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## Incorrect Identification of Recent HIV Infection in Adults in the United States Using a Limiting-Antigen Avidity Assay

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### Abstract

**Objectives**—To evaluate factors associated with misclassification by the limiting-antigen avidity (LAg-avidity) assay among individuals with long-standing HIV infection.

**Design**—Samples were obtained from the Multicenter AIDS Cohort Study (MACS), and AIDS Linked to the IntraVenous Experience (ALIVE) cohort (1089 samples from 667 individuals, 595 samples collected 2–4 years and 494 samples collected 4–8 years after HIV seroconversion). Paired samples from both time points were available for 422 (63.3%) of the 667 individuals.

**Methods**—Samples were considered to be misclassified if the LAg-Avidity assay result was  $\geq 1.5$  normalized optical density (OD-n) units.

**Results**—Overall, 4.8% (52/1089) of the samples were misclassified, including 1.8% (16/884, 95% confidence intervals [CI]: 1.09%–3.06%) of samples from individuals with viral loads  $>400$  copies/mL and 1.4% (10/705) of samples from individuals with viral loads  $>400$  copies/mL and CD4 cell counts  $>200$  cells/ $\mu$ l (95% CI: 0.68%–2.60%). Age, race, gender, and mode of HIV acquisition were not associated with misclassification. In an adjusted analysis, viral load  $<400$  copies/mL (adjusted odds ratio [aOR]: 3.72, 95% CI: 1.61–8.57), CD4 cell count  $<50$  cells/ $\mu$ l (aOR: 5.41, 95% CI: 1.86–15.74), and low LAg-Avidity result ( $\geq 1.5$  OD-n) from the earlier time point (aOR: 5.60, 95% CI: 1.55–20.25) were significantly associated with misclassification.

**Conclusions**—The manufacturer of the LAg-Avidity assay recommends excluding individuals from incidence surveys who are receiving antiretroviral therapy, are elite suppressors, or have

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AIDS (CD4 cell count <200 cells/ $\mu$ l). The results of this study indicate that those exclusions do not remove all sources of assay misclassification among individuals with long-standing HIV infection.

## Keywords

LAg-Avidity; incidence; MSM; PWID; HIV; misclassification

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## Introduction

The United States (US) Centers for Disease Control (CDC) recently introduced the limiting antigen avidity enzyme immunoassay (LAg-Avidity assay).[1, 2] This assay has been recommended as an accurate method for HIV incidence estimation,[1–5] and is commercially available from several sources. The LAg-Avidity assay measures the binding strength of antibodies to an immunodominant region of HIV-1.[2] The ability to use this assay to identify individuals with recent HIV infection is based on the premise that the strength of antibody binding is weak early in infection, and increases over time. A recent study evaluated the performance of the LAg-Avidity assay, alone and in multi-assay algorithms (MAAs), for cross-sectional HIV incidence estimation in the US.[6] In that study, the LAg-Avidity assay did not perform well in a single-assay format, regardless of the assay cutoff. However, MAAs that included the LAg-Avidity assay were identified that provided accurate incidence estimates.[6]

A limitation of serologic assays developed for cross-sectional incidence estimation is that these assays misclassify some individuals with long-term infection as assay positive (having recent infection).[7–12] This type of misclassification can lead to significant overestimation of HIV incidence.[13, 14] Several factors have been associated with misclassification by serologic incidence assays, include viral suppression,[15, 16] low CD4 cell count,[16–18] and long-term use of antiretroviral therapy (ART).[15–18] In this report, we identified factors associated with misclassification by the LAg-Avidity assay in adults in the US with long-standing HIV infection, including men who have sex with men (MSM) and persons who inject drugs (PWID).

## Materials and Methods

### Samples used for analysis

We analyzed 1089 plasma and serum samples from 667 individuals followed in the Multicenter AIDS Cohort Study (MACS) and the AIDS Linked to the IntraVenous Experience (ALIVE) cohort. MACS is a longitudinal study of the natural and treated history of HIV infection in MSM that has followed men semiannually since 1984.[19] ALIVE is a longitudinal study of HIV infection in PWID in Baltimore, Maryland that has been ongoing since 1988.[20] The samples analyzed in the present study were collected between 1987 and 2009 from individuals who had a last negative HIV test and first positive HIV test at study visits less than 1 year apart. The date of HIV seroconversion was defined as either: (1) the midpoint between the last negative HIV test and first positive HIV test, or (2) two weeks after a visit where acute HIV infection was diagnosed (HIV RNA positive, HIV antibody

negative). For each individual, samples were obtained either 2–4 years or 4–8 years after HIV seroconversion (595 and 494 samples, respectively). The time between the estimated date of infection and sample collection ranged from 2.0 to 8.3 years. Paired samples from 2–4 and 4–8 years after HIV seroconversion were available for 422 (63.3%) of the 667 individuals; 173 individuals had a single sample from 2–4 years after seroconversion, and 72 individuals had a single sample from 4–8 years after seroconversion. Previously collected epidemiological and laboratory data, including HIV viral load and CD4 cell count, were included in the analysis.

### Laboratory testing

Samples were analyzed using the LAg-Avidity assay (Sedia Biosciences Corporation, Portland, OR, USA).[2] Assay results are normalized using an internal calibrator and are reported as a normalized optical density (OD-n) values. The assay was performed according to methods of Duong et al.[1] If the initial test result was <2.0 OD-n, samples were retested in triplicate to obtain “confirmation” values, according to the manufacturer’s directions. The median of the confirmation values was used as the final result. The recently recommended assay cutoff of 1.5 OD-n (Sedia™ HIV-1 LAg-Avidity assay product insert [version LN6039-05]) was used for analysis in this report.

### Statistical analysis

Samples were stratified by time after HIV seroconversion (2–4 years versus 4–8 years). Age, race, HIV viral load, CD4 cell count, duration of ART at the time of sample collection, and year of sample collection were treated as categorical variables (Table 1). The association of categorical factors with misclassification was examined using the Fisher’s exact test or Chi square test. Logistic regression was performed using data stratified by duration of infection (2–4 years and 4–8 years) to determine the odds of misclassification for all factors analyzed. All factors associated with misclassification in the univariate analysis with  $p < 0.1$  were included in the multivariate logistic regression analysis. To account for individuals who had samples from two time points (2–4 years and 4–8 years after seroconversion), a visit-dependent variable with the 2–4 year result was used as a predictor of the 4–8 year result. All analysis was performed using STATA v11 (StataCorp, College Station, TX).

### Human subjects

All work was conducted in accordance with the Declaration of Helsinki, with informed consent from each participant and approval by appropriate institutional review boards.

### Results

We analyzed 1089 samples from 667 individuals who were HIV infected for 2–8 years; 52 (4.8%) of the samples had an OD-n  $\geq 1.5$  and thus were misclassified as assay positive. Overall, 5.8% (35/600) samples from MSM were misclassified as assay positive, and 4.5% (17/489) from PWID were misclassified as assay positive,  $p=0.07$ . The misclassified samples included 4.4% (26/595, 95% confidence intervals [CI]: 2.9%–6.3%) of the samples collected 2–4 years after seroconversion and 5.3% (26/494, CI: 3.5%–7.6%) of the samples collected 4–8 years after seroconversion. The misclassified samples included: 1.8% (16/884,

CI: 1.1%–3.1%) of the samples from individuals with viral loads >400 copies/mL; 17.6% (36/205, CI: 12.6%–23.5%) of the samples from individuals with viral loads <400 copies/mL; and 1.4% (10/705) of the samples from individuals with viral loads >400 copies/mL and CD4 cell counts >200 cells/ $\mu$ L (CI: 0.7%–2.6%). In addition, 12.2% (11/90, CI: 6.3%–20.8%) of the samples collected 5–8 years after seroconversion were from individuals whose samples were also misclassified as assay positive 2–4 years after seroconversion. When the analysis excluded individuals on ART who were not virally suppressed, elite suppressors, and individuals with AIDS (CD4 <200 cells/ $\mu$ L), 2.0% (12/592, CI: 1.1%–3.5%) of the remaining samples were still misclassified as assay positive.

In univariate analyses, the following factors were positively associated with misclassification 2–4 years after seroconversion: older age (40–74 years), viral suppression (viral load <400 copies/mL), more recent sample collection (during or after 1998) and on ART for  $\geq$  2 years, Table 1. Lower CD4 cell count (200–500 cells/ $\mu$ L) was negatively associated with misclassification, Table 1. All of these factors and associations, both positive and negative, were also associated with misclassification of samples collected 4–8 years after seroconversion, however, more recent sample collection (1994–present) was negatively associated with misclassification. Finally, for samples collected 4–8 years after seroconversion, misclassification was associated with prior misclassification (i.e., of the paired sample collected 2–4 years after seroconversion, Table 1).

In a multivariate model (Table 2, Model 1), the following factors were independently associated with misclassification: more recent sample collection (during or after 1998), lower viral load (<10,000 copies/mL), and lower CD4 cell count (either 200–500 or <50 cells/ $\mu$ L). ART was not associated with misclassification in this model. Similar results (odds, significance) were obtained when the model was not adjusted for ART and when a visit-dependent variable was included (Table 2, Model 2). Similar results (odds, significance) were also obtained when the model was not adjusted for CD4 cell count; however, in this model, a middle age range (40–44) was significantly associated with misclassification (Table 2, Model 3). In all models, when a visit-dependent variable was included, individuals who were misclassified at 2–4 years were 5.60 times more likely to be misclassified at 4–8 years. When samples with HIV viral load <400 copies/mL were excluded from the analysis (Table 2, Model 4), the only factors that were independently associated with misclassification were more recent sample collection (during or after 1998; adjusted odds ratio, aOR: 0.12,  $p$ <0.02) and lack of misclassification of the sample collected 2–4 years after seroconversion (aOR: 0.09,  $p$ <0.01).

## Discussion

Overall, the LAg-Avidity misclassified 4.8% of samples from individuals with long-standing infection as assay positive using the recently-recommended assay cutoff of 1.5 OD-n. This is considerably lower than the misclassification frequency of 10.3% that was previously obtained using the BED capture immunoassay (BED-CEIA), but higher than the misclassification frequency of 1.0% for the BioRad-Avidity assay using a 40% avidity index cutoff for the same sample set.[16, 21] The misclassification frequency obtained for samples from individuals who were likely to have subtype B HIV infection was 2.6% using the

AxSYM HIV 1/2 gO avidity assay,[22] 6.6% using the Architect HIV Ag/Ab Combo avidity assay,[22] and 2.4% using the V3 IDE assay.[23] HIV subtype can significantly affect misclassification; the misclassification frequencies for the BED-CEIA, LAg-Avidity assay, and BioRad-Avidity assay were 11.8%, 1.9%, and 1.9, respectively, for subtype A samples, and 15.1%, 4.4%, and 20.8%, respectively, for subtype D samples.[9] While this performance of the LAg-Avidity assay is clearly better than the performance of the BED-CEIA, these data suggest that the LAg-Avidity assay lacks the specificity required for use in a single-assay format. The strongest factors associated with misclassification of long-term HIV infections by the LAg-Avidity assay were more recent sample collection, viral suppression, and lower CD4 cell count (Table 1 and Table 2). When the analysis was adjusted for viral suppression, the impact of ART on misclassification was completely attenuated.

Current recommendations for the LAg-Avidity include exclusion of individuals on ART, elite suppressors, and individuals with AIDS (CD4 cell count <200 cells/ $\mu$ l, Sedia™ HIV-1 LAg-Avidity product insert [version LN6039-05]). However, persistent misclassification was observed using the LAg-Avidity alone, even after excluding individuals with viral suppression. Recent studies indicate that self-report of antiretroviral (ARV) drug use is unreliable.[24–27] Direct detection of ARV drugs in study or survey samples can be used to identify individuals on ART;[24, 25, 28] however, that testing will not identify elite controllers, who are also likely to be misclassified using serologic assays.[29] Elite controllers may represent a significant proportion of some study populations, for example 9% of HIV+ individuals surveyed at the Johns Hopkins Emergency Department in 2007 had viral loads <400 copies/ml without the presence of detectable ART.[21] For these reasons, it may be most appropriate to use the LAg-Avidity as part of a MAA that also includes HIV viral load.[6] While misclassification was observed using either the LAg-Avidity alone, or the LAg-Avidity assay with exclusions based on viral load and/or CD4 cell count (this report and [30]), a recent study demonstrates that MAAs that include the LAg-Avidity assay with a second serologic assay, as well as other biomarkers, can provide accurate HIV incidence estimates in populations in the US.[6, 31] Further studies are needed to evaluate MAAs that include the LAg-Avidity in study populations with different prevalent HIV subtypes.

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**Table 1**

Factors Associated with Misclassification of the LAg-Avidity assay in the ALIVE (1991–2007) and MACS (1987–2009) Cohorts.

	2 to 4 years after seroconversion		4 to 8 years after seroconversion	
	LAg-Avidity 1.5 % Misclassified	LAg-Avidity 1.5 OR (95% CI)	LAg-Avidity 1.5 % Misclassified	LAg-Avidity 1.5 OR (95% CI)
<b>All</b>	4.37 (26/595)		5.26 (26/494)	
<b>Cohort</b>				
MACS	5.49 (18/328)	1	6.25 (17/272)	1
ALIVE	3.00 (8/267)	0.53 (0.23–1.24)	4.05 (9/222)	0.63 (0.28–1.45)
<b>Sex</b>				
Female	3.08 (2/65)	1	1.61 (1/62)	1
Male	4.53 (24/530)	1.49 (0.35–6.47)	5.79 (25/432)	3.75 (0.50–28.16)
<b>Age at infection</b>				
23–34 years	1.63 (3/184)	1	1.10 (1/91)	1
35–39 years	1.39 (2/144)	0.85 (0.14–5.15)	2.42 (3/124)	2.23 (0.23–21.81)
40–44 years	5.69 (7/123)	3.64 (0.92–14.36)*	5.88 (7/119)	5.63 (0.68–46.56)
45–74 years	9.72 (14/144)	<b>6.50 (1.83–23.1)†</b>	9.38 (15/160)	<b>9.31 (1.21–71.69)†</b>
<b>Sample year</b>				
<1990	0.85 (1/118)	1	0.00 (0/5)	-
1990–1994	3.11 (6/193)	3.75 (0.45–31.58)	1.72 (2/116)	1
1994–1998	0.00 (0/170)	-	1.56 (3/192)	0.37 (0.03–4.75)
1998	16.67 (19/114)	<b>23.4 (3.1–177.99)†</b>	11.60 (21/181)	<b>5.78 (1.29–25.89)†</b>
<b>Race</b>				
Not White	4.06 (11/271)	1	4.91 (11/224)	1
White	5.07 (15/296)	1.26 (0.57–2.80)	5.98 (15/251)	1.23 (0.55–2.74)
<b>HIV viral load (copies/ml)</b>				
>10,000	2.34 (8/342)	1	3.10 (8/258)	1
10,000–400	0.00 (0/169)	-	0.00 (0/115)	-
<400	21.43 (18/84)	<b>11.39 (4.75–27.28)†</b>	14.88 (18/121)	<b>5.46 (2.30–12.96)†</b>



	2 to 4 years after seroconversion		4 to 8 years after seroconversion	
	LAg-Avidity 1.5	OR (95% CI)	LAg-Avidity 1.5	OR (95% CI)
<b>CD4 cell count (cells/mm<sup>3</sup>)</b>				
> 500	6.42 (14/218)	1	7.89 (12/152)	1
200–500	2.31 (6/260)	<b>0.34 (0.13–0.91)<sup>†</sup></b>	1.90 (4/210)	<b>0.23 (0.07–0.72)<sup>†</sup></b>
50–199	4.05 (3/74)	0.62 (0.17–2.21)	2.35 (2/85)	0.28 (0.06–1.29)
<50	6.98 (3/43)	1.09 (0.30–3.98)	17.02 (8/47)	2.39 (0.91–6.27) <sup>*</sup>
<b>On ART</b>				
No	2.83 (13/460)	1	3.94 (13/330)	1
Yes, <2 years	7.89 (3/38)	2.95 (0.80–10.83)	2.44 (1/41)	0.61 (0.08–4.79)
Yes, 2 years	10.31 (10/97)	<b>3.95 (1.68–9.30)<sup>‡</sup></b>	9.76 (12/123)	<b>2.64 (1.17–5.95)<sup>‡</sup></b>
<b>First LAg-Avidity 1.5<sup>d</sup></b>				
No	-	-	2.96 (12/405)	1
Yes	-	-	58.82 (10/17)	<b>46.79 (15.21–143.93)<sup>‡</sup></b>

\* Abbreviations: LAg-Avidity: limiting antigen avidity; OR: odds ratios; CI: confidence intervals; ART: antiretroviral therapy  
 LAg-Avidity assay results are reported as normalized optical density units (OD-n).

Statistically significant values are shown in bold text.;

<sup>\*</sup> p value <0.10,

<sup>†</sup> p value <0.05,

<sup>‡</sup> p value <0.01

**Table 2**  
Adjusted Odds of Misclassification by the LAg-avidity assay (OD-n 1.5) in the ALIVE (1991–2007) and MACS (1987–2009) Cohorts.

	Model 1	Model 2	Model 3	Model 4
<b>All</b>				
<b>Cohort</b>				
MACS	1	1	1	1
ALIVE	0.95 (0.42–2.13)	1.01 (0.46–2.22)	0.72 (0.34–1.55)	0.89 (0.38–2.09)
<b>Age at infection</b>				
23–34 years	1	1	1	1
35–39 years	1.09 (0.27–4.46)	1.09 (0.26–4.59)	1.27 (0.31–5.20)	1.18 (0.28–5.03)
40–44 years	2.32 (0.67–8.08)	3.09 (0.88–10.84)	<b>3.47 (1.01–11.91)<sup>†</sup></b>	2.92 (0.82–10.37)
45–74 years	2.27 (0.68–7.53)	2.09 (0.61–7.15)	2.83 (0.86–9.35)	2.12 (0.61–7.30)
<b>Sample year</b>				
<1990	1	1	1	1
1990–1994	2.41 (0.28–20.98)	3.20 (0.36–28.38)	3.83 (0.45–32.59)	3.40 (0.38–30.44)
1994–1998	1.07 (0.10–11.85)	1.34 (0.12–15.24)	1.65 (0.16–17.66)	1.70 (0.15–19.62)
1998	<b>10.15 (1.07–96.22)<sup>†</sup></b>	<b>11.60 (1.24–108.3)<sup>†</sup></b>	<b>10.61 (1.19–94.76)<sup>†</sup></b>	<b>16.70 (1.67–168)<sup>†</sup></b>
<b>HIV viral load (copies/ml)</b>				
>10,000	1	1	1	1
10,000–400	<b>0.18 (0.04–0.85)<sup>†</sup></b>	<b>0.14 (0.03–0.718)<sup>†</sup></b>	<b>0.15 (0.03–0.69)<sup>†</sup></b>	<b>0.14 (0.03–0.73)<sup>†</sup></b>
<400	<b>3.46 (1.52–7.86)<sup>†</sup></b>	<b>3.57 (1.56–8.15)<sup>†</sup></b>	<b>3.70 (1.69–8.07)<sup>†</sup></b>	<b>3.72 (1.61–8.57)<sup>†</sup></b>
<b>CD4 cell count (cells/mm<sup>3</sup>)</b>				
> 500	1	1	-	1
200–500	<b>0.38 (0.16–0.89)<sup>†</sup></b>	<b>0.38 (0.15–0.922)<sup>†</sup></b>	-	0.39 (0.15–0.97)
50–199	0.60 (0.18–1.93)	0.61 (0.18–2.04)	-	0.66 (0.19–2.27)
<50	<b>5.11 (1.80–14.49)<sup>†</sup></b>	<b>4.34 (1.56–12.1)<sup>†</sup></b>	-	<b>5.41 (1.86–15.74)<sup>†</sup></b>
<b>On ART</b>				
No	1	-	1	1
Yes, <2 years	0.31 (0.09–1.10)	-	0.33 (0.09–1.19)	0.26 (0.07–1.03)

	aOR (95% CI)			
	Model 1	Model 2	Model 3	Model 4
Yes, 2 years	1.39 (0.59–3.24)	-	1.09 (0.48–2.52)	1.22 (0.49–3.06)
<b>Visit-dependent variable</b>				
2–4 years after SC	-	1	1	1
5–8 years after SC, no 2–4 year sample	-	0.83 (0.23–2.96)	0.74 (0.21–2.59)	0.91 (0.22–3.00)
5–8 years after SC, not misclassified at 2–4 years	-	<b>0.33 (0.15–0.72)<sup>‡</sup></b>	<b>0.28 (0.13–0.62)<sup>‡</sup></b>	<b>0.27 (0.12–0.61)<sup>‡</sup></b>
5–8 years after SC, misclassified at 2–4 years	-	<b>8.10 (2.34–27.74)<sup>‡</sup></b>	<b>6.23 (1.66–23.38)<sup>‡</sup></b>	<b>5.60 (1.55–20.25)<sup>‡</sup></b>

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; ART, antiretroviral therapy; SC, seroconversion

<sup>†</sup> p value <0.05.

<sup>‡</sup> p value <0.01

\* Abbreviations: LAg-Avidity, limiting antigen avidity; aOR: adjusted odds ratios; CI: confidence intervals; ART: antiretroviral therapy; SC: seroconversion.

LAg-Avidity assay results are reported as normalized optical density units (OD-n).

Statistically significant values are shown in bold text; <sup>†</sup>p value <0.05, <sup>‡</sup>p value <0.01.