteria.

DISCUSSION

Supplement or confirmatory tests for HIV antibodies (western blot and immunofluorescence assay), in contrast to screening tests, 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 (Ravanshad et al., 2006). The recombinant HIV main structural antigens-based line immunoassay developed in this study, showed high sensitivity and specificity in testing anti-HIV antibodies and also fewer indeterminate results than commercial WB kits.

For lack of enough reactivity bands in strip, frequently, just a strong reactivity line with gag p24 protein no others env gene products, “indeterminate” results by WB were occasionally observed for some severe HIV-related disease, such as hepatitis C virus (HCV), hepatitis B virus (HBV), thrombocytopenia (TP) etc. Some of the “indeterminate” results were found to be associated with individuals having infections with Human T-lymphotropic virus (HTLV) or other retroviruses. A recent study determined that there were homologous regions in the surface glycoprotein sequences of HIV and the corresponding nucleotides of the lentivirus caprine arthritis-encephalitis virus (CAEV) (Tesoro-Cruz et al., 2003). The finding thus, revealed the basis for some of the cross-reactivity and suggested that human contact with CAEV could be yet another source of false reactivity and indeterminate results (Guan, 2007). Meanwhile, new diagnostic criteria are urgently needed (Lee et al., 2000; Mahé et al., 2002; Uneke et al., 2007).

In this work, the recombinant HIV antigens-based line immunoassay showed fewer indeterminate results when compared with commercial WB kit (Genelabs, Singapore), which was interpreted by CDC criteria. This most likely explained that recombinant antigens were designed to specific anti-HIV antibodies, then were expressed and purified. Furthermore, the recombinant antigens were safer than HIV virus and could test HIV-1 (O subtype) and HIV-2 in 1 antigen-coated strip.

Conclusion

In conclusion, we consider our line immunoassay approach to be highly accurate in distinguishing HIV seropositive from seronegative individuals. This line immunoassay benefited fewer indeterminate results, decreased the high proportion of the indeterminate results obtained from current western blot and required only very low serum volumes(10 µl). The results can be identified and interpreted easily, because control lines in the strips and the antigens are coated in identical district different with the abnormative bands in western blot. Moreover, the results of line immunoassay would be potentially used to quantitate and correlate levels of antibodies to different HIV gene products in relation to disease progression or therapy.

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Abbreviations

HIV, Human immunodeficiency virus; **WB,** western blot; **TBS,** tris buffered saline; **ELISA,** enzyme-linked immunosorbent assay; **EDTA,** ethylene diamine tetraacetic acid; IgG, immunoglobulin G; **AIDS,** acquired immunodeficiency syndrome; **PCR,** polymerase chain reaction; LB, lysogeny broth; **IPTG,** isopropylthio-β-D-galactoside; **BCIP,** 5-bromo-4-chloro-3'-indolyphosphate p-toluidine; **NBT,** nitro-blue tetrazolium; **CDC,** centers for disease control; **HCV,** hepatitis C virus; **HBV,** hepatitis B virus; **TP,** thrombocytopenia; **HTLV,** human T-lymphotropic virus; **CAEV,** caprine arthritis-encephalitis virus.

REFERENCES

Arens M (2000). Human immunodeficiency virus (HIV) and other human retroviruses. Churchill Livingston, USA. Essentials of Diagnostic. Virology.

Guan M (2007). Frequency, causes, and new challenges of indeterminate results in western blot confirmatory testing for antibodies to human immunodeficiency virus. Clin. Vaccine Immunol. 14: 649-659.

Ishikawa S, Hashinaka K, Hashida S, Oka S, Ishikawa E (1999). Use of indirectly immobilized recombinant p17 antigen for detection of antibodies to HIV-1 by enzyme immunoassay. J. Clin. Lab. Anal. 13: 9-18.

Lee MK, Martin MA, Cho MW (2000). Higher Western blot immunoreactivity of glycoprotein 120 from R5 HIV type 1 isolates compared with X4 and X4R5 isolates. Aids Res. Hum. Retrov. 16: 765-775.

Luft S, Seme K, Poljak M (2004). Laboratory diagnosis of human immunodeficiency virus infection. Acta. Dermatoven APA. 13: 43-49.

Ma AS, Irene O, Irene F, Rigmor T, Birger S (2004). A simple semi-rapid HIV-1& 2 confirmatory immunoassay using magnetic particles. J. Virol. Methods, 115: 191-198.

Mahé C, Kaleebu P, Ojwiya A, Whitworth JAG (2002). Human immunodeficiency virus type 1 Western blot: revised diagnostic criteria with fewer indeterminate results for epidemiological studies in Africa. Int. J. Epidemiol. 31: 985-990.

Mas A, Soriano V, Gutiérrez M, Fumanal F, Alonso A, González-Lahoz J (1997). Reliability of a new recombinant immunoblot assay (RIBA

HIV-1/HIV-2 SIA) as a supplemental (confirmatory) test for HIV-1 and HIV-2 infections. Transf. Sci. 18: 63-69.
Organization WH (2009). HIV/AIDS prevention, treatment and care in the health sector.
Ravanshad M, Sabahi F, Mahboudi F (2006). Evaluation of a new dot blot assay for confirmation of human immunodeficiency virus type 1 and 2 infections using recombinant. Antigens-p. 24, gp. 41, gp. 120, gp. 36. Saudi Med. J. 27: 31-36.
Schuepbach J, Gebhardt MD, Tomasik Z, Niederhauser C, Yerly S, Buergisser P, Matter L, Gorgievski M, Dubs R, Schultze D, Steffen I, Andreutti C, Martinetti G, Guentert B, Staub R, Daneel S, Vernazza P (2007). Assessment of recent HIV-1 infection by a line immunoassay for HIV-1/2 confirmation. Plos Med. 4: 1921-1930.
Tesoro-Cruz E, Hernández-González R, Kretschmer-Schmid R, Aguilar-Setién A (2003). Cross-reactivity between caprine arthritis-encephalitis virus and type 1 human immunodeficiency virus. Arch. Med. Res. 34: 362-366.
UNAIDS (2008). Report on the global AIDS epidemic.
Uneke CJ, Alo MN, Ogbu O, Ngwu BAF (2007). Western blot-indeterminate results in Nigerian patients HIV serodiagnosis: The clinical and public health implication. Aids Patient Care St, 21: 169-176.
Wang WK, Chen MY, Chuang CY, Jeang KT, Huang LM (2000). Molecular biology of human immunodeficiency virus type 1. J. Microbiol. Immunol. Infect. 33: 131-140.