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

Karl Stefic,1,2,5 Nadia Mahjoub,2 Céline Desouche,1,5 Marie Laure Néré,4 Damien Thierry,1,5 Constance Delaugerre,3,4,5 Francis Barin,1,2,5 and Marie Laure Chaix1,4,5

1Laboratoire de Virologie, CHU Bretonneau, Tours, France, 2INSERM U1259, Université de Tours, Tours, France, 3Laboratoire de Virologie, CHU Saint Louis, Paris, France, 4INSERM U944, Université de Paris, Paris, France, and 5Centre National de Référence du Virus de l’Immunodéficience Humaine (VIH), France

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
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) reac-
tivity. 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 charac-
terized 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 com-
pare the ability of confirmatory assays to identify early sero-
conversions. 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 serocon-
version, either a previous sample collected during acute infec-
tion (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
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 1/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 na-
ture and the number of the antigens used in each assay are dif-
ferent, 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 di-
agnostic 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 addi-
tion, 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 manufac-
turers (package insert). None of the tests mentioned interpre-
tative 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   |
| MP WB    | X     | X     |          | X    | X   | X   | X   |
| INNO-LIA |       | X     |          | X    | X   | X   |     |
| RecomLine|       |       |          | X    | X   |     | X   |
| Geenius  | 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
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

---

**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.
Therefore, the aim was not to compare deeply their performance 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.

Acknowledgments

Financial support. This study was supported by the Centre National de Référence du Virus de l’Immunodéficience Humaine (VIH).

Potential conflicts of interest. All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Hecht FM, Wellman R, Busch MP, et al. Identifying the early post-HIV antibody seroconversion period. J Infect Dis 2011; 204:526–533.
2. Grebe E, Facente SN, Bingham J, et al. Interpreting HIV diagnostic histories into infection time estimates: analytical framework and online tool. BMC Infect Dis 2019; 19:894.
3. Pilcher CD, Porco TC, Facente SN, et al. A generalizable method for estimating duration of HIV infections using clinical testing history and HIV test results. AIDS 2019; 33:1231–40.
4. Barin F, Meyer L, Lancar R, et al. Development and validation of an immunoblot assay for identification of recent human immunodeficiency virus type 1 infections and its use on dried serum spots. J Clin Microbiol 2005; 43:4441–7.
5. Guy R, Gold J, Calleja JMG, et al. Accuracy of serological assays for detection of recent infection with HIV and estimation of population incidence: a systematic review. Lancet Infect Dis 2009; 9:74–59.
6. Le Vu S, Le Strat Y, Barin F, et al. Population-based HIV-1 incidence in France, 2003–08: a modelling analysis. Lancet Infect Dis 2010; 10:682–7.
7. Kassanjee R, Pilcher CD, Busch MP, et al. Viral load criteria and threshold optimization to improve HIV incidence assay characteristics. AIDS 2016; 30:2361–71.
8. Parekh BS, Ou C-Y, Fonjungo PN, et al. Diagnosis of human immunodeficiency virus infection. Clin Microbiol Rev 2018; 32.
9. Murphy G, Pilcher CD, Keating SM, et al. Moving towards a reliable HIV incidence test – current status, resources available, future directions and challenges ahead. Epidemiol Infect 2017; 145:925–41.
10. Fiebig EW, Wright DJ, Rawal BD, et al. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS 2003; 17:1871–9.
11. Dong KL, Moodley A, Kwong DS, et al. Detection and treatment of Fiebig stage I HIV-1 infection in young at-risk women in South Africa: a prospective cohort study. Lancet HIV 2018; 5:e35–44.
12. Colby DJ, Trautmann L, Pinyakorn S, et al. Rapid HIV RNA rebound after anti-retroviral treatment interruption in persons previously suppressed in Fiebig I acute HIV infection. Nat Med 2018; 24:923–926.
13. Ananworanich J, Fletcher JL, Pinyakorn S, et al. A novel acute HIV infection staging system based on 4th generation immunoassay. Retrovirology 2013; 10.
14. Vissieux B, Assoumou L, Mahjoub N, et al. Surveillance of HIV-1 primary infections in France from 2014 to 2016: toward stable resistance, but higher diversity, clustering and virulence? J Antimicrob Chemother 2020; 75:183–93.
15. Wong CC, Lim SH, Tan CT, Lui SY, Lee YL, Chan KP. Performance of the HIV Blot 2.2, INNO-LIA HIV I/II test, and Geenius HIV 1/2 confirmatory assay for use in HIV confirmation. PLoS One 2018; 13:e0199502.
16. Serhir B, Desjardins C, Doualla-Bell F, Simard M, Tremblay C, Longtin J. Evaluation of the bio-rad Geenius HIV 1/2 assay as part of a confirmatory HIV testing strategy for Quebec, Canada: comparison with Western blot and INNO-LIA assays. J Clin Microbiol 2019; 57:e01398–18.
17. Armstrong WS, Guarnier J, Kraft CS, Caliendo AM. Human immunodeficiency virus. Microbiol Spectr 2016; 4:DMH2-0024–2015.
18. Cornett JK, Kirn TJ. Laboratory diagnosis of HIV in adults: a review of current methods. Clin Infect Dis 2013; 57:712–8.
19. Taillon E, Sansooyan A, Pisoni A, Liscouët J, Makinson A, de Perre PV. Staging of recent HIV infection using Geenius rapid confirmatory assay compared to INNO-LIA, New Lav and Blot 2.2 assays. J Clin Virol 2017; 95:47–51.