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► To cite this version:

Vincent Guiraud, Jonathan Bocobza, Marion Desmonet, Florence Damond, Jean-Christophe Plantier, et al.. Are confirmatory assays reliable for HIV-1 / HIV-2 infection differentiation? A multicenter study. *Journal of Clinical Microbiology*, In press, 10.1128/jcm.00619-23 . hal-04164703

HAL Id: hal-04164703

<https://hal.sorbonne-universite.fr/hal-04164703v1>

Submitted on 18 Jul 2023

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1 **Are confirmatory assays reliable for HIV-1 / HIV-2 infection differentiation? A**
2 **multicenter study**

3

4 **Vincent Guiraud,^{&a,b} Jonathan Bocobza,^{&a} Marion Desmonet,^a Florence Damond,^{c,d} Jean-Christophe**
5 **Plantier,^e Ghislaine Moreau,^a Marc Wirden,^{a,b} Karl Stefic,^f Francis Barin,^f Agnès Gautheret-**
6 **Dejean^{*a,g}**

7 Affiliations:

8 ^aAP-HP, Hôpitaux Universitaires La Pitié Salpêtrière-Charles Foix, Service de Virologie, F-75013 Paris,
9 France

10 ^bSorbonne Université, INSERM U1136, Institut Pierre Louis d'Epidémiologie et de Santé Publique
11 (IPLESP)

12 ^cAP-HP, University Hospital Bichat-Claude Bernard, Service de Virologie, F-75018 Paris, France

13 ^dUniversité Paris cité, IAME, INSERM, F-75018 Paris, France

14 ^eUniversité de Rouen Normandie, Inserm UMR1311 DYNAMICURE, et CHU de Rouen, Laboratoire de
15 virologie associé au CNR du VIH, F-76000 Rouen, France

16 ^fUniversité de Tours, UMR Inserm 1259, and CHU de Tours, Laboratoire associé au CNR du VIH, F-
17 37000 Tours, France

18 ^gUniversité Paris cité, INSERM UMR-S 1139, 3PHM, F-75006 Paris, France

19

20 * Corresponding author, E-mail: agnes.gautheret@aphp.fr

21 & These authors contributed equally to this work.

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23

1 **ABSTRACT**

2 Immunoblots remain the gold standards for HIV-1/HIV-2 infection confirmation. However, their
3 ability to differentiate HIV-1 from HIV-2 infection on an antigenically diversified HIV-1 and HIV-2
4 panel remain scarce. We performed a multicenter study on 116 serum samples accounting for most
5 HIV-1 (9 different subtypes in group M, 17 CRFs, 3 group O) and HIV-2 (groups A and B) diversity,
6 evaluating seven confirmatory assays (six commercially available assays and one in-house assay) with
7 genotyping as reference. The assays were INNO-LIA HIV I/II Score, HIV-2 Blot 1.2, HIV Blot 2.2, New
8 Lav Blot I and II, Geenius and an in-house Serotyping ELISA. Among HIV-1 samples, INNO-LIA, HIV Blot
9 2.2, New Lav Blot I, Geenius and Serotyping had comparable high sensitivities, from 98% to 100%,
10 whereas HIV-2 Blot 1.2 and New Lav Blot II had a high undetermined rate (85% and 95%,
11 respectively). HIV-2 Blot 1.2 and New Lav Blot II misclassified 7% and 5% HIV-1 samples as HIV-2,
12 respectively, and HIV-2 Blot 1.2 had a 8% false-negative rate. Among HIV-2 samples, INNO-LIA, New
13 Lav Blot II, HIV-2 Blot 1.2 and Serotyping had high sensitivities, from 96% to 100%. HIV Blot 2.2
14 misclassified 17% HIV-2 samples as HIV-1/HIV-2 dual infections. New Lav Blot I misclassified 19% of
15 HIV-2 samples as HIV-1 with a high (81%) undetermined rate, and Geenius misclassified 2% as HIV-1
16 and 7% as untypable HIV-positive. For HIV-1/HIV-2 dual infection, results were less sensitive with at
17 most 87.5% for INNO-LIA and Geenius, and 75% for HIV Blot 2.2 and Serotyping. Overall,
18 confirmatory assays remain useful for most cases, with the exception of HIV-1/HIV-2 dual infection
19 suspicion.

20 **KEYWORDS** HIV confirmation, Western blot, INNO-LIA, Geenius, HIV-1, HIV-2, differentiation

21 **Fundings**

22 This work received no specific funding

23

24 **Declaration of Competing Interests**

25 The authors declare that they have no competing financial interests or personal relationships that
26 could have influenced the work reported in this paper.

27

28 **Acknowledgments**

29 The authors thanks ADEBIOPHARM ER28 association for the financial participation which allowed us
30 to present our results in congresses. The authors thank Bio-Rad, MP Biomedicals and Innogenetics

1 for providing free kits for this study. Laboratories that gave HIV detection kits had no part on study
2 design, data collection, data analyses, data interpretation nor manuscript writing.

3

4 According to the World Health Organization data, around 38 million people are living with HIV-1
5 worldwide, of whom 26 million in Africa (1). HIV-2 is much less prevalent, with an estimated 2 million
6 people infected (2), mainly in West African countries with prevalence up to 2.8 % (3–5). HIV-2 is
7 infrequent in other parts of the world, accounting for new HIV cases from 0.1 % in the United States
8 to 2% in France and 5 % in Portugal (6–8). Misclassifying HIV-2 can have deleterious consequences
9 for the follow-up of infection since all HIV-1 viral load commercial assays fail to detect HIV-2 (9), and
10 for antiretroviral therapy due to a natural resistance to all non-nucleoside reverse transcriptase and
11 fusion inhibitors (10), and a lower efficacy of both some protease inhibitors (11) and the newly
12 developed capsid inhibitor lenacapavir (12). For instance, the recent analysis of a cohort of
13 Guinean pregnant women noticed that three quarters of infants from HIV-2 positive mothers
14 received ineffective antiretroviral therapy at birth (4) due to the use of Nevirapine, a non-nucleoside
15 inhibitor, as a first line treatment for newborns (13). Diagnosis of HIV-1 or HIV-2 infection is based on
16 the detection of antibodies to HIV by enzyme immunoassays (ELISA) or rapid lateral flow assays
17 followed by a specific confirmatory assay, or combination of rapid assays, which may differentiate
18 HIV-1 from HIV-2 (14). Western blot have been the historical gold standard for differentiation (15),
19 but only few studies have compared them to the more accurate molecular HIV-1 and HIV-2 RNA/DNA
20 genotyping.

21 The objective of this study was to assess the accuracy of seven confirmatory assays to
22 discriminate between an infection by either HIV-1 or HIV-2, or a dual infection (HIV-1/HIV-2), using a
23 large panel of well characterized serum or plasma samples accounting for a large part of HIV-1 and
24 HIV-2 diversities.

25

26 MATERIALS AND METHODS

27 **Study population.** Samples were originated from the routine of the lab. They corresponded to
28 chronically untreated HIV-infected patients from four French University Hospitals (Bichat-Paris, Pitié
29 Salpêtrière-Paris, Rouen and Tours) were included. Selection of HIV-1, HIV-2 or HIV-1/HIV-2
30 infections were assessed respectively by the detection of HIV-1 RNA (Cobas® AmpliPrep/Cobas
31 TaqMan® HIV-1 Test, v2.0; Roche Diagnostics, Mannheim Germany, or Real-Time HIV-1, Abbott
32 Molecular, Rungis France) or HIV-2 RNA as previously described (16). In case of undetectable plasma
33 viral load, typing was performed by quantification of HIV-1 proviral DNA (Biocentric generic DNA cell

1 for HIV-1, Biocentric, Bandol France) or HIV-2 DNA by an in-house method (17). Subtypes and
2 recombinant forms of HIV-1 strains were determined using nucleotide sequences of the protease and
3 reverse transcriptase genes and a Basic Local Alignment Search Tool (18) with the Los Alamos
4 laboratory HIV-1 references , except for HIV-1 group O or HIV-2 for which in-house methods were
5 used (19, 20). Serum samples were stored frozen at -20°C. Each sample was aliquoted to avoid
6 several freeze-thawing cycles and was tested within the same thawing cycle for each assay.

7 **Assays evaluated.** Assays tested were INNO-LIA HIVI/II Score (Innogenetics, Gent, Belgium), HIV-
8 2 Blot 1.2 and HIV Blot 2.2 (MP Biomedicals, Illkirch, France), New Lav Blot I, New Lav Blot II and
9 Geenius (Bio-Rad laboratories, Marnes-la-Coquette, France), and a Serotyping ELISA (SS-ELISA), an in-
10 house method used by the French National Reference Center for HIV-1 Serotyping (21). Each assay is
11 described in Table 1. Commercial assays were performed and interpreted according to the
12 manufacturers' recommendations. For INNO-LIA and Geenius, samples reactive for both HIV-1 and
13 HIV-2 specific antibodies are classified as "positive for HIV antibodies (untypable)". As previously
14 described for INNO-LIA (22), to ease readability in this paper, we termed this category as "HIV-1/HIV-
15 2 dual reactive". Sensitivities for HIV-1, HIV-2 and HIV-1/HIV-2 dual infection were defined as the
16 ability to identify respectively HIV-1, HIV-2 or HIV-1/HIV-2 dual infection. All confirmatory assays
17 were prospectively performed at the Pitié Salpêtrière Hospital except for Serotyping, which was
18 prospectively performed in Tours University Hospital. Reading and assessment of HIV-1, HIV-2 and
19 HIV-1/HIV-2 infections were done by 2 blinded operators. Results from Geenius and INNO-LIA were
20 assessed using their respective readers.

21 **Statistical analysis.** Statistical analysis was conducted using R version 4.2.1 software (23). The
22 95% confidence intervals (95% CI) were calculated using Wilson confidence interval for proportions
23 (24). We determined a priori that considering a 95% sensitivity for each test, with 61 samples (for
24 HIV-1) we would have about 7% accuracy, and with 47 samples (for HIV-2) about 8% accuracy. We
25 considered that these precisions would be enough to be clinically relevant.

26 **Ethics.** This work was approved by the French Infectious Disease Research Ethics Board
27 (IRB00011642), no. 2023-0108. Patients samples were anonymized prior to the study in accordance
28 with local ethics guidelines.

29

30 RESULTS

31 **Samples panels.** In this study, 116 samples were gathered from patients with chronic HIV
32 infections: 61 samples from people infected with HIV-1 (except 47 for Geenius), 47 samples from
33 people infected with HIV-2 (except 45 for Geenius), and 8 samples from those with HIV-1/HIV-2 dual
34 infection. Subtypes and recombinant forms (genotypes) of HIV-1 strain accounted for most of its

1 antigenic diversity within HIV-1 group M: subtypes A (n=5), B (n=8), C (n=5), D (n=3), F (n=2), G (n=2),
2 H (n=2), J (n=2), K (n=2) and Circulating Recombinant Forms (CRF)
3 05/06/11/12/13/14/15/18/19/22/27/30/36/42 (1 each), CRF01 and CRF09 (n=2 each), and CRF02
4 (n=9). We used three serum samples from HIV-1 group O infected patients. Of the 47 HIV-2 infected
5 patients, seven were related to the group A and 13 to the group B, while 27 remained undetermined.

6 **Accuracies for HIV-1 infection only.** Assays designed to confirm HIV-1 infection specifically
7 (INNO-LIA, HIV Blot 2.2, New Lav Blot I, Geenius and Serotyping) had similar high sensitivities (98% to
8 100%) with a low undetermined rate and no misclassification (Table 2). Of note, the only sample with
9 undetermined result for HIV-Blot 2.2 corresponded to one of the three HIV-1 group O samples.
10 Otherwise, tests designed to confirm HIV-2 infection only (HIV-2 Blot 1.2 and New Lav Blot II)
11 exhibited very high rate of undetermined results (85% to 95%). This phenomenon is linked to cross-
12 reactivities of HIV-1 antibodies toward HIV-2 GAG (mostly p26 for HIV-2 Blot 1.2 and New Lav Blot II),
13 POL (34 and 66 for HIV-2 Blot 1.2 and New Lav Blot II), and ENV (gp105 for HIV-2 Blot 1.2 and New
14 Lav Blot) specific antigens.

15 **Accuracies for HIV-2 infection only.** Assays designed to confirm HIV-2 infection specifically (HIV-
16 2 Blot 1.2, New Lav Blot II and HIV Blot 2.2, INNO-LIA Geenius and serotyping) had HIV-2 sensitivities
17 ranging from 83% to 100% (Table 3). INNO-LIA and Geenius classified respectively 2% (95% CI, 0.3%
18 to 11%) and 7% (95% CI, 2% to 18%) of the samples as HIV-positive without differentiation. Geenius
19 misclassified 2% (95% CI, 0.4% to 11%) samples as HIV-1 infection. New Lav Blot I misclassified 19 %
20 (95% CI, 10% to 33%) samples as HIV-1 infection. HIV Blot 2.2 misclassified as possible HIV-1/HIV-2
21 dual infection 17% (95% CI, 8% to 30%) samples. These phenomena are due to cross-reactivities of
22 HIV-2 antibodies toward HIV-1 GAG (p24, p40, and p55 proteins for New Lav Blot I, p24 and p40 for
23 HIV Blot 2.2), POL (p34 and p68 proteins for New Lav Blot I, p34 for HIV Blot 2.2) specific antigens.

24 **Accuracies for HIV-1/HIV-2 dual infection.** Only four methods, INNO-LIA, HIV Blot 2.2, Geenius
25 and Serotyping, were designed to identify HIV-1/HIV-2 dual infections, due to the presence of specific
26 proteins for both viruses. INNO-LIA and Geenius performed well, with 7 of 8 samples being HIV-
27 1/HIV-2 dually reactive. HIV Blot 2.2 and Serotyping accurately identified 6 dual infections among 8
28 samples. HIV-2 Blot 1.2, New Lav Blot I and New Lav Blot II were positive for all samples for their
29 corresponding HIV type, HIV-1 or HIV-2 (table 4).

30

1 DISCUSSION

2 This study aimed to evaluate the accuracy of seven HIV confirmatory assays to identify an
3 infection with HIV-1, HIV-2, or HIV-1/HIV-2, on a panel of serum or plasma samples designed to
4 account for most HIV-1 and HIV-2 diversity (25). To date, few studies have already addressed this
5 issue, as most used Western blot or INNO-LIA as the gold standard, instead of genotypes
6 identification (22, 26–34). This study analyzed samples from 9 HIV-1 group M subtypes and 17
7 different HIV-1 CRFs, HIV-1 group O, and at least 2 of the main HIV-2 groups (35).

8 Considering HIV-1 infection only, INNO-LIA, HIV Blot 2.2, New Lav Blot I, Geenius and
9 Serotyping had similar sensitivities, from 98% to 100%. On the opposite, assays designed only to
10 identify HIV-2 infection, HIV-2 Blot 1.2 and New Lav Blot II, exhibited high rates of undetermined
11 results and misclassified as HIV-2 some HIV-1 only infections (7% and 5%, respectively), a
12 consequence of cross-reactivity between HIV-1 antibodies and HIV-2 proteins. These results are
13 consistent with previous studies regarding HIV-1 infection sensitivities and undetermined results for
14 INNO-LIA (27, 36, 37), HIV Blot 2.2 (36, 38), New Lav Blot I and II (39, 40) and Geenius (27, 36–39, 41,
15 42), summarized in Fig. 1.

16 For HIV-2 infection only, sensitivities ranged from 91% to 100% with methods designed to
17 confirm HIV-2 infection (INNO-LIA, HIV-2 Blot 1.2, New Lav Blot II, Geenius and serotyping). Overall,
18 results were consistent with previous studies. New Lav Blot II and HIV-2 Blot 1.2 had reported
19 sensitivities of at least 95% (32, 43). INNO-LIA had a reported sensitivity of 100% (36, 37), while
20 sensitivity ranged from 85% to 100% for Geenius (27, 39–42, 44). However, most of these studies
21 included a very limited number of patients. HIV Blot 2.2 had an intermediate sensitivity of 83%,
22 which corresponded to the HIV-2 specific antigen positivity, an antigen designed by the manufacturer
23 to alert for a possible HIV-2 infection. New Lav Blot I misclassified a high proportion of HIV-2 infection
24 as HIV-1 infections (19%) and exhibited a particularly high undetermined rate (80%), a result in
25 accordance with previous studies (39, 43). Thus it should not be used alone for differentiation but in
26 combination with a HIV-2 specific lateral flow or confirmatory assay. When considering the infections
27 with HIV-1 or HIV-2 only, a higher occurrence of cross-reactivity was noted when HIV-2 antibodies
28 interacted with HIV-1 proteins, as opposed to HIV-1 antibodies binding to HIV-2 proteins. This
29 phenomenon has been described by Damond et al for HIV-2 group B (43). Our results, along with an
30 extensive comparison to previous studies that used genotyping as the gold standard are summarized
31 Fig. 1.

32 For HIV-1/HIV-2 dual infections, INNO-LIA and Geenius reacted with both HIV-1 specific and HIV-
33 2 specific antigens for 87% of the samples. However, no assay exhibited a perfect concordance with

1 genotyping as the gold standard for this scarce situation, a finding consistent with Tchounga et al
2 who used both Immunocomb BiSpot and Serotyping as the reference assay (45). In the event of a
3 suspected dual infection, we advise to consider a genetic assay to confirm the diagnosis.

4 We must consider that our study has some limitations. Firstly, since by design the study focused
5 on samples selected from patients having either HIV-1, or HIV-2 or HIV-1/HIV-2 infection, we could
6 not determine predictive positive and negative value of each test. Secondly, we have not been able
7 to identify the HIV-2 group concerned in half samples, not allowing to see if a group exhibited more
8 cross-reactivities toward HIV-1 than others, as it was already observed for HIV-2 group B (43) . Lastly,
9 the number of HIV-1/HIV-2 dual infections was low, even in a context of a multicentric study,
10 reflecting the scarcity of such cases in France (46). As a consequence, assay accuracies presented for
11 HIV-1/HIV-2 infection should be considered with caution, and further studies should be conducted on
12 this population.

13 Our study demonstrated that commercial assays designed to confirm specifically either HIV-1 (New
14 Lav Blot I) or HIV-2 (New Lav Blot II, HIV-2 Blot 1.2) infection exhibited non negligible cross-reactivity
15 with HIV-2 or HIV-1, respectively, leading to misclassification. They should not be used alone for
16 differentiation. On the opposite, assays designed to detect both HIV-1 and HIV-2 (HIV-Blot 2.2, INNO-
17 LIA, Geenius, serotyping) performed well for both infections, although most assays exhibited some
18 cross-reactivity, especially for HIV-2 positive samples. Overall, unresolved differentiation should alert
19 to HIV-2 or dual infection, requiring further investigation, but misclassification of HIV-1 or HIV-2
20 single infection should be exceptional. However, no assay aligns perfectly with genotyping as the
21 gold standard, especially for dual infections.

22 In conclusion, confirmatory assays have been the historical gold standard for HIV-1 and HIV-2
23 differentiation, and they remain useful and reliable for HIV-1/HIV-2 differentiation for most cases,
24 with the exception of HIV-1/HIV-2 dual infection suspicion. However, they remain time-consuming,
25 with a relative high cost. Altogether, these factors raise the question of the most suitable gold
26 standard for differentiating HIV-1 from HIV-2, especially in the suspicion of dual infection or in the
27 realm of clinical trials and research.

28

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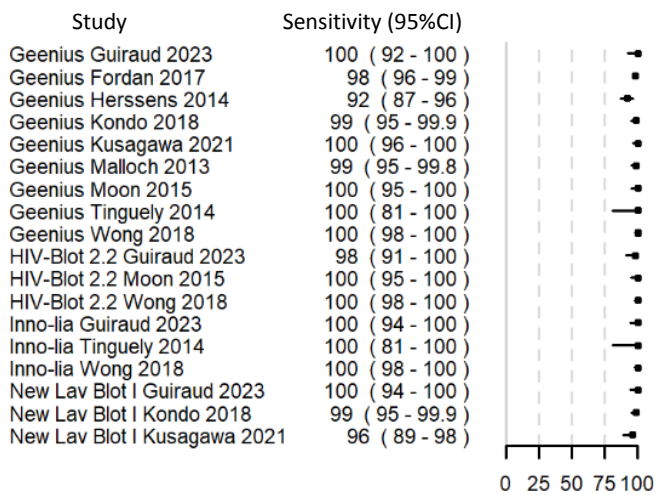
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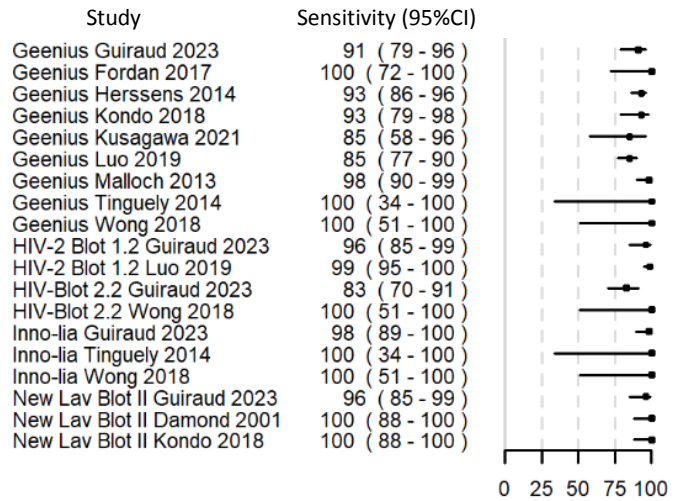
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23

A. Sensitivities to detect HIV-1 infection

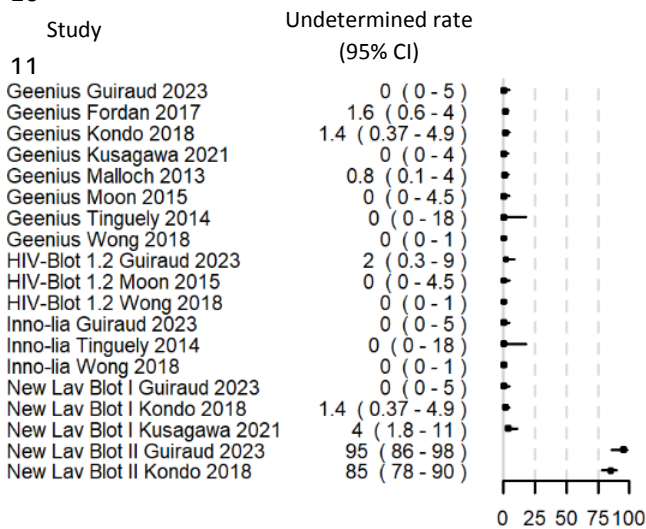


B. Sensitivities to detect HIV-2 infection

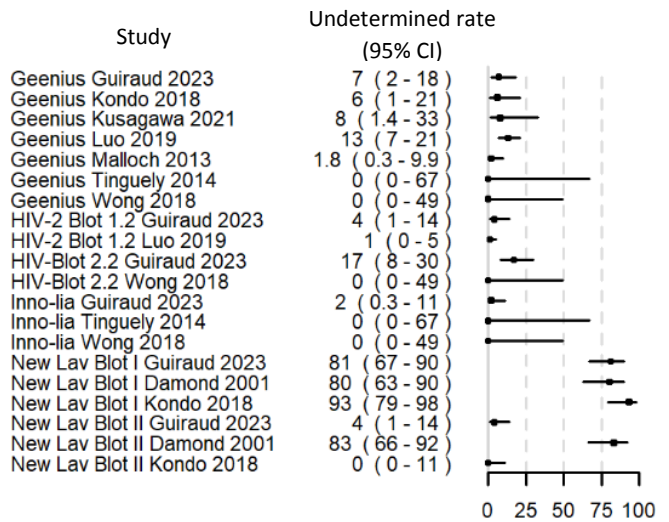


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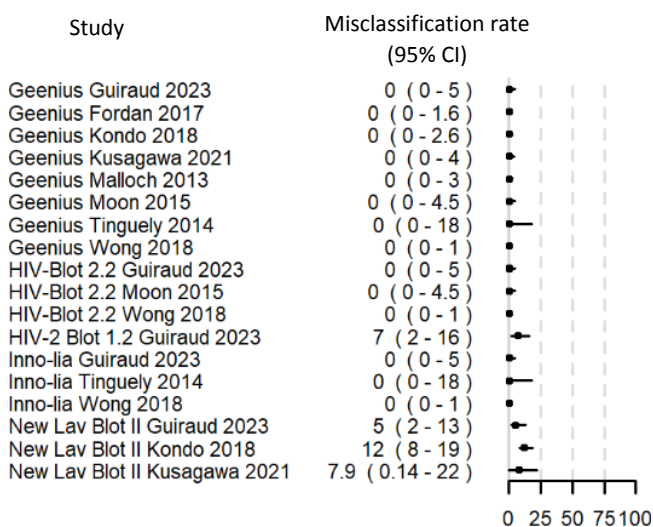
C. Undetermined rate to detect HIV-1 infection



D. Undetermined rate to detect HIV-2 infection



E. Percentage of HIV-1 infection misclassified as HIV-2 infection



F. Percentage of HIV-2 infection misclassified as HIV-1 infection

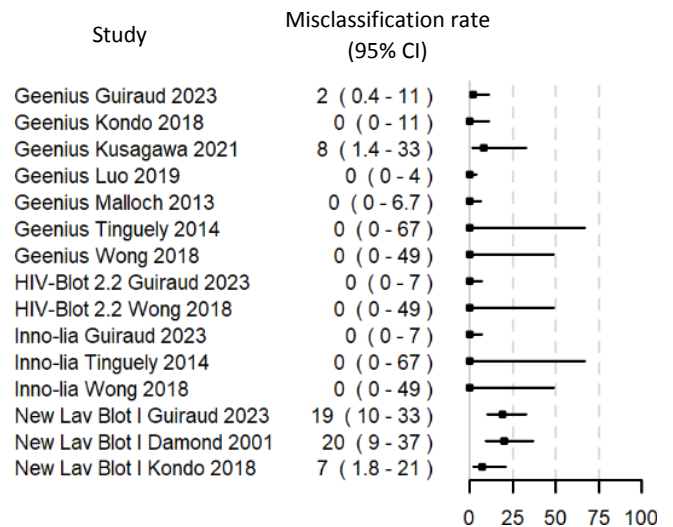


FIG 1 Comparison for the different tests among studies to accurately identify or misclassify HIV-1 or HIV-2 infection, with sensitivity for HIV-1 (A), HIV-2 (B) infection, undetermined rate for HIV-1 (C) and HIV-2 (D) samples, and percentage of HIV-1 samples misclassified as HIV-2 (E) or percentage of HIV-2 samples misclassified as HIV-1 (F).

1 **TABLE 1** Description of kits and methods used

Kit / method evaluated	Antigens used	Technology	Matrix	Test duration	Cost per sample^c
INNO-LIA HIV I/II Score	Sgp120 ^b , gp41 ^b , p31 ^a , p24 ^a , p17 ^a , gp36 ^b , and sgp105 ^b	Line immunoblot	Serum, plasma	18h	11 US\$
HIV Blot 2.2	HIV-1 viral lysate and peptide from HIV-2 envelope	Western blot	Serum, plasma	3h	28 US\$
HIV-2 Blot 1.2	HIV-2 viral lysate	Western blot	Serum, plasma	3h	22 US\$
New Lav Blot I	HIV-1 viral lysate	Western blot	Serum, plasma	4h	18 US\$
New Lav Blot II	HIV-2 viral lysate	Western blot	Serum, plasma	4h	29 US\$
Geenius	Gp160 ^a , gp41 ^b , p31 ^b , p24 ^a , gp140 ^b , and gp36 ^b	Immunochromatography	Serum, plasma, whole blood	25 min	20-25 US\$
Serotyping (SS-ELISA)	HIV-1 and HIV-2 peptides	ELISA	Serum, plasma	2h	1 US\$

2 ^aRecombinant protein.

3 ^bSynthetic peptide.

4 ^cReagents only.

5

Methods							
HIV-1 positive panel (<i>n</i> = 61)	INNO-LIA ^a	HIV-2 Blot 1.2 ^b	HIV Blot 2.2	New Lav Blot I ^c	New Lav Blot II ^b	Geenius ^a	Serotyping (SS-ELISA)
No. correctly identified/no. tested	61/61	NA	60/61	61/61	NA	47/47	61/61
Sensitivity to detect HIV-1 only % [95% CI] ^d	100 [94-100]	NA	98 [91-100]	100 [94-100]	NA	100 [92-100]	100 [94-100]
No. misclassified as HIV-2 (%) [95% CI]	0 (0) [0-5]	4 (7) [2-16]	0 (0) [0-5]	NA	3 (5) [2-13]	0 (0) [0-5]	0 (0) [0-5]
No. false negative (%) [95% CI]	0 (0) [0-5]	5 (8) [3-18]	0 (0) [0-5]	0 (0) [0-5]	0 (0) [0-5]	0 (0) [0-5]	0 (0) [0-5]
No. undetermined (%) [95% CI]	0 (0) [0-5]	52 (85) [74-92]	1 (2) [0.3-9]	0 (0) [0-5]	58 (95) [86-98]	0 (0) [0-5]	0 (0) [0-5]

1

2 **TABLE 2** Performance characteristics of INNO-LIA, HIV-2 Blot1.2, HIV Blot 2.2, New Lav Blot I, New Lav Blot II, Geenius and Serotyping methods for HIV-1
3 positive panel

4 ^aFor INNOLIA and Geenius, results using an automatic reading are presented.

5 ^bHIV-2 Blot 1.2 and New Lav Blot II are designed to confirm only HIV-2 infection.

6 ^cNew Lav Blot I is designed to confirm only HIV-1 infection.

7 ^d95% CI: Confidence Interval (95% CI).

8

HIV-2 positive panel (<i>n</i> = 47)	Methods						Serotyping (SS-ELISA)
	INNO-LIA ^a	HIV-2 Blot 1.2 ^b	HIV Blot 2.2	New Lav Blot I ^c	New Lav Blot II ^b	Geenius ^a	
No. correctly identified/no. tested	46/47	45/47	39/47	NA	45/47	41/45	47/47
Sensitivity to detect HIV-2 [95% CI] ^d	98 [89-100]	96 [85-99]	83 ^e [70-91]	NA	96 [85-99]	91 [79-96]	100 [92-100]
No. misclassified as HIV-1 (%) [95% CI]	0 (0) [0-7]	NA	0 (0) [0-7]	9 (19) [10-33]	NA	1 (2) [0.4-11]	0 (0) [0-7]
No. misclassified as HIV positive (%) [95% CI] ^f	1 (2) [0.3-11]	NA	8 (17) [8-30]	NA	NA	3 (7) [2-18]	0 (0) [0-7]

1 **TABLE 3** Performance characteristics of INNO-LIA, HIV-2 Blot1.2, HIV Blot 2.2, New Lav Blot I, New Lav Blot II, Geenius and Serotyping methods for HIV-2
2 panel

No. undetermined (%)	0 (0)	2 (4)	0 (0)	38 (81)	2 (4)	0 (0)	0 (0)
[95% CI]	[0-7]	[1-14]	[0-7]	[67-90]	[1-14]	[0-7]	[0-7]

1 ^aFor INNOLIA and Geenius, results using an automatic reading are presented.

2 ^bHIV-2 Blot 1.2 and New Lav Blot II are designed to confirm only HIV-2 infection.

3 ^cNew Lav Blot I is designed to confirm only HIV-1 infection.

4 ^d95% CI: Confidence Interval (95% CI).

5 ^eAt least positive for HIV-2 specific antigen

6 ^fHIV-1 and HIV-2 positive without differentiation.

7

8

HIV-1 and HIV-2 positive panel (n = 8)	Methods						Serotyping (SS-ELISA)		TABLE
	INNO-LIA ^a	HIV-2 Blot 1.2 ^b	HIV Blot 2.2	New Lav Blot I ^c	New Lav Blot II ^b	Geenius ^a			
No. correctly identified/no. tested	7/8 ^d	NA	6/8 ^d	NA	NA	7/8 ^d	6/8	4	4 Perfor mance charac teristic s of INNO- LIA, HIV-2 Blot1.
Sensitivity to detect HIV-1 and HIV-2 % [95% CI] ^e	87.5 [53-98]	NA	75 [41-93]	NA	NA	87.5 [53-98]	75 [41-93]	5 6 7	
No. misclassified as HIV-1 only (%) [95% CI]	0 (0) [0-32]	NA	1 (12.5) [2-47]	8 (100) [67-100]	NA	0 (0) [0-32]	1 (12.5) [2-47]	8 9 10	
No. misclassified as HIV-2 only (%) [95% CI]	1 (12.5) [2-47]	8 (100) [67-100]	1 (12.5) [2-47]	NA	8 (100) [67-100]	1 (12.5) [2-47]	1 (12.5) [2-47]	11 12	
								13	

14 2, HIV Blot 2.2, New Lav Blot I, New Lav Blot II, Geenius and Serotyping methods for dual HIV-1 and HIV-2
15 positive panel

16

17 ^aFor INNOLIA and Geenius, results using an automatic reading are presented.

18 ^bHIV-2 Blot 1.2 and New Lav Blot II are designed to confirm only HIV-2 infection.

19 ^cNew Lav Blot I is designed to confirm only HIV-1 infection.

20 ^dReactive for both HIV-1 and HIV-2 specific antibodies.

21 ^e95% CI: Confidence Interval (95% CI).

22

23

24