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Validation of Anti-Hiv 1/2 Confirmatory Immunoblot Assay and Development of Algorithm For Anti-Hiv Testing At A Specialized Healthcare Facility In Pakistan

Ali Raza, Noor Fatima Shahid, Muhammad Numan, Anam Yousaf

Pakistan Kidney and Liver Institute and Research Center Lahore, Pakistan

ABSTRACT

Objective: To validate the Anti-HIV 1/2 confirmatory, immunoblot assay and develop an optimized diagnostic algorithm and enhance Human Immunodeficiency Virus (HIV) detection by integrating serological and molecular methods.
Study Design: Cross-sectional analytical study.
Place and Duration of Study: Pakistan Kidney and Liver Institute and Research Center (PKLI&RC), Lahore, Pakistan, from Feb to Jul 2024.
Methodology: This study evaluated the diagnostic performance of the Anti-HIV 1/2 immunoblot assay and proposed a multi-step algorithm for HIV detection. Participants included hospitalized patients and blood donors. Initial screening were confirmed using Elecsys® HIV Combi PT assay through Electrochemiluminescence Immunoassay (ECLIA). Reactive samples were confirmed using the Bio-Rad Geenius™ HIV 1/2 confirmatory assay and Nucleic Acid Testing (NAT). Additional testing was performed using the Bioline™ HIV Immunochromatographic Test (ICT).
Results: Sixty participants were enrolled. Both immunoblot and ICT assays exhibited 100.00% sensitivity and specificity. NAT Polymerase Chain Reaction (PCR) demonstrated 100.00% sensitivity and 92.30% specificity, detecting acute infections missed by serological tests.
Conclusion: Integrating serological and molecular diagnostic improves HIV detection accuracy. The proposed algorithm, including ECLIA, immunoblot, and NAT, proved reliable. Modifying the ECLIA cutoff to ≥ 75.215 improved specificity from 57.70% to 100.00% while maintaining 100.00% sensitivity.

Keywords: HIV testing, Anti-HIV antibody, Immunoblotting, Predictive Value of Tests Sensitivity and Specificity.

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INTRODUCTION

Human Immunodeficiency Virus (HIV) is a significant public health challenge which has claimed 42.3 million lives to date.[1] The highest burden is in Africa, with an estimated 20.8 million cases (53.00% of global infections).[2] HIV pathogenesis progresses through acute, chronic, and advanced stages, with early symptoms often absent or nonspecific, making clinical diagnosis and early detection difficult.[3] The World Health Organization (WHO) and diagnostic guidelines from Europe [4], the United Kingdom (UK) [5], and the United States (US) recommend a diagnostic algorithm that includes a fourth-generation antigen-antibody screening assay, followed by a confirmatory test capable of differentiating HIV-1 and HIV-2.[6] These advanced diagnostics allow simultaneous detection of p24 antigen and HIV antibodies, offering improved sensitivity and earlier diagnosis while, if results are indeterminate, Nucleic Acid Testing (NAT) is advised as a third-tier confirmatory step.[7] As of 2024, there are 297,052 reported cases in Pakistan.[8] The highest prevalence is among young individuals aged 18-30, with males being more vulnerable than females.[9] In Pakistan, the National AIDS Control Program (NACP) is responsible for developing HIV testing policies, however, standardized diagnostic algorithms are inconsistently implemented, and reliance on single assay testing risks false results.[10] Despite the availability of advanced serological and molecular assays, Pakistan lacks a validated, multi-assay HIV diagnostic algorithm that is both accurate and cost-effective. This study aims to evaluate the diagnostic performance of the Anti-HIV 1/2 immunoblot assay and develop optimized testing by combining ECLIA, NAT PCR, and ICT to ensure greater reliability in HIV detection and diagnosis.

METHODOLOGY

A cross-sectional analytical research design was used for the evaluation of the anti-HIV 1/2 confirmatory immunoblot assay. The study was conducted in full compliance with the ethics

Correspondence: Dr Ali Raza, Pakistan Kidney and Liver Institute and Research Center Lahore, Pakistan
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guidelines, which were reviewed and approved by the Institutional Review Board (IRB) of the PKLI&RC on January 29, 2024 (PKLI-IRB/AP/179). As the suspected cases are less frequent in our setup therefore suspected patients who visited during February 1 to July 31, 2024 (six months) at PKLI&RC, Lahore, Pakistan. The sample size was calculated according to the Clinical and Laboratory Standards Institute (CLSI) directive, that is, EP12-A2, giving user guidelines on the assessment of performance of qualitative tests especially the sensitivity and specificity.[11] A sufficient number of positive (30) and negative (30) samples were aimed at to comply with the requirements of the recommended confidence interval of 95%. Through 5,158 screening tests, 33 cases of HIV-positivity were noted, with the overall frequency being about 0.63 % in the period of the study. Using this, a one-proportion sample size calculation that was based on a 95% level of confidence and a 3% percentage margin of error confirmed the minimum and required sample size to be 27 and was rounded off to 30. Convenience sampling was done on 30 selected out of the confirmed HIV positive cases, as three borderline cases were rejected. Also, 30 samples that tested negative to HIV were taken randomly to act as controls. The research assessed the diagnostic accuracy of serological (ECLIA, ICT, and Immunoblot) and molecular (NAT PCR) HIV tests by comparing sensitivity, specificity, and predictive values. The study population was divided into patients (n=28) and voluntary blood donors (n=32) to ensure a diverse dataset representing a healthy population.

Inclusion Criteria: Samples belonging to male and female patients, of any age, tested with anti-HIV antibody testing via Elecsys HIV Combi PT assay (Roche Diagnostics, Switzerland) for ECLIA.

Exclusion Criteria: Incomplete clinical records or confirmed HIV cases.

Plasma/serum samples were collected through venipuncture and centrifuged at 5000 rpm for 10 minutes following standard protocols and stored at -80°C until analysis. Initial HIV screening was performed using the Elecsys HIV Combi PT assay (Roche Diagnostics, Switzerland), which detects HIV antigens and antibodies through a sandwich electrochemiluminescence immunoassay (ECLIA) on the Roche Cobas-C601 platform. Reactive samples were confirmed using the Bio-Rad Geenius™ HIV 1/2 Confirmatory Assay (Bio-Rad Laboratories, USA), an immunoblot-based assay that distinguishes between HIV-1 and HIV-2. This assay targets six HIV proteins including gp41, p24, gp160, gp31 (HIV-1) and gp140, gp36 (HIV-2). Band results were interpreted by one specialist and verified by another, per manufacturer guidelines. All samples were further evaluated using the BiolineTM HIV 1/2 ICT rapid test. A third confirmatory step involved nucleic acid testing (NAT PCR) using a multiplex PCR kit on the Cobas 5800 (Roche Molecular Systems, USA) for detecting HIV RNA. This multi-assay approach was used to assess diagnostic accuracy and validate a refined testing algorithm suitable for resource-limited clinical setting. Data was analyzed using SPSS (Statistical Package for the Social Sciences) v25.00 (IBM Corp., USA). Categorical data like gender and HIV status were presented in the form of frequencies and percentages. The association between standardized and modified values by confirmatory tests was assessed by Chi square test, in case of ≤5expected count Fisher's Exact. Shapiro Wilk Test showed that age (p-value <0.001) and ECLIA results (p-value <0.001) were not normally distributed. Therefore, numerical data like age and ECLIA were represented in Median and Interquartile Range (IQR). Mann Whiteny U test was applied to determine significant differences of age and ECLIA regarding patient and donors. Receiver Operating Characteristic (ROC) curve analysis assessed immunoblot and NAT PCR performance, the Area Under the Curve (AUC) evaluating ECLIA sensitivity and specificity.

RESULTS

Of the 60 participants, 51(85.00%) were male and 9(15.00%) were female. The median age was 29 (IQR 38.75-23.25) years, while the median age of males was 27(IQR 37-22) years and that of females was 41 (IQR 59-30.5) years. Among the males, 19(37.30%) were patients, and 32(62.70%) were donors, whereas all the female participants were patients. Nine individuals (15.00%) were identified as HIV-positive, and all were from the patient group. These findings highlight the demographic and clinical differences between the patient and blood donor groups. The baseline characteristics of the study participants are shown in Table I.

Table I indicates, age was statistically similar in both donors and Patients whereas all the donors were male (p <0.001) and HIV status was positive in patients only (p < 0.001) which strongly correlates with patient status, ensuring the reliability of results.

Table-I: General Characteristics of Study Participants (N=60)

Characteristic	Total (n=60)	Patients (n=28)	Blood Donors (n=32)	*p*-value*
Age (years) Median(IQR)	29.0(38.8-23.3)	34(43.3-25.3)	26.0(32.0-22.0)	0.055Ψ
Gender				
Male	51(85.00%)	19(67.90%)	32(100.00%)	<0.001*
Female	9(15.00%)	9(32.10%)	0(0.00%)	
HIV Status				
Positive (Confirmed)	9(15.00%)	9(32.10%)	0(0.00%)	<0.001*
Negative	51(85.00%)	19(67.90%)	32(100.00%)	

**p-value based on Fisher's Exact Test, Ψp-value based on Mann Whitney U test*

Table II summarizes the gender-wise comparison of reactive/detected patients based on confirmatory tests and ECLIA. Out of 60, immunoblot and ICT identified 8(13.30%) reactive patients, of whom 5 patients (9.80%) were male and 3(33.30%) patients were female. Whereas NAT PCR detected 9(15.00%) positive patients, of which 5 patients (9.80%) were male and 4 (44.40 %) were female.

Table-II: Gender-wise Comparison of Reactive/Detected Patients Based on Confirmatory Tests and ECLIA (n=60)

HIV Confirmatory test	Gender		Total (n=60)	*p*-value
	Male (n=51)	Female (n=9)		
Bio-Rad GeeniusTM HIV 1/2 Immunoblot	5(9.80%)	3(33.30%)	8(13.30%)	0.090
BiolineTM HIV ICT	5(9.80%)	3(33.30%)	8(13.30%)	0.090
NAT PCR	5(9.80%)	4(44.40%)	9(15.00%)	0.022*
ECLIA (Standard ≥ 1.00)	24(47.10%)	6(66.60%)	30(50.00%)	0.236
ECLIA (Modified ≥ 75.215)	5(9.80%)	3(33.30%)	8(13.30%)	0.090
ECLIA				
Median (IQR)	0.24 (0.19-5.97)	1.83(0.56-353.15)	1.03(0.20-7.68)	0.126
Range	0.16-931.70	0.19-1238	0.16-1238.00	-

**Fisher's Exact test, p-value significant at <0.05*

Immunoblot and ICT exhibited 100% sensitivity, specificity, and accuracy with 8(100%) true positives and 52 (100%) true negatives. Comparing immunoblot with NAT PCR, 8(100%) true positives and 51(98.5%) true negatives were observed. The accuracy was 98.36%, sensitivity was 100% and specificity was 92.30%.

Figure 1 illustrates the ROC for immunoblot and NAT PCR based on ECLIA values.

According to the ECLIA standard cut-off (≥1.00), the sensitivity regarding the immunoblot confirmatory test was 100%, and the specificity was 57.70%. There was a statistically significant association between ECLIA standard results and immunoblot (*p*-value = 0.002). With a modified cut-off (≥75.2), the sensitivity and specificity reached 100%, showing a highly significant (*p*-value <0.001) as shown in Table III.

When using the ECLIA standard cut-off (≥1.00), sensitivity for NAT PCR was 30%, and specificity was 100%. With a modified cut-off (≥75.2), sensitivity was raised to 100%, and specificity to 98.10%, showing a significant association (*p*-value <0.001).

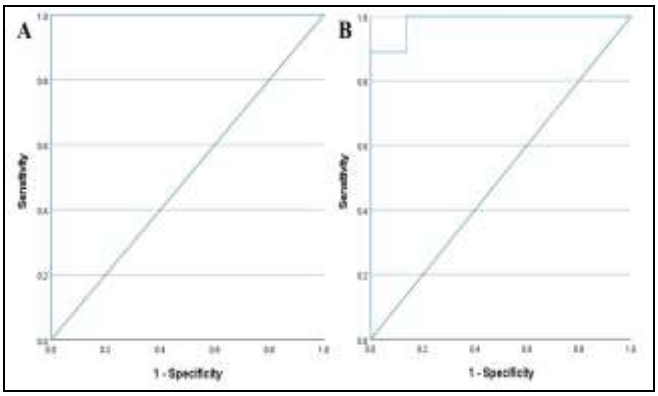

Figure-1: Roc For Immunoblot (A) & Nat Pcr (B) Based on Eclia Values (N=60)

Table-III: Association of The Standardized and the Modified Values According To Confirmatory Tests In Hiv Diagnosis (n=60)

Parameters		ECLIA			
		Standard (≥1.00)		Modified (≥75.2)	
		Positive n=30	Negative n=30	Positive n=8	Negative n=52
Immunoblot	Positive (n=8)	8(26.70%)	-	8(100.00%)	-
	Negative(n=52)	22(42.30%)	30(100.00%)	-	52(100.00%)
	p-value	0.005*		<0.001*	
NAT PCR	Positive (n=9)	9(30.00%)	-	8(100.00%)	1(1.90%)
	Negative(n=51)	21(70.00%)	30(100.00%)	-	51(98.10%)
	p-value	0.002*		<0.001*	

**Fisher's Exact test, p-value significant at <0.05*

DISCUSSION

This study validated the Geenius HIV 1/2 confirmatory immunoblot assay and ICT (Elecsys HIV Combi PT) for HIV detection in transplant facilities in Pakistan. The Geenius assay exhibited 100% sensitivity and specificity compared to BiolineTM HIV ICT. Against NAT PCR (Cobas MPX), it had 100% sensitivity, 92.30% specificity and 93.70% accuracy. Among 30 seropositive cases, 8 were Geenius positive, displaying specific HIV-related bands, while 9 were confirmed through NAT PCR (p=0.022). Adjusting the ECLIA cut-off to ≥75.2 increased specificity from 57.7% to 100% while maintaining 100% sensitivity. Another study reported a significantly higher false positive rate (1.30%, with a 95% Confidence Interval of 0.66-2.22) of HIV cases among the individuals who were previously diagnosed with COVID-19 which was attributed to the cross-reactivity of IgG antibodies targeting the viral epitope proteins of SARS-CoV-2.[12]

A study on HIV diagnostics highlighted challenges in relying solely on serological screening, as some ELISA-reactive samples yielded false positives; an issue also seen in our study while their findings reinforced the need for HIV-1 NAT testing to confirm acute infections, similar to our study, it demonstrated that integrating ECLIA, immunoblot, and NAT PCR improved specificity and reduced false positives while both studies support incorporating NAT earlier in the workflow to enhance accuracy, streamline diagnosis, and enable early detection, underscoring the need for continuous refinement of HIV testing strategies, particularly in resource-limited settings.[13] In a similar study conducted in Brazil, 8 rapid tests for HIV detection were evaluated using 200 samples. Among the 5 tests (AlereDetermine HIV 1 & 2, DPP rapid test HIV1/2, DS rapid test HIV, Interkit HIV 1/2, and bioeasy HIV) achieved 100% sensitivity, while 1 showed 99% sensitivity (HIV 1/2 /0 Tri-line). However, 2 kits (Immunerapido HIV 1/2 and imunocrom HIV 1/2) demonstrated lower performances, each with 92% sensitivity.[14] One patient tested positive for HIV RNA via NAT PCR, while both Geenius HIV-1/2 and ICT assays were non-reactive, highlighting NAT PCR's ability to detect acute infections before sero-conversion. A similar study reported cross-reactivity in 2 of 130 HIV-negative samples, and 1 HIV NAT-negative sample exhibited HIV-1 specific bands in Geenius.[15] Another study assessing Rapid Enzyme Immunoassay (rEIA)-Western blot, and ELISA-Western blot algorithms for HIV reported 100% sensitivity and specificity for HIV-1 detection, consistent with our results. However, it noted a high proportion (50–60.7%) of indeterminate results for HIV-2 and 7.1–10.7% classified as HIV-1/HIV-2 co-infections, highlighting Western blot's limitations in certain cases.[16] A study on the ARCHITECT-Multispot-viral load (AR-MS-VL) algorithm highlighted the benefits of a multi-tiered HIV diagnostic approach, improving accuracy and status resolution. Initial reactive fourth-generation assay results had a low positive predictive value (PPV) of 0.44 in a hospital setting, but added confirmatory viral load testing reliability. Similarly, our study's multi-step algorithm (ECLIA, immunoblot, and NAT PCR) improved specificity and sensitivity. Their research also found that S/CO ratios under [15] led to false-positive results, supporting our result of modifying the ECLIA cut-off.[17] NAT PCR can serve as a third-step confirmatory test for serologically positive Geenius-negative samples. Nevertheless, where HIV NAT is unavailable, the HIV 1/2 immunoblot assay remains a reliable alternative. Implementing reflex testing would further enhance Pakistan's National AIDS Control Program (NACP) diagnostic algorithm.[18]

LIMITATION OF STUDY

The small sample size (n = 60) and male-dominated population limit generalizability. Further studies with larger populations need to validate the diagnostic algorithm for it to prove its effectiveness. The brief research period might prevent the identification of potential diagnostic variations.

CONCLUSION

The combined usage of NAT with ECLIA cut-off adjustments and anti-HIV 1/2 immunoblot assay improved test results during this study while the combination of serological tests with molecular tests enhances acute infection detection while decreasing procedural mistakes.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

AR & MFS: Data acquisition, data analysis, critical review, approval of the final version to be published.

MN & AY: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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