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STANDARDIZATION AND VALIDATION OF A WESTERN BLOT FOR THE DIAGNOSIS OF HUMAN IMMUNODEFICIENCY VIRUS

ESTANDARIZACIÓN Y VALIDACIÓN DE UN WESTERN BLOT PARA EL DIAGNÓSTICO DEL VIRUS DE INMUNODEFICIENCIA HUMANA

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ABSTRACT

Objectives: To standardize and validate a western blot test for the diagnosis of human immunodeficiency virus. **Methods:** A prospective observational study was carried out during 2017 and 2018. The western blot test was standardized, using the polyacrylamide gel electrophoresis technique with sodium dodecyl sulfate (SDS PAGE), being the nitrocellulose blot strips prepared with an Optimal HIV-1 antigen concentration of 2.71 µg / mm. The western blot was validated in the laboratory against 400 reference samples (300 sera and 100 plasmas): 200 positive and 200 negatives for antibodies against HIV-1, being the reference test the Immunoblot of the Fujirebio brand. Diagnostic performance parameters were estimated using Epidat v3.1 and Excel. **Results:** Eight important bands of the HIV-1 antigen were identified: p17, p24, p31, p39, gp41, p55, p66, and gp120. According to the Consortium for the normalization of serology for retroviruse, those that were taken as specific diagnostic bands were: p24, p31, gp41, and gp120. The sensitivity, specificity, positive and negative predictive value and validity index against sera were: 96.7%, 96.0%, 96.0%, 96.6%, 96.3%; and against plasmas: 98.0%, 100.0%, 100.0%, 98.0%, 99.0% respectively. No false positives and negatives were found, but some were undetermined. **Conclusions:** The development of this western blot test with proprietary technology presented similar diagnostic performance to the reference test, without showing cross-reactions, being useful for confirming HIV.

Key words: Western Blotting; HIV; Diagnosis; Sensitivity and Specificity (source: MeSH NLM).

RESUMEN

Ojetivos: Estandarizar y validar una prueba de western blot para el diagnóstico del virus de inmunodeficiencia humana. **Métodos:** Se realizó un estudio observacional prospectivo durante el 2017 y 2018. Se estandarizó la prueba de western blot, usando la técnica de electroforesis en gel de poliacrilamida con dodecil sulfato de sodio (SDS PAGE), siendo las tiras blot de nitrocelulosa preparadas con una concentración óptima de antígeno de VIH-1 de 2,71 µg/mm. Se validó el western blot en laboratorio, frente a 400 muestras referentes (300 sueros y 100 plasmas): 200 positivas y 200 negativas a anticuerpos contra VIH-1, siendo la prueba de referencia el Inmunoblot de la marca Fujirebio. Se estimaron los parámetros de rendimiento diagnóstico usando el programa Epidat v3.1 y Excel. **Resultados:** Se logró identificar ocho bandas importantes del antígeno de VIH-1: p17, p24, p31, p39, gp41, p55, p66 y gp120. De ellas, las que se tomaron como bandas diagnósticas específicas según el Consorcio de normalización de serología para los retrovirus, fueron: p24, p31, gp41 y gp120. La sensibilidad, especificidad, valor predictivo positivo y negativo e índice de validez frente a sueros fueron: 96,7%, 96,0%, 96,0%, 96,6%, 96,3%; y frente a plasmas: 98,0%, 100,0%, 100,0%, 98,0%, 99,0% respectivamente. No se encontraron falsos positivos y negativos, pero si algunos indeterminados. **Conclusión:** El desarrollo de esta prueba western blot con tecnología propia, presentó similar rendimiento diagnóstico a la prueba de referencia, sin mostrar reacciones cruzadas; siendo útil para la confirmación del VIH.

Palabras clave: Western blot; VIH; Diagnóstico; Sensibilidad y Especificidad (fuente: DeCS BIREME).

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INTRODUCTION

In Peru, within the strategies for the approach to infection by the human immunodeficiency virus (HIV) is the coverage of the diagnosis[1], so much so that there are serological screening tests (ELISA, chemiluminescence and rapid tests) and confirmation: indirect immunofluorescence (IIF) and immunoblot (IB) or western blot (WB)[2,3]. The IIF is a test with its technology[4], relatively inexpensive and constitutes 95% of the confirmations at the national level, however when the result is indeterminate or nonspecific, the IB or WB is used for having superior diagnostic performance[3].

The commercial IB of the Fujirebio brand INNO-LIA HIV I / II Score (Online Immunoassay: sensitivity 100.0% and specificity 96.7%) is the kit most used as a second opinion test for samples that did not resolve IIF[5], being at the same time applied in numerous studies as a gold standard[6-10], but its main disadvantage is that its costs are high, approximately $800 for 20 determinations[5].

Trademarks available from WB include MP Diagnostics HIV BLOT 2.2 (sensitivity: 100.0%; specificity: 91.9%)[11], Biorad NEW LAV-BLOT I (sensitivity: 87.0%; specificity: 99 , 5%)[12] and Biokit bioblot HIV-1 Plus (sensitivity: 100.0%; specificity: 91.9%)[13]. Although these three brands do not report false positive and negative results (only indeterminate), their use is very limited due to the expensive prices attributed to their kits (1,000 to 1,200 dollars for 18 determinations)[11-13].

The western blot, immunoblot, or immunoblotting is a useful test in identifying antibodies against HIV. It consists of separating the viral antigens by electrophoresis in polyacrylamide gels, then transferred to a nitrocellulose membrane, which will be subsequently exposed with the antibodies of the serum or problem plasma. When they come into contact with an anti-immunoglobulin labeled with an enzyme, they will react immunologically, giving rise to a pattern of bands, which will be interpreted under some of the criteria described by international organizations[14].

It should be said that, in Peru, around 1,200 annual samples are resolved by immunoblot/western blot[15], whose expenses reach more than 60,000 dollars per year. Therefore, due to the need for a low-cost western blot with our technology and the high prices of commercial kits, we set ourselves the following objective: Standardize and validate a western blot to diagnose human immunodeficiency virus.

METHODS

Design and study area

Prospective observational study of standardization and validation of a diagnostic test carried out during 2017 and 2018 at the National Institute of Health (INS) of Peru. Under this approach, the design is appropriate to estimate the capacity of a measure that allows discriminating between people with the disease and without the disease.

Population and sample

The population consisted of sera and plasmas that belonged to the serum and plasma library of the National Reference Laboratory of STV-HIV / AIDS of the INS during the years 2016 and 2017. The estimation of the sample size was defined by convenience, using a non-probabilistic sampling, with 400 reference samples: 150 HIV-1 positive sera; 150 HIV-1 negative sera (30 from healthy individuals and 120 from individuals with the following conditions: twenty with syphilis, twenty from pregnant women, twenty with rheumatoid disease (RD), twenty with hepatitis B, twenty with HTLV-1, ten with cytomegalovirus and ten with dengue); 50 HIV-1 positive and 50 negative plasmas. The Reference or Gold Standard test was the commercial IB (INNO-LIA ™ * HIV I / II Score; Fujirebio, Belgium)[5].

Variables and instruments

The standardization and validation of a diagnostic test implied considering the following variables: Sensitivity, Specificity, Validity Index, Positive Predictive Value (PPV), Negative Predictive Value (NPV), Youden Index, and Negative Likelihood Ratio. Three stages were previously carried out to measure these variables.

Optimization in the preparation of the HIV-1 antigen. - The cell cultures of the H9 / HTLV-IIIB line infected with HIV-1 were prepared, following the methodology described by Romero -Ruiz et al. (16). The cell supernatant was washed and centrifuged three times with 0.9% physiological saline solution (SSF) at 4,400 rpm for 15 minutes at 2 to 8 ° C. The sediment or pellet was then resuspended with SSF. A sonicator was used to carry out the viral and cell lysis at 6 periods of 60 decibels with 2 minutes per period and one minute of rest between periods, maintaining the cold chain (2 to 8 ° C).

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The sonicated product was centrifuged at 1,500 rpm for 10 minutes, and its supernatant was registered as HIV-1 antigen. Finally, the Bradford method[17] was used to quantify the HIV-1 antigen proteins.

Standardization in the preparation of Western blot strips.- This step was standardized following electrophoresis methodology in polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE)[18]. The Mini-Protean vertical electrophoresis chamber (Bio-rad) was used for 1.0 mm gels (gel size: 8.3 by 7.3 cm). The percentage of acrylamide was optimized to prepare the resolving gel, establishing two concentrations (mixed) 9% (3.8 cm) and 15% (2 cm). The antigen treatment, separation, and electrophoretic transfer were carried out following the methodology described by Miranda et al. (19). Finally, the optimal concentration of the HIV-1 antigen was established to prepare the WB strips, being 2.71 µg / mm.

Optimization in the Immunoenzymatic reaction of the Western blot test.- The optimum volume that was established in all the steps of this stage was 1 mL. The blot strips were incubated for three hours in PBS / tween-milk (PBS / 0.1M NaCl, 0.05 M Na2 PO4, pH 7.2; Tween 20 / 0.3% tween 20; milk / 5% milk) containing the sera or plasmas of the study at a 1/40 dilution. Next, the nitrocellulose strips were washed five times with PBS / Tween-20 and incubated for one hour in PBS / tween-milk containing the conjugate Anti-human IgG linked to a peroxidase, at a dilution of 1/1000. The strips were again washed three times with PBS / Tween-20 and twice with PBS. Bands were developed against a substrate solution (30% hydrogen peroxide at 1uL / mL and diaminobenzidine at 0.5 mg / mL in PBS pH 7.2). The reaction was stopped by washing the strips with distilled water.

Procedures

Reading, interpretation, and validation of the Western blot test.- For the positivity criterion, the Consortium guidelines for the normalization of retrovirus serologies (CRSS)[11-14,20] were considered. POSITIVE: A band of p24 or p31 and an ENV band (gp41 or gp120). NEGATIVE: No specific viral band present. UNDETERMINED: Any specific viral band present, but the pattern does not meet the criteria for positive. With these criteria, the WB was validated, performing the readings and interpretations of the 400 reference samples incorporated into the study.

Statistical analysis

The analysis was estimated using a contingency table, the Epidat v3.1 program, and Excel. The percentages of Sensitivity, Specificity, Validity Index, Positive Predictive Value (PPV), Negative Predictive Value (NPV), Youden Index, and Negative Likelihood Ratio were reported, considering a 95% confidence level (95% CI).

Ethical considerations

The Institutional Research Ethics Committee approved the study protocol of the National Institute of Health of Peru with the code: OI-022-14 and with Directorial Resolution N °: 418-2014-DG-OGITT-OPE / INS. Likewise, the General Office for Research and Technology Transfer approved the final report of this study with MEMORANDUM N ° 027-2019-OGITT / INS.

RESULTS

Among our findings, we were able to show eight important bands corresponding to the HIV-1 viral proteins: p17, p24, p31, p39, gp41, p55, p66, and gp120. Of these, those that were taken as specific diagnostic bands according to the CRSS were: p24, p31, gp41, and gp120 (Figure 1).

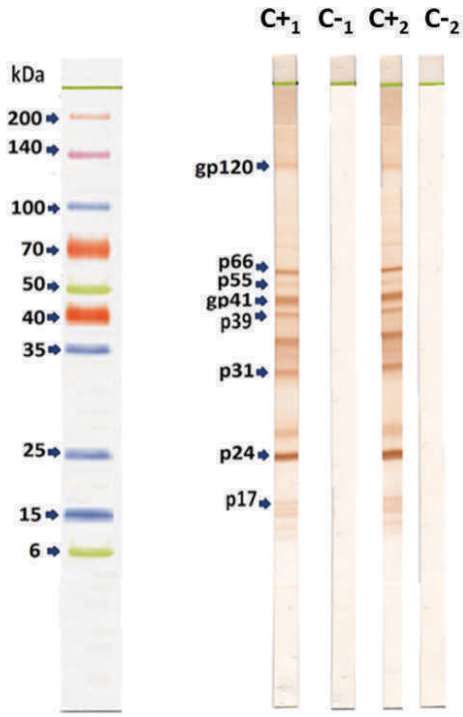

Figure 1. Reactivity pattern of the western blot test for the detection of antibodies: (C +1) HIV positive in serum; (C-1) HIV negative in serum; (C +2) HIV positive in plasma; (C-2) negative for HIV in plasma.

Among other findings, we highlight that against the serum samples, there were eleven indeterminate results (5 from the positive panel and 6 from the negative panel), and against the plasma samples, there was an indeterminate from the positive panel. Said results of the WB test are shown in the flow diagram of samples incorporated into the study (Figure 2).

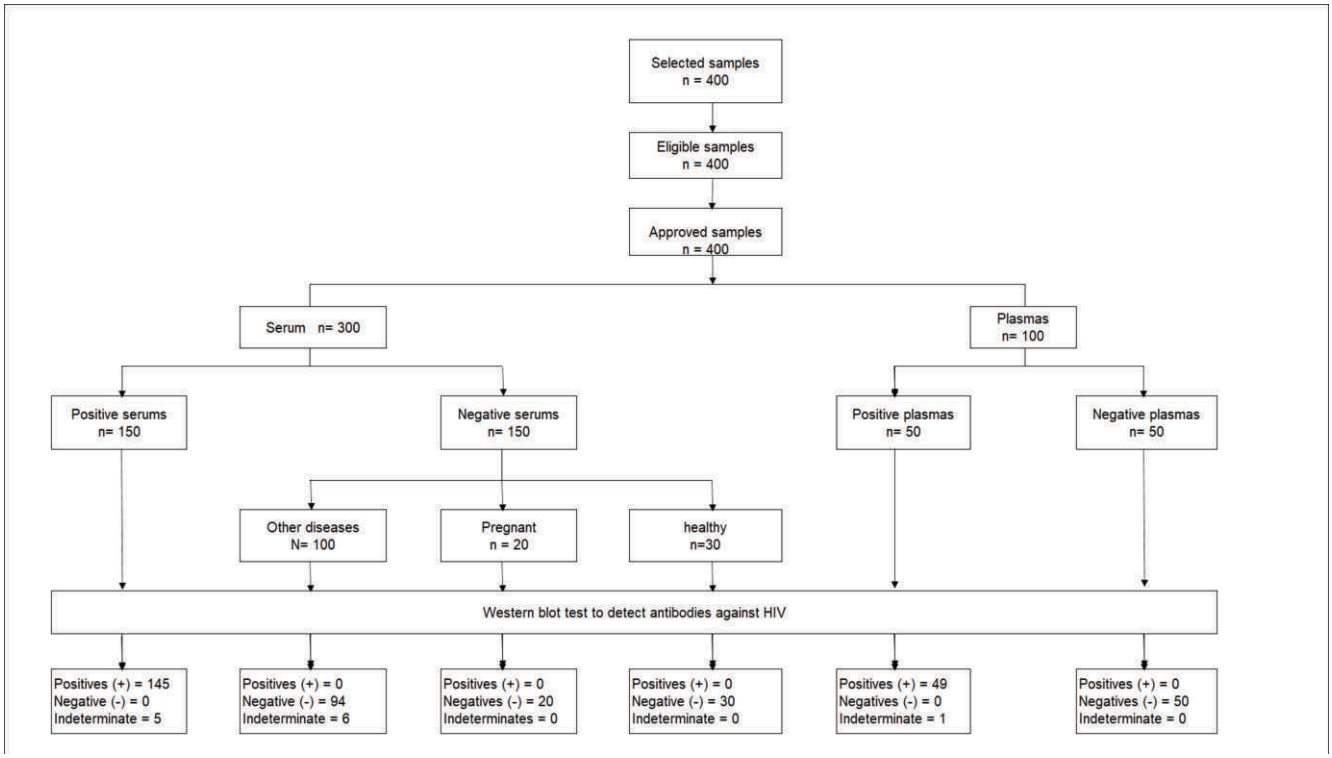

Figure 2. The flow of samples incorporated into the study.

An important highlight of this WB test is that it did not show false positives and negatives. However, only some serum samples had indeterminate results: syphilis (2/20), hepatitis B (2/20), ER (1/20), and HTLV-1 (1/20) (Table 1).

Table 1. Validation of the Western blot test to detect antibodies against HIV using positive negative and interfering samples.

Condition	Quantity	Western Bloth VIH				
		True positives	False positives	True negatives	False negatives	Undetermined
Serums						
HIV-1	150	145	-	0	0	5
Syphilis	20	-	0	18	-	2
Pregnant	20	-	0	20	-	0
ER*	20	-	0	19	-	1
Hepatitis B	20	-	0	18	-	2
HTLV-1	20	-	0	19	-	1
CMV**	10	-	0	10	-	0
Dengue	10	-	0	10	-	0
Healthy	30	-	0	30	-	0
Plasmas						
HIV-1	50	49	-	0	0	1
HIV-1 negative	50	-	0	50	-	0
Total	400	194	0	194	-	12

*ER: Rheumatoid disease. **CMV: Cytomegalovirus

Among the main achievements, we show that the results of the validation parameters (sensitivity, specificity, PPV, NPV and Validity Index) for our WB test were higher than 96.0%. In the same way, the Youden index with the values of 0.93 and 0.98, confirms a minimal possibility of obtaining false positives or false negatives; as well as a low negative likelihood ratio of 0.03 and 0.02, which is consistent with the other results (Table 2).

Table 2. Parameters of the Western blot test for the detection of antibodies against HIV against serum and plasma samples

Parameters	Western Bloth VIH	
	Serum	Plasma
	CI value (95%)	CI value (95%)
Sensitivity (%)	96,7 (93,5 - 99,9)	98,0 (93,1-100,0)
Specificity (%)	96,0 (92,5 - 99,5)	100,0 (99,0 - 100,0)
Validity Index (%)	96,3 (94,0 - 98,6)	99,0 (96,6 - 100,0)
Predictive value + (%)	96,0 (92,6 - 99,5)	100,0 (99,0 - 100,0)
Predictive value - (%)	96,6 (93,4 - 99,9)	98,0 (93,3 - 100,0)
Youden index	0,93 (0,88 - 0,97)	0,98 (0,94 - 1,02)
Likelihood ratio	0,03 (0,01 - 0,08)	0,02 (0,0 - 0,14)

IC 95%: Confidence interval at 95%

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DISCUSSION

The reference test used in the present study is the most used in Peru[2,3,6,15], and other countries[5, 7-10], the insert reports a sensitivity of 100% and specificities of: 96.7% (blood bank) and 96.1% (clinical samples). This kit incorporates recombinant proteins and synthetic peptides from HIV-1 and HIV-2[5]. The sensitivity and specificity of our WB using antigenic proteins from HIV-1 lysis were higher than 96.0%; even so, we did not have false negatives and positives, which allows us to deduce that our results for the same parameters are similar.

Western blot kits NEW LAV BLOT I (Biorad)[12] and bioblot HIV-1 plus (Biokit)[13] using the CRSS positivity criteria, report sensitivities of 99.5% and 94.9% and specificities 87.0% and 91.9%, respectively. At the same time, they report that they did not have false negatives and positives, only indeterminate[12,13]. Likewise, the Immunoblot Recom Line HIV-1 & HIV-2 IgG kit (Mikrogen diagnostik)[21], reports a sensitivity of 100% and specificities of: 99.3% (blood bank), 98.5% (samples clinical) and 96.4% (interfering). Our WB test for the same parameters showed results comparable to the three western blot / Immunoblot brands described.

Due to the indeterminate results obtained, a sensitivity of 100.0% was not achieved; This could be due to the fact that the antibodies against p24 and p31 decrease during the course in the AIDS phase, which causes a displacement of the interpretation from positive to indeterminate[11]; however, we do not know if the samples came from patients in this phase.

To establish specificity (sera), indeterminate results represented 4.0% (6/150); It should be said that, for the calculations in other validation studies, indeterminates are not considered in the category of false positives[22], being this way, we would have a global specificity of 100.0%, in the 144 samples (144/144). In general, the indeterminate (12/400) that we obtained (Table 1) does not affect the diagnostic efficiency of our WB since the Peruvian technical standard indicates to perform the HIV-1 viral RNA PCR test (viral load) on the indeterminate or proviral-HIV-1 DNA PCR[2].

Our standardized and validated test followed a WB design developed for parasites, which had excellent results in Peru[19,23-25]. The different commercial brands of immunoblot/western blot for HIV use a conjugate containing the enzyme alkaline phosphatase, the substrate 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium[5,11-13,21]. While in our WB we used a conjugate containing peroxidase, the substrate hydrogen peroxide and diaminobenzidine

as a chromogen, similar to these reagents they were used in the WB DAVIH-BLOT kit from Cuba, which showed good diagnostic performance against samples serum, urine and oral fluid[26].

In addition, the strip size format and the volume to be used are similar to the Reference test[5], while the other commercial brands use larger blot strips and consequently require double the volume of reagents. The aforementioned conditions are important to highlight because our methodology saves, since it uses fewer reagents. At the same time, we prepare our viral antigen, having the advantage that the INS has a Biosafety Level III Laboratory, where it is performing the maintenance and development of cell cultures of the H9 / HTLV-IIIB line infected with HIV-1. Consequently, our WB test would cost up to ten times less than commercial kits.

The WB test was standardized and validated in the laboratory, being a limitation of the study in completing its validation in the field. It can be implemented in the reference laboratories of Peru.

CONCLUSION

The results of the parameters obtained in the WB test for the detection of antibodies against HIV qualify it as a test of good diagnostic performance and make it useful for serological confirmation. Consequently, we recommend its use in the National Reference Laboratory of the INS Sexually Transmitted Virus Test as an alternative assay to commercial confirmatory tests in addition to significantly reducing costs in HIV confirmation.

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