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Immunoblots may not be effective in confirming the recency of HIV-1 infection

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Abstract

Recently, immunoblots (IBs) have tended to substitute Western blots (WBs) for HIV infection diagnosis. Several studies have confirmed IBs' high sensitivity to confirm HIV infection for every stage. Since the nature and pattern of the antigens of IBs are different from those of WB, the abilities of IBs and WBs to distinguish the stages of recent seroconversion and open-ended chronic infection might differ. We aimed to evaluate the performance of two IBs (INNO-LIA™ HIVI/II, Fujirebio, and Geenius™ HIV1/2 Confirmatory assay, Bio-Rad) to define the stage of infection. We studied 53 patients from the French ANRS CO6 PRIMO cohort. IBs have higher positive rates than WB. However, Geenius was less sensitive than WB and INNO-LIA to detect antibodies to p31 (0% vs 22.6% and 15.1%, respectively), so it could wrongly label late Fiebig stage and open-ended chronic infections as recent infections (n=5/53). For the first time, we provide evidence that centralized WBs associated with an enzyme immunoassay for the identification of recent HIV-1 infection support the establishment of a more accurate diagnosis of primary HIV infection to improve the accuracy of enrollments in cohorts of recent HIV infections useful for epidemiological studies, pathogenesis studies or therapeutic trials.

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Keywords:

HIV primary infection
Immunoblot
Western blot
HIV diagnosis
Geenius
INNO-LIA.

Highlights :

- Evaluation of Immunoblots to precise the recency of HIV infection.
- 53 patients from the ANRS PRIMO cohort.
- Geenius is not efficient at detecting anti-p31 antibodies in Fiebig VI.
- Immunoblots could wrongly label late Fiebig stages as more recent infections.
- Centralized WBs improve the accuracy of enrollments in primary infections cohorts.

Introduction:

Diagnosis of human immunodeficiency virus (HIV) infection as soon as the primary HIV infection (PHI) occurs is essential for early initiation of combined antiretroviral therapy (cART) to preserve immune function, limit the viral reservoir, and prevent transmission. The biological characterization of PHI is also useful for epidemiological studies as well as enrollment in cohorts dedicated to pathogenesis studies or therapeutic trials (Colby et al., 2018; Dong et al., 2018).

For the laboratory diagnosis of HIV infection, reactive serological testing with a 4th generation enzyme immunoassay (EIA) must be confirmed. The confirmatory assays can be Western blots (WBs) using viral antigens, which are considered the gold standard to confirm HIV infection or immunoblots (IBs) using recombinant or synthetic antigens. In case of discrepancy between EIA and confirmatory assays, HIV-RNA plasma detection is recommended.

PHI infection usually refers to acute HIV infection and the early stage of HIV infection up to about six months but definitions and biological markers may differ across study. French guidelines defined PHI as detectable HIV-RNA associated with a negative 4th generation EIA or a positive EIA with ≤ 5 HIV-specific antibodies on WB (Morlat, 2019). WB results are then useful to precisely determine the stage of PHI based on Fiebig's classification (Fiebig et al., 2003). Six stages are defined according to the dynamics of HIV-RNA, p24 antigen and antibody seroconversions observed from WB results. This broadly used classification distinguishes acute infection (stages I–III), recent seroconversion (stages IV–V), and open-ended chronic infection (stage VI). Stages I–III are defined by a negative WB. Stage IV is defined as the presence of HIV-1-specific bands that fail to meet criteria for a reactive WB, identified by the US Food and Drug Administration as reactivity to 2 of the following

antigens: p24, gp41, and gp120/160. Fiebig stage V is defined as a reactive pattern (presence of at least 2 antigens) but lacking p31 reactivity. Stage VI is defined as full reactivity, including a p31 band.

In recent years, several new IBs, which are faster to use than WBs, have tended to substitute WBs as confirmation assays. IBs assess fewer antibodies than WBs, but their high sensitivity to confirm HIV infection has been reported in several studies (Kondo et al., 2018; Lindman et al., 2019; Serhir et al., 2019; Tinguely et al., 2014). However, their ability to differentiate PHI from later stages needs to be largely evaluated.

We aimed to evaluate two IBs frequently used at preinclusion in the French ANRS (Agence Nationale de Recherches sur le SIDA et les Hépatites Virales) CO6 PRIMO cohort, to determine if they are performant to consider a patient as recently HIV infected.

Materials and methods

Fifty-three patients from the ANRS PRIMO cohort between November 2018 and May 2019 who had frozen blood plasma at inclusion in the cohort participated in this study. The cohort was approved by the Ile-de-France-3 Ethics Committee, and patients gave their informed consent to participate. Participants presenting with acute or recent HIV-1 infection, symptomatic or not, were included if they presented one of the following criteria: 1) detectable p24 antigenemia or plasma viral load, associated with an incomplete confirmatory assay (i.e., indeterminate or positive confirmatory assay with absence of antibodies to p31 and/or p68) during the six weeks before inclusion, 2) detectable p24 antigenemia or plasma viral load, with either a negative or weakly reactive EIA or a negative WB or IB during the six weeks before inclusion, or 3) an interval of <3 months between a negative and a positive EIA (Goujard et al., 2006). Preinclusion was based on clinical investigation and routine

laboratory assays, which could vary depending on the laboratory. Subsequently, centralized Vidas® HIV-DUO Ultra and WBs were systematically performed on inclusion samples (APHP, Hôpital Necker-Enfants Malades, Paris, France). Vidas® HIV-DUO Ultra, which is an Enzyme Linked Fluorescent Assay (ELIFA), was applied to determine if antibodies against HIV-1 or HIV-2 or p24 antigen were detectable.

Bio-Rad HIV-1 WB uses the inactivated HIV-1 native viral antigens Env (gp160, gp120 and gp41), Pol (p68/65, p51 and p31) and Gag (p55, p40/39, p24, and p18/17). The assay was performed according to the manufacturer's recommendations using a visual reading. We interpreted the WBs according to the WHO's recommendations (WHO, 1991): they were considered positive if at least two antibodies against the envelope antigens were detected. The WBs were indeterminate if antibodies were detected without the above criteria of positivity. As a result, we were able to precisely determine the pattern of HIV-specific antibodies on WB to classify the stage of infection with centralized tools.

The two IB assays INNO-LIA™ HIVI/II (Fujirebio) and Geenius™ HIV1/2 Confirmatory assay (Bio-Rad) were performed by the same operator on the same inclusion samples and interpreted according to manufacturer recommendations. The INNO-LIA assay is a single-use line immunoassay (LIA) which can detect antibodies against five HIV-1 proteins (gp120, gp41, p31, p24, and p17) and two against HIV-2 envelope proteins (gp105 and gp36). Patients samples were incubated all night with the test strips. Each line's intensity was read visually and compared to control lines. INNO-LIA was interpreted as HIV-1 positive when there were at least two positive anti-HIV-1 antibodies, including one directed against the HIV-1 envelope antigens. The Geenius assay is a single-use immunochromatographic test. It is composed of four HIV-1 antigens (Env gp120 and gp41, Pol p31 and Gag p24 and p17) and two HIV-2 antigens (gp36 and gp140). The duration of the assay is less than one hour. In our study,

bands were observed visually. Geenius was interpreted HIV-1 positive if the antibody to at least one envelope protein and antibody to another HIV antigen were positive.

Fiebig's classification (Fiebig et al., 2003) was used to differentiate acute and early infection according to Vidas® HIV-DUO Ultra, WB and IB assays.

Finally, a centralized EIA was also performed for the identification of recent HIV-1 infection (EIA-RI) to clarify doubtful cases. It was based on the quantification of antibodies binding to synthetic antigens of the immunodominant epitope of gp41 and the V3 region of gp120 (Barin et al., 2005). A score <0.5 was in favor of infection of fewer than 180 days, as previously described (Barin et al., 2005).

Fisher and χ^2 tests were used to compare WB and IBs and to evaluate the relationship between WB or IB results and Fiebig stages (Prism version 8.0, GraphPad).

Results

Fifty-three patients were tested with WB and two IBs. The positive rate of WB (13.2%) was lower than that of INNO-LIA (66.0%, $p<0.0001$) and Geenius (62.3%, $p<0.0001$). INNO-LIA and Geenius showed positive results for most of the indeterminate WB samples, 87.1% ($n=27/31$) and 77.4% ($n=24/31$), respectively (Table 1). Seven samples showed concordant positive results with the three assays ($n=7/53$, 13.2%).

Antibody patterns help to distinguish acute, recent, and established infections. Antibodies to p24 and envelope glycoproteins are the earliest to develop during seroconversion. WB and INNO-LIA better detected antibodies to p24 than Geenius (66.0% vs 9.4%, $p<0.0001$ and 62.3% vs 9.4%, $p<0.0001$, respectively). Detection of antibodies to Env antigens was higher with Geenius than with WB (gp120/160: 62.3% vs 30.2%, $p=0.0017$; gp41: 84.9% vs 13.2%, $p<0.001$). Antibodies to gp120/160 were more often detected with Geenius than with INNO-LIA (62.3% vs 32.1%, $p=0.0033$). Antibodies to p31 appear later during the course of

infection and are used to define Fiebig stage VI. WB detected antibodies to p31 in twelve samples (n=12/53, 22.6%), including eight samples that were also p31 positive with INNO-LIA (n=8/53, 15.1%). Both WB and INNO-LIA better detected antibodies to p31 than Geenius, which did not detect any antibodies to p31 in any sample (WB vs. Geenius: $p = 0.0002$; INNO-LIA vs Geenius: $p=0.0059$) (Table 1).

Fiebig classification applied to Vidas® HIV-DUO Ultra combined with either WB, INNO-LIA, or Geenius showed different distributions of Fiebig stages ($p<0.0001$) (Table 1). Differences were particularly observed for the latest Fiebig stages IV to VI. Fiebig classification according to WB ranked more samples in Fiebig stage IV (n=31/53, 58.5%) than both INNO-LIA (n=9/53, 17.0%) and Geenius (n=12/53, 22.6). Conversely, the IBs ranked more samples in Fiebig stage V than WB (INNO-LIA n=27/53, 50.9%, Geenius n=33/53, 62.3% vs WB n= 1/53, 1.9%). Due to the difference in detecting antibodies to p31, WB and INNO-LIA classified some samples in Fiebig stage VI (n=6/53, 11.3%, and n=8/53, 15.1%, respectively), whereas Geenius did not.

We further performed EIA-RI to assess discrepancies. Out of 53 samples, 48 were classified as recent infection shorter than 180 days (median score: 0.01, range [0.01-0.25]), one equivocal and four long-term (>180 days). These five cases are detailed on Table 2. The only sample presenting Fiebig stage V characteristics on WB showed an index of EIA-RI at 0.99 in favor of an infection longer than 180 days. The IBs also classified this sample in Fiebig stage V. INNO-LIA had been used to confirm the diagnosis at preinclusion for this participant and presented negative for antibodies to p31 and positive for antibodies to p24. There was no previous negative HIV serology and no p24 detection, and the CD4/CD8 T cell ratio was 0.2, with 4.7 log copies HIV-RNA/mL at inclusion. Immunosuppression in late chronic infection leading to loss of antibodies to Pol and Gag might have distorted the confirmatory assay and mislead the Fiebig classification.

Three patients out of six classified in Fiebig stage VI with WB had an elevated index of EIA-RI >0.8 in favor of infection longer than 180 days. They all had been diagnosed on the basis of the results of a Geenius assay at preinclusion (no antibodies to p24 or p31), and two of them were positive for p24 antigen at preinclusion. Curiously, one patient had very low HIV-RNA and HIV-DNA and a CD4/CD8 T cell ratio conserved >1 at inclusion fourteen days later. This patient could be a natural HIV controller. The fifth patient presented a doubtful EIA-RI index (0.44) and was positive for all three assays but with antibodies to p31 detected only with WB. The patient had no past serology and no p24 antigen detected, and WB was complete except for gp110.

Discussion

Different manufacturers have approved several IB assays as confirmation assays, without any doubt regarding their performance (Kondo et al., 2018; Serhir et al., 2019; Tinguely et al., 2014), but few studies concern their ability to distinguish infection stages. Therefore, our aim was to focus on acute and recent stages.

The present study on a large number of samples clearly shows that the ability to detect each antibody differed between the IBs and WB. The higher ability of the IBs to detect anti-Env antibodies led to a higher positive rate of these assays than that of WB, which is advantageous to confirm HIV diagnosis as soon as the earliest Fiebig stages of seroconversion. WBs were then more often indeterminate, with a profile suggesting recent infection. Moreover, Gag and Pol antibodies are essential to distinguish acute from recent and chronic infections, particularly anti-p31 antibodies, which characterize Fiebig stage VI. Our study showed that Geenius is not efficient at detecting antibodies to p31 in Fiebig stages VI, so Geenius could wrongly label late Fiebig stage and chronic infections as more recent infections. These data

are in agreement with the lower capacity of Geenius to detect anti-p24 and anti-p31 antibodies observed during chronic HIV infection (Tuaille et al., 2017). An alternative method based on the quantitative measurements of antibody band intensities determined by the automated Geenius optical reader may have an interest to distinguish between recent and long-standing HIV infection, as previously suggested (Keating et al., 2016).

Overall, we provide evidence that Geenius and INNO-LIA may not be assays of choice to stage infections following Fiebig classification. As the latest stages or some rare cases, such as natural HIV controllers, can be confusing for both WB and the IBs, the EIA-RI result, interpreted in association with other assays, can be an additional argument to confirm or reject recent infection.

In conclusion, our study highlights the difficulties of providing consistent results for determining the stage of HIV infection when antibodies are already detectable. Clinical laboratories must keep in mind that Geenius might mistakenly refer to recent infection, so if a recent infection is suspected, additional investigations are needed. In our study, we showed that a centralized WB confirmation with a complementary assay of recent infection combined with assays realized at preinclusion are beneficial to confirm the recency of HIV infection and reassure enrollment criteria in PHI cohorts.

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Declarations of interest: none

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1 Table 1. Comparison of positive antibodies and stages of Fiebig's classification between Western blot (WB), Geenius, and INNO-LIA

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N (%)	Assay results			Positive antibodies				Fiebig stages					
	Positive	Indeter- minate	Negative	GP160/120	GP41	P24	P31	I	II	III	IV	V	VI
Western blot, Bio-Rad	7 (13.2)	31 (58.5)	15 (28.3)	16 (30.2)	7 (13.2)	35 (66.3)	12 (22.6)	0 (0.0)	10 (18.9)	5 (9.4)	31 (58.5)	1 (1.9)	6 (11.3)
Immunoblot, INNO-LIA HIV 1/2	35 (66.0)	9 (17.0)	9 (17.0)	17 (32.1)	44 (83.0)	33 (62.3)	8 (15.1)	0 (0.0)	7 (13.2)	2 (3.8)	9 (17.0)	27 (50.9)	8 (15.1)
Immunoblot, GEENIUS HIV 1/2	33 (62.3)	12 (22.6)	8 (15.1)	33 (62.3)	45 (84.9)	5 (9.4)	0 (0.0)	0 (0.0)	6 (11.3)	2 (3.8)	12 (22.6)	33 (62.3)	0 (0.0)
EIA-RI index for Fiebig's stages defined with WB: Median (Ranges)									0.01 (0.01-0.04)	0.01 (0.01-0.01)	0.02 (0.01-0.19)	0.99	0.63 (0.16-0.99)
EIA-RI: Enzyme immunoassay for identification of recent HIV-1 infections, threshold to discriminate between < or > 180 days: 0.50.													

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Fiebig's Stages defined according to each confirmation assay	Preinclusion		Inclusion										
	p24 antigen	Confirmation test	Delay from preinclusion	CD4 T cell count (/mm ³)	CD4/CD8 ratio	HIV-RNA (log copies/mL)	HIV-DNA (log copies/10 ⁶ PBMCs)	Antibodies, DUO ULTRA VIDAS	p24 antigen, DUO ULTRA VIDAS	WB, VIH1	INNO-LIA	Geenius	EIA-RI index
WB: V INNO-LIA: V Geenius: V	nr	INNO-LIA: gp120, gp41, P24	22	247	0.2	4.7	3.57	21.08 Positive	Negative	Positive gp160, gp110, p68, p55, gp41, p24+, p18±	Positive gp120, gp41, p24	Positive gp160, p24±, gp41	0.99
WB: VI INNO-LIA: VI Geenius: V	Positive	Geenius: gp160, gp41	14	602	1.5	1.53	1.0	13.71 Positive	Negative	Positive complete	Positive gp120, gp41, p31, p24, p17	Positive gp160, p24, gp41	0.83
WB: VI INNO-LIA: VI Geenius: V	Positive	Geenius: gp160, gp41	13	nr	nr	4.85	3.09	10.03 Positive	Negative	Positive gp160, p68, p55, gp41, p31, p24,	Positive gp120, gp41, p31, p24±	Positive gp160, gp41	0.99
WB: VI INNO-LIA: VI Geenius: V	nr	Geenius: gp160, gp41	13	307	0.6	6.09	3.41	13.92 Positive	Negative	Positive gp160, gp110, p68, p55±, p31, p24±, p18	Positive gp120, gp41, p31, p24±	Positive gp160, gp41	0.99
WB: VI INNO-LIA: V Geenius: V	nr	Geenius: gp160, gp41, p24	15	451	0.3	5.57	3.21	8.36 Positive	Negative	Positive gp160, p68, p55, gp41, p40, p31, p24, p18	Positive gp120, gp41, p24, p17	Positive gp160, p24, gp41	0.44

nr: not realized.

WB: Western blot.

Antibodies, DUO ULTRA VIDAS: relative fluorescence value (RVF) of antibodies ≥ 0.25: positive.

EIA-RI: Enzyme immunoassay for identification of recent HIV-1 infections, threshold to discriminate between < or > 180 days: 0.50.