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Original Article

Evaluation of a New Anti-HIV1/2 ELISA–HIV 1/2 REC Diagnostic Kit Based on *E. coli* Derived Soluble Recombinant Proteins: Experience of an International Study

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Abstract

Background/Objective: Development of a new enzyme-linked immunosorbent assay (ELISA) for screening human blood serum and plasma for antibodies to human immunodeficiency virus type 1 (HIV1) and type 2 (HIV2) as HIV1/2REC ELISA diagnostic kit based on *E. coli* derived soluble recombinant proteins.

Methods: Polypeptides corresponding to HIV1 gp41 and HIV2 gp36 immunodominant regions and HIV1 gag were expressed in *E. coli* in fusion with thioredoxin (Trx) to obtain a highly purified (>98%) soluble refolded proteins, which was used as solid phase antigens for ELISA.

Results: The sensitivity and specificity of anti-HIV1/2 antibody detection were evaluated with representative panels of positive and negative sera. Positive panels included HIV1-positive Western-blot (WB)-confirmed specimens collected in Iran, Russia, and Uganda. Commercially available HIV1 and HIV2 seroconversion low titer and performance panels were also used. Negative panel was collected from random volunteer blood donors, risk group members, HCV-infected patients and individuals with non-HIV related conditions potentially influencing test results. The sensitivity of antibody detection with new kit was determined to be 100%. Specificity was determined to be 99.82%. It was shown than thioredoxin (Trx) did not change the immunodominant epitopes of HIV. These fusion proteins are recognized by human native antibodies. In addition, thioredoxin (Trx) would help natural refolding of HIV proteins by *E. coli*.

Conclusion: These characteristics of the new assay are comparable to those of majority of FDA-licensed and officially approved European diagnostic kits, which are currently available in the United States and Europe.
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Keywords • HIV ▪ enzyme-linked immunosorbent assay ▪ recombinant proteins

Introduction

Antibodies against HIV are found with high frequency in patients with HIV infection.[1] Screening of blood and blood products for antibodies to HIV has minimized the risk of transfusion-acquired HIV infection.[2] Most of the first generation ELISA tests is based on whole-cell viral lysate antigens. Second generation ELISA utilizes recombinant proteins and/or synthetic peptides. The latter offers the advantage of decreasing nonspecific reactions resulting from cross-reactivity with host cell antigens in cell-culture derived virus and is more sensitive.[3-6] Among antibodies appearing in blood as a response to HIV infection, those against *env* and *gag* encoded proteins are the most important for serodiagnosis.[7-9] In order to meet the needs of blood transfusion service in sensitive, specific and affordable HIV test we developed a new indirect ELISA based on HIV1 gp41, gag and HIV2 gp36 recombinant proteins (HIV1/2 REC diagnostic kit). Recombinant proteins containing highly conserved immunodominant fragments of HIV1 gp41, HIV2 gp36, and HIV1 gag expressed in *E. coli* higher than 98% purified soluble refolded proteins, and used as solid phase antigens for indirect ELISA.[10-11] In this report, we present the results of the evaluation of a HIV1/2 REC diagnostic kit using fusion proteins with thioredoxin (Trx).

Materials and Methods

Sera

Positive panel included **1)** Iranian collection of 603 confirmed HIV1 positive samples, collected in 1998–1999. **2)** Russian collection of 20 confirmed HIV1 positive samples, collected in 1986–1994 by the Institute of Immunology, Moscow. **3)** Ugandan collection of 150 HIV1 WB confirmed positive samples, collected in 1988. **4)** Russian Reference Low Titer Standard Panel, comprising 16 HIV1 positive samples and one HIV2 positive sample. **5)** BBI , HIV1 seroconversion panels AF (PRB931) and AI (PRB934). **6**) Anti-HIV2 Performance Panel, PRF 202, 15 members, including 14 HIV2 positive samples. HIV1 seroconversion panels AF (PRB931) and AI (PRB934) and Anti-HIV2 performance panel (PRF 202) were obtained from Boston Biomedica, Inc. (BBI, 375 West Street, West Bridgewater, MA 02379, USA). Iranian positive collection included the samples that were repeatedly reactive using licensed HIV1/2 ELISA's and further tested positive in Western blot assay.

Negative panel included: **1)** Iranian collection comprising 2800 random blood donors, that were nonreactive with commercial ELISA **2)** Russian collection of 510 random blood donors; 390 individuals with risk of HIV behavior, 32 patients with allergy and asthma, 16 patients with oncological diseases, 12 patients with autoimmune diseases, 73 HCV positive samples, total=1033; **3**) Russian Reference Negative Standard Panel, n=20; **4)** Ugandan collection 50 HIV negative blood donors, collected in 1988). A ground total of 3903 negative samples.

Expression of gp36, gag and gp41 fusion proteins with thioredoxin in E. coli

The *E. coli* k12 BL21 (DE3) (Novagen Company, USA) strains were transformed with pET expression system (Novagen Company, USA) containing gp36, gag and gp41. The immunogenic peptides of gp36 gene, gag, and gp41 were selected for expression and purification.[12]

Reference kits

ELISA kits—Following ELISAs were used as reference kits: Biotest based on HIV1 gp41, HIV1 p24 and HIV2 gp36 recombinant proteins (Germany); Genelavia Mixt based on HIV1 gp41 HIV2 gp36 synthetic peptides and HIV1 gp160 protein (Sanofi Diagnostics Pasteur, France); and GenScreen based on HIV1 gp41, HIV1 gp41 subtype O and HIV2 gp36 synthetic peptides and HIV1 gp160 protein (Sanofi Diagnostics Pasteur, France); Peptoscreen-2 based on HIV1 gp41 and HIV2 gp36 synthetic peptides (AmerCard, Russia); Vironostika HIV UNI-FORM II (Organon Teknika, Belgium) based on HIV1 gag and gp160 and synthetic peptides mimicking immunoreactive fragments of gp41HIV1 subtype O (ANT70) and HIV2 gp36.

Western blotting

New Lav Blot I (Sanofi Pasteur, France) and LiaTek HIV III (Organon Teknika, Belgium) were used for the confirmation of antibodies to HIV1 and HIV2. All assays were performed according to manufacturers' instructions.

Data analysis

Sensitivity (accuracy) of antibody detection was estimated as the percentage of Western blot confirmed HIV antibody positive samples found to be reactive by HIV1/2REC. Specificity was defined as the percentage of HIV negative samples that were determined as negatives by the assay under evaluation.

Results

Sensitivity

The results of evaluation of the sensitivity with Iranian, Russian and Ugandan collections of HIV1-positive samples are shown in Table 1. Sera were collected from individuals in different regions in

Table 1: Detection of antibodies to HIV1 and HIV2 with HIV1/2 REC in confirmed–positive samples from diverse geographic areas

Panel HIV1 positive, WB* positive	No. of specimen tested	No. of positive cases	No. of negative cases	No. of positive results / No. of positive samples	Sensitivity (accuracy), %
Iranian collection (1998–1999)	603	603	0	603/603	100 %
Russian collection (1986–1994)	20	20	0	20/20	100 %
Ugandan collection (1988)	150	150	0	150/150	100 %
Total	773	773	0	773/773	100 %

Table 2: Comparative sensitivity of HIV1 antibody detection by HIV1/2 REC and commercially available kits

	Day of the first positive test result[a] for the panel PRB931	PRB934
EIA test kit		
HIV 1/2 REC	28	7
Biotest [b]	28	–
Abbott HIV1 [b]	33	7
Abbott HIV1/2 [b]	28	7
Gen. Sys. HIV [b]	35	neg at day 11
Gen. Sys. HIV 1/2 [b]	33	11
Org Tek HIV [b]	33	7
Org Tek Uni–Form II HIV [b]	–[c]	–
Org Tek Uni–Form Plus[b]	–	7
Syva [b]	–	–
Diag. Past. Genelavia [b]	28	7
Diag. Past. Genscreen [b]	28	0
Abbott 3rd Gen. Plus [b]	28	7
Abbott AxSYM [b]	28	7
Abbot ImxPlus	28	7
Abbot PRISM	28	7
Behring ENZ Plus [b]	28	7
Behring [b]	–	–
Ortho Capture [b]	28	–
Wellco [b]	–	–
CBC HIV [b]	–	–
CPI HIV [b]	–	–
BioChem [b]	–	–
Biochem Detect [b]	28	–
Boeh. Mann. Enzymun Gen 3 [b]	28	7
Innogenetics Innotest [b]	–	7
Murex/Well. HIV1/2 [b]	–	7
Murex ICE HIV 1.O.2 [b]	–	7
Roche DAGS [b]	–	7
Fujirebio Serodia HIV1/2 [b]	–	7
Western Blot kit		
Bio Rad Western Blot [b]	35	ind. At day 11
Ortho/Cambridge Western Blot [b]	33	7

[a] Data appear as the days after first blood drawing, according with BBI data sheet. Days of sampling are shown in table 3.
[b] Data provided by BBI
[c] no data

HIV1 were detected by HIV 1/2 REC in all 773 of 773 HIV1 infected individuals. Infection was confirmed by commercially available ELISAs and western blot assays. No false negative cases were found. Sensitivity of HIVl/2 REC was reported as 100 %.

BBI Anti-HIV2 performance panel (PRF202) was used to evaluate the ability of HIV1/2 REC to identify anti-HIV2 antibodies. The assay detected antibody in all fourteen positive samples, and one negative sample was detected as negative.

A critical parameter of assay sensitivity is its ability to identify the cases of early infection and low titers of antibodies. HIV1/2 REC ELISA was evaluated for its sensitivity in detecting low titer of antibodies to HIV1 and HIV2 with the use Russian Reference Low Titer Standard Panel. All 16 HIV1–positive samples and one HIV2 positive sample were identified as positive (data not shown). Table 2 shows the days of first positive result of HIV1/2 REC, in comparison with US FDA licensed and European kits when evaluated against HIV1 Seroconversion Panels AF (PRB931) and AI (PRB934). HIV1/2 REC and 12 other licensed kits have shown the earliest positive result with panel PRB931 on day 28 subsequent to the first bleed, as well as. Three other tests detected antibody at day 33, and one at day 35. HIV1/2 REC and other 17 tests detected antibody on day 7. Diagnostics Pasteur Genscreen detected antibody on day 0,which was 7 days earlier than HIV1/2 REC whereas antibody detected by Genetic Systems HIV1/2 on day 11 after bleeding, none of these samples were tested positive by Genetic Systems HIV1 ELISA. Thus the efficiency of HIV1/2 REC parallels other licensed HIV ELISA kits.

Specificity

Table 3 shows the results of evaluation of test specificity. The HIV1/2 REC in the random–donor population (2800 samples from Iran and 510 sam-

Table 3: Detection of antibodies to HIV1 and HIV2 with HIV1/2 REC in negative samples

	Panel source	No. of specimen tested	No. (%) of repeatedly reactive specimens	No. of negative cases	n(–) results/n(–) samples	No. of specimens positive in Genscreen EIA	No. of Western blot positive specimens	Specificity %
1	Iranian collection (random blood donors, total 2800)	2800	6 (0.22 %)	2794	2794/2800	0	0	99.78
2	Russian collection (total 1033)							
	random blood donors	510	1 (0.20 %)	509	509/510	0	0	99.80
	risk of HIV behavior	390	0	390	390/390	0	0	100
	patients with allergy and asthma	32	0	32	32/32	0	0	100
	patients with oncological diseases	16	0	16	16/16	0	0	100
	patients with autoimmune diseases	12	0	11	11/12	0	0	100
	HCV positive patients	73	0	73	73/73	0	0	100
3	Russian Reference Negative Standard Panel	20	0	20	20/20	0	0	100
4	Ugandan collection (random blood donors, total 50)	50	0	50	50/50	0	0	100
	Total	3903	7	3896	3896/3903	0	0	99.82

ples from Russia, total 3360 samples) gave a repeatedly reactive rate of 0.21 % (7 samples). None of fifty blood donors from Uganda who were tested negative earlier, showed positive result when tested by HIV1/2 REC. None of 390 samples from patients with risk behavior was reactive. Additional data on specificity of detection were obtained by testing samples from individuals with clinical conditions potentially interfering with the results (allergy and asthma, autoimmune conditions, oncological diseases, HCV infection). All 20 samples Russian Reference Negative Standard Panel members were detected as negatives. No false positive results were obtained. Thus the specificity was 99.82%.

Discussion

The HIV1/2 REC ELISA is the indirect assay for the detection of both HIV1 and HIV2 antibodies. The test utilizes *E. coli* derived soluble recombinant proteins corresponding to HIV1gp41, HIV2 gp36 immunodominant regions and HIV1 gag as the antigens adsorbed onto the solid phase. In this report, we present the results of the evaluation of HIV1/2 REC ELISA's sensitivity and specificity.

The sensitivity of anti HIV1 antibody detection was estimated to be 100 % (773/773) in the populations from geographically diverse regions (Russia, Iran and Uganda), where multiple distinct genotypes of HIV1 have been documented.[12-15] The HIV1/2 REC detected HIV1 positive samples independently of circulating viral variant, population and region. The sensitivity of anti HIV2 antibody detection was estimated to be 100 % (14/14) with Anti–HIV2 PRF202 performance panel.

Additional studies of sensitivity were conducted on seroconversion panels PRB931 and PRB934. HIV1/2 REC ELISA and 12 other licensed kits detected antibody to HIV1 in the specimen PRB931–

06 (day 28 since first bleed). Two assays based on whole–cell viral lysate detected antibody on day 33, and one on day 35. The earliest detection of antibody for panel PRB934 by HIV1/2 REC and 17 other tests was on day 7, whereas Genetic Systems HIV 1/2 based on whole–cell viral lysate has showed positive result only on day 11, and Genetic System HIV ELISA showed negative results for the samples drawn on days 7 and 11.

Specificity of HIV1/2 REC was determined as 99.82 % thus being in the range of the most FDA approved commercial kits. (WHO acceptance level is 95% for HIV1/2 diagnostic kit.[16] The six repeatedly reactive samples from Iranian donor's population were also false positive when Biotest Anti HIV1/2 was used. However these samples were reported as negative when tested by Vironostika HIV UNI–FORM II and GenScreen ELISA's and New Lav Blot I. We tested the falsely positive samples by ELISA with recombinant antigens rec gp41 HIV1, rec gp36 HIV2 and rec gag HIV1 adsorbed onto separate wells of solid phase. The resultant non–specific reactivity of the test was found to be due to cross–reactivity with recombinant gag antigen.

Tropical parasitic diseases such as malaria, shistosomiasis and leishmaniasis are known to be accompanied by hypergammaglobulinemia, which is a potential cause of false reactivity.[17]..Iran and Uganda are endemic areas for *Leishmania, Shistosoma* and malaria infections. However none of 50 samples from Ugandan blood donors showed false positive, and the specificity of the test value obtained for Iranian donor's population was as high as for the Russian donors, due to the purity of recombinant antigens used.

Evaluation of HIV1/2 REC with sera samples obtained from 73 HCV–positive patients (majority of whom were hemophiliacs with multiple blood transfusions) showed 100 % specificity, same rate was observed for the panel of samples from persons with clinical entities other than HIV infection and for samples containing potentially interfering substances (Table 3).

The sensitivity of the antibody detection with HIV1/2 REC ELISA determined with representative panel of 773 positive sera collected in geographically diverse areas were found to be 100 %. The assay demonstrated 99.82 % specificity when evaluated by panel of 3903 negative samples, including those obtained from individuals with conditions potentially interfering with the test results. Thus the sensitivity and specificity of the test were shown to be comparable with those of the majority of commercially available kits in the USA and Europe.

Whereas the seroconversion panel PRB934 tested with HIV1/2 REC ELISA and 17 other kits demonstrated the first positive results on day 7, Genscreen (Sanofi Diagnostics Pasteur) detected antibody in samples drawn on day 0. While blood samples–seroconversion panel members–were not drawn daily, and thus the exact period prior to antibody detection could not be precisely determined, Genscreen demonstrated higher sensitivity than other tests (Table 2). This assay is based on the double–antigen sandwich format and detects of all classes of antibodies, and such third generation ELISA constitute the most sensitive tests.[18-20]

Iran and Russia are the countries with low HIV prevalence. Thus it is especially important to use a diagnostic kit with high sensitivity, even at the expense of lower specificity. Since our preparation of recombinant antigens, especially that of gag, retained their native and naturally folded configurations, they can easily be used for sandwich format, which provides excellent sensitivity and specificity. Similar international studies can facilitate HIV investigations and allow the evaluation of the test with sera collected from genetically different populations and geographically diverse areas with different circulating HIV subtypes. This would expedite the development of sensitive, specific and affordable assays.

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