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MAJOR ARTICLE

Difficulties of Identifying the Early HIV Antibody Seroconversion Period Depending on the Confirmatory Assay

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Background. Identification of HIV infection at the early stage is valuable for patient management, for prevention, and for research purposes. In practice, identification of a recent HIV infection at diagnosis proves challenging after HIV antibody seroconversion but can be suspected using Western blots (WBs) or immunoblots (IBs) as confirmatory assays.

Methods. Five commercially available confirmatory assays were compared using 43 samples from recently infected individuals. This included 2 WBs (New LAV Blot I, Biorad, and HIV Blot 2.2, MP Biomedicals), 2 IBs (INNO-LIA HIV I/II, Fujirebio, and RecomLine HIV-1 & HIV-2, Mikrogen Diagnostik), and 1 immunochromatographic single-use assay (Geenius HIV1/2 supplemental assay, Biorad).

Results. Following the manufacturer's recommendations for interpretation, the 2 WBs led to indeterminate results for 30% and 42% of the samples, suggesting recent infection, compared with 2%–7% for the 3 other assays. When interpreted based on the Fiebig classification, concordant stages were observed in 42% of samples, and only 49% were classified as early seroconversion by all 5 assays. For the remaining specimens, the distinction with chronic infection was highly variable depending on the assay (5%–100%).

Conclusions. Clinical laboratories must consider this variability, which must be kept in mind both for initial diagnosis and for multicenter studies for which inclusion criteria refer to serological profiles by confirmatory assays.

Keywords. HIV; HIV antibodies; recent HIV infection; seroconversion; Western blotting.

Although it is now recommended that any individual newly diagnosed as infected with HIV-1 must be treated whatever the stage of infection, identifying persons who are within weeks of HIV-1 antibody seroconversion remains useful for both clinical decision-making and prevention, as well as for pathogenesis studies and epidemiological surveillance [1]. Acute HIV-1 infection, defined biologically as the period from HIV blood detection until seroconversion, is usually easily diagnosed based on the presence of p24 antigen (p24 Ag) and/or HIV RNA in serum or plasma in the absence of detectable HIV antibodies. Therefore, during this brief window of time, methods based on interpreting HIV test results allow an accurate estimation of timing of infection [2, 3]. However, detecting the early postseroconversion stage is much more challenging. Assays for identification of recent infection based on antibody level or antibody avidity have been developed, but due to a substantial false recency rate, their use has been limited to epidemiological studies for incidence estimates and has not been recommended for diagnosis at the individual level [4–9]. Therefore, besides the diagnosis of acute infection such as that described above, the identification of a recent seroconversion necessitates interpretation of a confirmatory assay, either a classical "historical" Western blot (WB) using viral antigens or a more recent immunoblot assay (IB) using recombinant or synthetic antigens.

The laboratory staging of HIV-1 infection initially described by Fiebig et al. remains a reference that allows, through 6 stages, identification of acute infection (stages I–III), recent seroconversion (stages IV–V), or open-ended chronic infection (stage VI) [10]. It is broadly used, particularly for enrollment in cohorts dedicated to pathogenesis studies or therapeutic trials [11, 12]. Stages I–III are defined by a negative Western blot. Stage IV is defined as the presence of HIV-1-specific bands that fail to meet criteria for reactive WB identified by the US Food and Drug Administration (FDA) as reactivity to 2 of the following antigens: p24, gp41, gp120/160. The definition of acute infection is primarily clinical, but the biological delineation may differ across studies. Biologically speaking, it generally refers to

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the period before the detection and/or confirmation of HIV-specific antibodies: Fiebig stage I–III or IV [13]. Fiebig stage V is defined as a reactive pattern, that is, the presence of at least 2 of the antigens listed above, but lacking p31 (integrase) reactivity. Stage VI is defined as full reactivity including a p31 band [10]. Based on this classification, stages IV and V correspond to the early seroconversion period spanning ~1–3 months postexposure [1, 10].

Because methods that could identify individuals who are still in early infection, albeit in the presence of antibodies characterized by a supplemental assay, would allow clinicians to target these persons for appropriate interventions and/or enrollment in clinical studies, the aim of the present study was to compare the ability of confirmatory assays to identify early seroconversions. Five commercially available confirmatory assays that could readily be performed in clinical laboratories were evaluated.

METHODS

Serum Samples

Forty-three serum samples from HIV-1 seroconverters were selected in 2 clinical laboratories (St-Louis Hospital, Paris, and Bretonneau Hospital, Tours, France). The selection criteria were based on an incomplete or weakly reactive Western blot (either New LAV Blot I in Paris or HIV Blot 2.2 in Tours; see "HIV Immunoassays" below) and additional proof of early seroconversion, either a previous sample collected during acute infection (p24 Ag positive and/or HIV RNA positive in the absence of antibodies detected by Western blot) or an evolving Western blot profile on a subsequent serum sample. The serum samples were collected between 2014 and 2019 and stored frozen until used for the present study. Patients were part of an ongoing multicenter study whose primary aim is to characterize the viruses identified during acute/early HIV-1 infection in France [14]. This study was approved by the required ethics committee (Comité Consultatif de Traitement de l'Information dans la Recherche Scientifique et Médicale) and by the national data confidentiality watchdog organization (Commission Nationale Informatique et Liberté), in keeping with French law. Patients received full information on their participation in the study and did not oppose the use of the data.

Among the 43 individuals, 40 were men, of whom 33 were men who have sex with men (MSM) and 3 were women. Based on the sequence of the reverse transcriptase gene, 23 were infected by subtype B variants and 18 were infected by non-B variants (6 CRF02_AG, 1 D, 2 F, 1G, 2 CRF06_cpx, 2 CRF19_cpx, 1 CRF60_BC, and 3 U). The subtype was not determined for the remaining 2.

HIV Immunoassays

Five immunoassays were evaluated. There were 2 Western blots (New LAV Blot I, Biorad, Marnes-la-Coquette, France; and HIV Blot 2.2, MP Biomedicals, Singapore), 2 immunoblots (INNO-LIA HIV I/II, Fujirebio, Ghent, Belgium; and RecomLine HIV-1 & HIV-2, Mikrogen Diagnostik, Neuried, Germany), and 1 immunochromatographic single-use assay (Geenius HIV1/2 supplemental assay, Biorad, Marnes-la-Coquette, France). All are approved for confirmation of HIV seropositivity by the FDA and/or the Commission of the European Union. Both the nature and the number of the antigens used in each assay are different, the Western blots using all the HIV antigens present in virions produced in cell culture, whereas the 3 other assays use a limited number of recombinant or synthetic antigens. As shown in Figure 1, only 3 antigens are shared by all assays: gp41, p31, and p24. At least 1 additional Env antigen is included in each assay: gp160 in Geenius, gp120 in INNO-LIA and Recomline, and both gp160/gp120 in the Western blots.

All assays were performed following the recommendations of the manufacturers. All samples were tested simultaneously with only 1 freeze-thawing between performing the initial diagnostic assay and the present study. Interpretation was done independently by 3 readers, affecting a score for each antigen depending on the intensity (negative, ±, +, ++, or +++). The final score corresponded to the mean of the 3 readings. In addition, the Geenius assay was read using the Geenius reader and software, which interpret the bands as positive or negative without a quantification score.

Interpretation was done following 2 strategies. First, we strictly followed the criteria recommended by the manufacturers (package insert). None of the tests mentioned interpretative criteria for recent infection, but all proposed to conclude as negative, indeterminate, or positive, based on the presence/absence of a selection of bands, which could differ from FDA

	gp160	gp120	RT 64/51	gp41	p31	p24	p17
Biorad WB	X	X	X	X	X	X	X
MP WB	X	X	X	X	X	X	X
INNO-LIA		X		X	X	X	X
RecomLine		X	X	X	X	X	X
Geenius	X			X	X	X	

Figure 1. Antigenic composition of the confirmatory assays. X means presence in the assay. Abbreviation: WB, Western blot.

criteria. Second, for stratification and standardization, Fiebig stage was attributed to every sample based on the profile observed for each assay: stage I/II/III (no band), stage IV (only 1 band among p24, gp41, and gp120/160), stage V (at least 2 bands among p24, gp41, gp120/160; ie, FDA criteria for HIV seropositivity, but without p31), stage VI (full reactivity including a p31 band).

All samples were further tested with an assay for recent infection (EIA-RI) previously developed in our laboratory that combines standardized measures of antibody binding with the immunodominant epitope (IDE) of gp41 and the V3 region of gp120 [4]. Level of antibody to IDE (ratio of absorbance/mean absorbance of negative controls), which is the most discriminant for recency [6], was used to classify the samples by ascending order of magnitude.

RESULTS

The detailed comparisons of the 5 immunoassays for all samples are represented in Figure 2. Although we considered the mean of 3 visual readings, it must be said that there were only a few minor differences between the readings by different persons. Similarly, although an automatic interpretation was done for Geenius, there was no qualitative difference between reading with the Geenius reader and visual interpretation. In other words, there was perfect concordance between automatic reading and visual reading to conclude a positive or negative band. We first analyzed the results by strictly following the criteria recommended by the manufacturer for each assay, as would be done in field practice. The 2 WBs Biorad and MP led to the highest number of indeterminate results, for 18 (42%) and 13 (30%) samples, respectively (Figure 3). In contrast, 42 (98%), 41 (95%), and 40 (93%) samples would have been interpreted as positive by Geenius, INNO-LIA, and RecomLine, respectively, without suggestion of recency. In these cases, a recent seroconversion profile would not have been identified, leading to classification as long-lasting infection.

In the analysis based on Fiebig classification, concordant staging between the 5 assays was observed only for 18 of the 43 samples (42%), which were all stage V and were therefore correctly classified as early seroconversions (Figure 3). Three other samples were also correctly classified as early seroconversions, but with different staging: sample #23 was classified as Fiebig II/III by RecomLine and Geenius but Fiebig IV by both WBs and INNO-LIA, sample #10 was classified as Fiebig IV by INNO-LIA and RecomLine but Fiebig V by the 3 other assays, and sample #22 was classified as Fiebig IV by RecomLine but Fiebig V by the 4 other assays. Taken together, 21 of 43 samples were correctly classified as early seroconversions (49%). On the

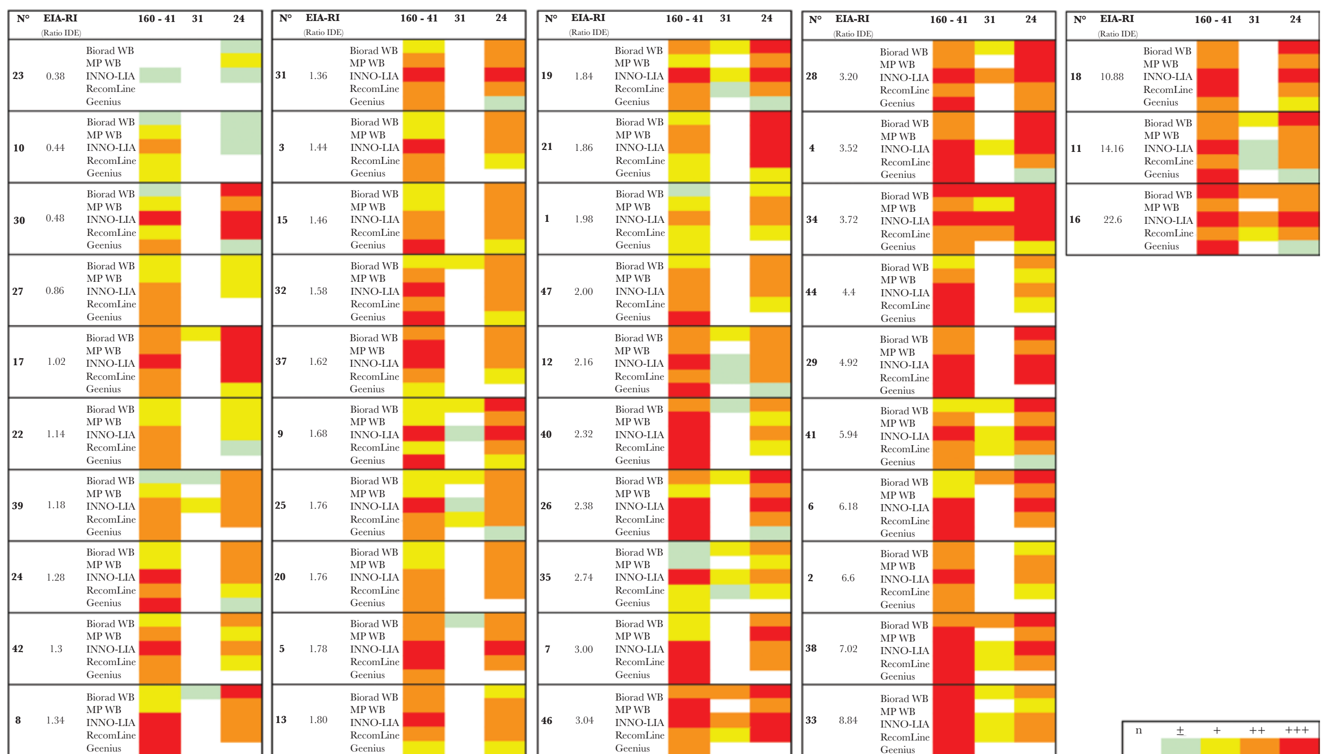

Figure 2. Synthesis of the detailed serological profiles observed for every sample in each assay. Boxes are colored according to the legend (intensity score: negative, ±, +, ++, or +++) for each antigen. Data are shown only for antigens common to all assays (gp160-41, p31, p24). Samples were classified following the ascending order of antibody reactivity toward the gp41 immunodominant epitope [4, 6]. Abbreviations: EIA-RI, enzyme immunoassay for recent HIV-1 infections; IDE, immunodominant epitope; Ratio, ratio of absorbance/cutoff value; WB, Western blot.

Sample	EIA-RI (Ratio IDE)	Biorad WB	MP WB	INNO-LIA	RecomLine	Geenius	Biorad WB	MP WB	INNO-LIA	RecomLine	Geenius
		Results interpretation (Manufacturer's criteria)					Fiebig stage (FDA criteria)				
23	0.38	IND	IND	IND	NEG	NEG	IV	IV	IV	II / III	II / III
10	0.44	IND	IND	IND	IND	POS	V	V	IV	IV	V
30	0.48	IND	IND	POS	POS	POS	V	V	V	V	V
27	0.86	IND	IND	POS	POS	POS	V	V	V	V	V
17	1.02	IND	POS	POS	POS	POS	VI	V	V	V	V
22	1.14	IND	IND	POS	IND	POS	V	V	V	IV	V
39	1.18	IND	POS	POS	POS	POS	VI	V	VI	V	V
24	1.28	IND	IND	POS	POS	POS	V	V	V	V	V
42	1.30	IND	POS	POS	POS	POS	V	V	V	V	V
8	1.34	POS	POS	POS	POS	POS	VI	V	V	V	V
31	1.36	POS	POS	POS	POS	POS	V	V	V	V	V
3	1.44	IND	POS	POS	POS	POS	V	V	V	V	V
15	1.46	POS	IND	POS	POS	POS	V	V	V	V	V
32	1.58	POS	POS	POS	POS	POS	VI	V	VI	VI	V
37	1.62	POS	POS	POS	POS	POS	V	V	V	V	V
9	1.68	POS	POS	POS	POS	POS	VI	V	V	V	V
25	1.76	POS	IND	POS	POS	POS	VI	V	V	VI	V
20	1.76	IND	IND	POS	POS	POS	V	V	V	V	V
5	1.78	POS	POS	POS	POS	POS	VI	V	V	V	V
13	1.80	POS	POS	POS	POS	POS	V	V	V	V	V
19	1.84	POS	IND	POS	POS	POS	VI	V	VI	V	V
21	1.86	POS	POS	POS	POS	POS	V	V	V	V	V
1	1.98	IND	IND	POS	POS	POS	V	V	V	V	V
47	2.00	IND	POS	POS	POS	POS	V	V	V	V	V
12	2.16	POS	POS	POS	POS	POS	VI	V	V	V	V
40	2.32	POS	POS	POS	POS	POS	VI	V	V	V	V
26	2.38	POS	IND	POS	POS	POS	VI	V	V	V	V
35	2.74	IND	IND	POS	POS	POS	VI	V	VI	V	V
7	3.00	IND	POS	POS	POS	POS	V	V	V	V	V
46	3.04	POS	POS	POS	POS	POS	VI	VI	VI	VI	V
28	3.20	POS	POS	POS	POS	POS	VI	V	VI	VI	V
4	3.52	POS	POS	POS	POS	POS	V	V	VI	V	V
34	3.72	POS	POS	POS	POS	POS	VI	VI	VI	VI	V
44	4.40	IND	POS	POS	POS	POS	V	V	V	V	V
29	4.92	POS	POS	POS	POS	POS	V	V	V	V	V
41	5.94	POS	POS	POS	POS	POS	VI	V	VI	VI	V
6	6.18	IND	POS	POS	POS	POS	VI	V	V	V	V
2	6.60	POS	POS	POS	POS	POS	V	V	V	V	V
38	7.02	POS	POS	POS	POS	POS	VI	V	VI	VI	V
33	8.84	POS	POS	POS	POS	POS	VI	V	VI	VI	V
18	10.88	IND	POS	POS	POS	POS	V	V	V	V	V
11	14.16	POS	POS	POS	POS	POS	VI	V	V	V	V
16	22.60	POS	POS	POS	POS	POS	VI	V	VI	VI	V

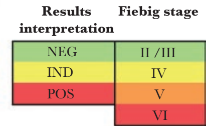

Figure 3. Confirmatory test results following manufacturers' recommendations (left) and interpretation of the stage of infection according to Fiebig stage (right) for 43 specimens from recently infected HIV-1 seroconverters. Boxes are colored according to the legend. Samples were classified following the ascending order of antibody reactivity toward the gp41 immunodominant epitope [4, 6]. Abbreviations: EIA-RI, enzyme immunoassay for recent HIV-1 infections; FDA, Food and Drug Administration; IDE, immunodominant epitope; Ratio, ratio of absorbance/cutoff value; WB, Western blot.

contrary, 22 samples (51%) collected during the early post-HIV antibody seroconversion period provided discrepant results, as they were classified as Fiebig V by some assays but Fiebig VI by others (Figures 2 and 3). All 22 samples were Fiebig V by Geenius, and 20 of them by MP WB, suggesting that these 2 assays diagnose early seroconversions with accuracy. In contrast, 21 (95%), 12 (55%), and 9 (41%) of these 22 samples were Fiebig VI by Biorad WB, INNO-LIA, and RecomLine, respectively. The discrepancies were clearly attributed to the ability of the assays to detect antibody to p31. Indeed, 21 (49%), 12 (28%), 9 (21%), and 2 (5%) of the 43 samples were positive for antibody to p31 by Biorad WB, INNO-LIA, RecomLine, and MP WB, respectively, leading to classification of these samples as Fiebig stage VI, whereas they corresponded to a recent seroconversion. All were classified correctly by Geenius. The proportion of B vs non-B viruses was not associated with an earlier Fiebig stage, nor with concordance between the 5 assays (P = .53 and .76, respectively, Fisher exact test), suggesting that the viral subtype had no significant effect on the results.

DISCUSSION

Whereas diagnosis of acute infection corresponding to Fiebig stages I–III, before detection of anti-HIV antibodies by Western blot, is relatively easy, identifying Fiebig stages IV and V may be more challenging using serological tests. However, identifying patients at these early stages of HIV infection may be critical for appropriate interventions and/or enrollment in clinical studies. This diagnosis relies on the use of supplemental assays that dissect the antibody profile, that is, the description of the antibody specificities directed at the main HIV antigens. The aim of the present study was to evaluate the ability of 5 commercially available confirmatory assays to identify patients during the early HIV-1 antibody seroconversion period, corresponding only to Fiebig stages IV and V. These supplemental assays have been

broadly validated for confirmation of HIV infection previously, without any doubt regarding their performance [8, 15–18]. Therefore, the aim was not to compare deeply their performance in different situations of HIV infection, but just to focus on the short period following the antibody-negative window.

The present study shows clearly that they do not behave similarly when the question is to identify a recent seroconversion corresponding to the few weeks or months following acute infection. When using the manufacturers' criteria, WBs allowed suspicion of a recent seroconversion more easily than immunoblots or the immunochromatographic single-use assay due to the "indeterminate" status. A majority of cases would have been classified as long-lasting infection, especially with the latter assays, missing the information of recency. Using Fiebig classification to homogenize interpretation, up to half of our panel of serum samples collected during this early phase were misclassified as long-lasting HIV-1 infection, depending on the assay. The discrepancies were related to the sensitivity of detection of antibody to p31 but not to the nature of the assay. Indeed, one could hypothesize that assays using antigens isolated from cultured virions would behave differently from those using recombinant or synthetic antigens. This is not the case, as, for instance, the MP WB misclassified only 5% of our sample compared with 45% for the Biorad WB. The difference between 2 WBs was already reported, the median time from estimated date of seroconversion to positivity of the p31 band being 41 days for Biorad WB compared with 63 days for Ortho WB [1]. Although the Ortho WB was not included in our study, previous results appear similar to our observations, that is, that time to detection of anti-p31 appears longer for both MP WB and Geenius than for Biorad WB. A lower reactivity to p31 is not without consequences, however, as it has been shown to increase misclassification of chronic HIV infection as recent infection [19].

A limitation could have been that the 2 Western blots included in our retrospective study were those used for confirmation at the time of initial screening. However, because each laboratory used either the Biorad WB exclusively or the MP WB exclusively, the studied panel was not biased for selection by a single assay, restricting this limitation.

Our study highlights the difficulties of providing consistent results for identification of recently infected individuals when antibodies are already detectable, particularly when different confirmatory assays and/or different clinical laboratories are involved. This can be the case when enrollment in cohorts necessitates multicenter studies. Consequently, confirmation should be performed a second time using a single assay in a centralized laboratory. Alternatively, an algorithm combining a confirmatory assay and a so-called "incidence assay" could be evaluated in order to pave the way to more consistent and reliable results.

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