Differential Specificity of HIV Incidence Assays in HIV Subtypes A and D-Infected Individuals from Rakai, Uganda

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Abstract

Assays to determine HIV incidence from cross-sectional surveys have exhibited a high rate of false-recent misclassification in Kenya and Uganda where HIV subtypes A and D predominate. Samples from individuals infected with HIV for at least 2 years with known infecting subtype (133 subtype A, 373 subtype D) were tested using the BED-CEIA and an avidity assay. Both assays had a higher rate of false-recent misclassification for subtype D compared to subtype A (13.7% vs. 6.0%, $p=0.02$ for BED-CEIA; 11.0% vs. 1.5%, $p<0.001$ for avidity). For subtype D samples, false-recent misclassification by the BED-CEIA was also more frequent in women than men (15.0% vs. 5.6%, $p=0.002$), and for samples where that had an amino acid other than lysine at position 12 in the BED-CEIA peptide coding region ($p=0.002$). Furthermore in subtype D-infected individuals, samples misclassified by one assay were 3.5 times more likely to be misclassified by the other assay. Differential misclassification by infecting subtype of long-term infected individuals as recently infected makes it difficult to use these assays individually to estimate population level incidence without precise knowledge of the distribution of these subtypes within populations where subtype A and D cocirculate. The association of misclassification of the BED-CEIA with the avidity assay in subtype D-infected individuals limits the utility of using these assays in combination within this population.

Introduction

ACCURATE ESTIMATES OF HIV incidence are needed to monitor transmission dynamics, to assess the efficacy of interventions for HIV prevention.¹ HIV incidence can be estimated from cross-sectional surveys by use of biomarkers that evolve during the course of HIV infection^{2,3} (reviewed in Guy *et al.*⁴ and Murphy and Parry⁵). Two assays that have been developed for cross-sectional estimation of HIV incidence are the BED Capture Enzyme Immunoassay (BED-CEIA), which measures the proportion of anti-HIV IgG to total IgG,⁶ and an avidity assay based on the BioRad HIV-1/HIV-2 PLUS O EIA, which measures the strength of anti-HIV antibody binding.⁷

One obstacle to using serologic assays for HIV incidence estimation is the misclassification of individuals with long-

standing infection as recently infected (i.e., false-recent misclassification). Low HIV viral load, low CD4 cell count, and long-term use of antiretroviral therapy (ART) have been associated with false-recent misclassification with the BED-CEIA and the avidity assay.^{8–12} Also, the frequency of false-recent misclassification varies in different regions of Africa,^{13,14} with high rates of false-recent misclassification in Eastern Africa, where subtypes A and D are the predominant circulating strains.^{15,16}

In this study, we compared the frequency of false-recent misclassification of individuals from Rakai, Uganda who were infected >2 years with subtype A and D HIV using the BED-CEIA and the avidity assay.¹⁷ We also examined the association of gender, age, and differences in the genetic sequence of HIV in samples that produced false-recent misclassification.

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Materials and Methods

Study population

Samples from individuals with nonrecent HIV infection were obtained from 517 individuals in the Rakai Community Cohort Study (RCCS, 2002–2003, Table 1)¹⁸ who were infected with HIV for at least 2 years and were previously analyzed to determine the HIV sequence of the gp41 region. The median (and interquartile ranges) for the lengths of infection was 5.52 (3.36–7.74) and 5.70 (3.35–8.45) for those infected with subtype A and D, respectively. All samples came from individuals who were treatment naive.

Laboratory methods

The BED-CEIA was performed according to the manufacturer's directions (Calypse Biomedical Corporation, Lake Oswego, OR), with one exception: samples were run in duplicate and results were reported as an average of normalized optical density units (OD-n).⁶ The BED-CEIA cut-off for recent infection was defined as <0.8 mean OD-n.¹⁹ The avidity assay was performed using a modified Bio-Rad HIV-1/HIV-2 PLUS O EIA with 0.1 M diethylamine (DEA) as the chaotropic agent.²⁰ The avidity index was calculated as the optical density (OD) of the DEA-treated well divided by the OD of the wash buffer-treated well, expressed as a percentage. The avidity assay cut-off for recent infection was defined as an avidity index of <40%.¹⁷

Sequence analysis

HIV from individuals in the RCCS was sequenced as previously described,¹⁸ though subtype assignments were limited to the gp41 envelope region, since this region of env is the basis for BED-CEIA antigenicity. The HIV subtypes of the 517 samples were 133 (25.7%) subtype A, 373 (72.1%) subtype D infection, and 11 (2.1%) subtype C. Subtype C samples were excluded from further analysis. DNA sequences were aligned and translated into amino acid sequences using BioEdit v7.0.0.²¹ The number of amino acid differences between the sample sequence and the three immunodominant sequences in the gp41 region of the BED-CEIA target antigen (corresponding to HBX2 nucleotides 7992–8045)²² was calculated using Mega v.5.0.²³

Statistical analysis

Chi-square tests were used to compare the frequency of individuals with recent HIV infection who had BED-CEIA or

avidity assay results above the cut-off for recent HIV infection. A two sample test of proportions was used to compare false-recent misclassification rates in individuals with subtype A vs. D. The associations of false-recent misclassification with age and gender and misclassification using either the BED-CEIA or avidity assay were assessed using logistic regression. A multivariate logistic regression was performed to evaluate the association of these factors with false-recent misclassification, after adjusting for age, subtype, gender, and misclassification using the other assay. A z-test was used to assess whether false-recent misclassification was associated with the number of amino acid differences at specific positions in HIV samples and the BED-CEIA target antigen. Combinations of amino acid differences were also examined and adjusted for multiple comparisons using the Holm–Bonferroni method. All statistical analysis was performed using Stata v11 (Stata Corp, College Station, TX).

Human subjects

All work was conducted in agreement with the Declaration of Helsinki. Written informed consent for sample storage and testing was provided by participants. The study was approved by the Science and Ethics Committee of the Uganda Virus Research Institute, the Uganda National Council for Research and Technology, the Western Institutional Review Board (Olympia WA), and the Committee on Human Research at Johns Hopkins Bloomberg School of Public Health.

Results

False-recent misclassification by the BED-CEIA in individuals with subtype A vs. D HIV infection

We evaluated the frequency of false-recent misclassification by BED-CEIA by analyzing samples from individuals (one sample per individual) who were HIV infected for at least 2 years. Individuals with subtype D infection had a higher frequency of false-recent misclassification than those with subtype A infection [51/373 (13.7%) vs. 8/133 (6.0%)] $p=0.02$, Table 2).

One possible explanation for the higher frequency of false-recent misclassification for subtype D is that the amino acid sequences of the HIV strains (which stimulated the antibody response) might differ from the amino acid sequence of the BED-CEIA, which includes target antigens from the gp41 region (HBX2 nucleotides 7992–8045) of HIV subtypes B, E (currently referred to as CRF01_AE), and D.²² We compared the sequences of HIV from individuals in the Rakai Community Cohort Study (Table 1) to the sequence of the BED-CEIA target antigen. We detected an amino acid difference in both subtype A and subtype D strains. For subtype A, amino acid differences at the BED-CEIA target antigen site were not associated with false-recent misclassification. In contrast, for subtype D, a higher number of amino acid differences between HIV in the test sample and the target antigen was associated with false-recent misclassification (comparison to the subtype B target antigen: $p=0.004$; comparison to the CRF01_AE target antigen: $p=0.01$; comparison to the subtype D target antigen: $p=0.03$). Specifically, test samples that had arginine (R) at position 12 had significantly lower BED-CEIA values (median OD-n=1.28, $n=86$) than samples that had a lysine (K) at that position (median OD-n=1.85, $n=451$,

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION (RAKAI, UGANDA)

	Rakai community cohort study	
	Subtype A	Subtype D
Number of individuals	133	373
Gender		
Female	90 (67.7 %)	237 (63.5%)
Male	43 (32.3%)	136 (36.5%)
Age (years)		
<30	36 (27.1%)	98 (26.3%)
30 to 35	35 (26.3%)	98 (26.3%)
35 to 40	32 (24.1%)	100 (26.8%)
>40	30 (22.5%)	77 (20.6%)

TABLE 2. FACTORS ASSOCIATED WITH FALSE-RECENT MISCLASSIFICATION FOR SUBTYPES A AND D USING THE BED-CEIA AND AN AVIDITY ASSAY

	BED-CEIA			Avidity assay		
	Subtype A		Subtype D	Subtype A		Subtype D
	% Misclassified	OR (95% CI)	% Misclassified	% Misclassified	OR (95% CI)	% Misclassified
All	6.0% (8/133)	—	13.7% (51/373)	1.5% (2/133)	—	11.0% (41/373)
Gender						
Female	6.7% (6/90)	1	18.1% (43/237)	2.2% (2/90)	—	9.7% (23/237)
Male	4.7% (2/43)	0.68 (0.13–3.53)	5.9% (8/136)	0.0% (0/43)	—	13.24% (18/136)
Age (years)						
<30	8.3% (3/36)	1	16.3% (16/98)	2.8% (1/36)	1	9.2% (9/98)
30 to 35	0.0% (0/35)	—	14.3% (14/98)	2.9% (1/35)	1.03 (0.06–17.13)	11.2% (11/98)
35 to 40	6.3% (2/32)	0.73 (0.11–4.69)	10.0% (10/100)	0.0% (0/32)	—	13.0% (13/100)
>40	10.0% (3/30)	1.22 (0.23–6.6)	14.3% (11/77)	0.0% (0/30)	—	10.4% (8/77)
Misclassified						
Avidity assay	0.0% (0/2)	—	29.3% (12/41)	—	—	—
BED-CEIA	—	—	—	0.0% (0/8)	—	23.5% (12/51)
						3.11 (1.47–6.59)

BED-CEIA, BED capture immunoassay; OR, odds ratio; CI, confidence intervals. Statistically significant values are shown in bold text.

TABLE 3. MULTIVARIATE ANALYSIS OF FACTORS ASSOCIATED WITH FALSE-RECENT MISCLASSIFICATION USING THE BED-CEIA AND AN AVIDITY ASSAY

	BED misclassification	Avidity misclassification
	aOR (95% CI)	aOR (95% CI)
Subtype		
A	1	1
D	2.34 (1.06–5.16)	7.11 (1.69–30.03)
Gender		
Female	1	1
Male	0.28 (0.14–0.60)	1.62 (0.81–3.24)
Age (years)		
<30	1	1
30 to 35	0.78 (0.36–1.67)	1.20 (0.49–2.97)
35 to 40	0.68 (0.30–1.50)	1.33 (0.54–3.27)
>40	1.31 (0.60–2.89)	0.89 (0.32–2.47)
Misclassified		
Avidity	3.57 (1.65–7.71)	
BED-CEIA		3.48 (1.60–7.57)

BED-CEIA, BED capture immunoassay; aOR, adjusted odds ratio; CI, confidence intervals. Statistically significant values are shown in bold text.

adjusted $p < 0.001$). In addition, samples that had histidine (H) at position 13 and arginine (R) at position 12 had lower BED-CEIA values (median OD- $n = 1.24$, $n = 80$) than samples that had leucine (L) and lysine (K) at those positions (median OD- $n = 1.92$, $n = 166$, adjusted $p < 0.0001$). For subtype D, 37.25% (19/51) of the samples that were misclassified had arginine (R) at position 12, compared to 19.25% (62/322) of the samples that were not misclassified ($p = 0.002$). For subtype A, 3.0% (4/133) of the samples had arginine (R) at position 12, which was not associated with misclassification ($p = 0.607$).

False-recent misclassification by the avidity assay in individuals with subtype A vs. D HIV infection

Samples were also tested with the avidity assay. Samples from individuals with subtype D infection were significantly more likely to be misclassified as recently infected than individuals with subtype A infection [41/373 (11.0%) vs. 2/133 (1.5%), $p < 0.001$]. When used jointly (BED-CEIA < 0.8 and avidity $< 40\%$) the misclassification for subtype A was 0% (0/133) and D was 3.2% (12/373).

Factors associated with false-recent misclassification by the BED-CEIA and avidity assay

In univariate models, false recent misclassification using the BED-CEIA was associated with female gender for subtype D, but not for subtype A (Table 2). Using the BED-CEIA 18.1% of women with subtype D infection were misclassified as recently infected, compared to 6.7% of women with subtype A infection ($p < 0.001$). There was no difference in the false-recent misclassification rate among men with subtype A vs. D infection ($p = 0.8$). Age was not associated with false-recent misclassification by the BED-CEIA for either subtype. Gender and age were not associated with false-recent misclassification by the avidity assay (Table 2).

In a multivariate analysis subtype D and female gender were independently associated with false-recent misclassification with the BED-CEIA (Table 3). False-recent misclassification

with either assay was independently associated with false-recent misclassification with the other assay in this multivariate model (Table 3).

Discussion

HIV subtype affected the performance of the BED-CEIA and avidity assays. Among those infected with HIV for at least 2 years, individuals with subtype D infection were two and seven times more likely to be misclassified as recently infected by the BED-CEIA and avidity assay, respectively, than individuals with subtype A infection. False-recent misclassification was significantly more frequent in women than men using the BED-CEIA, but there was no difference in assay performance by gender using the avidity assay. The high prevalence of subtype D HIV infections in Uganda and Kenya likely explains the high false-recent misclassification rates observed with these assays in populations from Eastern African.¹⁶ This may limit the utility of serologic HIV incidence assays in populations where subtype D is prevalent, even when these assays are used in parallel or in serial testing algorithms.

One factor associated with false-recent misclassification with the BED-CEIA was a sequence difference between the test sample and the BED-CEIA target antigens. Presumably, these sequence differences impacted the specificity of the antibody response to HIV infection in the test subject; antibodies directed toward dissimilar target antigens may not bind as well to the BED-CEIA target antigens, giving lower BED-CEIA values. Interestingly, the number of amino acid differences between the test sample and the BED-CEIA target peptide was significantly associated with false-recent misclassification for subtype D, but for not subtype A. In subtype D, the higher frequency of arginine (R) at position 12 may contribute to the higher rate of false-recent misclassification in this subtype. However, other factors associated with subtype D infection must also impact assay performance, since 32 of the 51 individuals with subtype D infection who were misclassified as recently infected using the BED-CEIA did not have R at position 12.

The higher false-recent misclassification rate observed for subtype D using both the BED-CEIA and the avidity assay suggests that there may be another, more general subtype-specific factor associated with false-recent misclassification. It is possible, for example, that the immune response to infection may be blunted in some individuals with subtype D infection. Studies have shown that individuals with subtype D HIV have more rapid CD4 T cell decline and progress to death or AIDS at a faster rate than individuals with subtype A infection.^{24–26} The association of gender with false-recent misclassification by the BED-CEIA in individuals with subtype D infection suggests that there may be differences in humoral immune responses in men and women with subtype D HIV. The finding that misclassification with either assay (the BED-CEIA or the avidity assay) is associated with misclassification by the other assay for this population may limit the utility of assays even when applied in parallel or serial testing algorithms in areas with subtype D infection.

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Author Disclosure Statement

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References

1. Brookmeyer R: Measuring the HIV/AIDS epidemic: Approaches and challenges. *Epidemiol Rev* 2010;32(1):26–37.
2. Brookmeyer R and Quinn TC: Estimation of current human immunodeficiency virus incidence rates from a cross-sectional survey using early diagnostic tests. *Am J Epidemiol* 1995;141(2):166–172.
3. Janssen RS, *et al.*: New testing strategy to detect early HIV-1 infection for use in incidence estimates and for clinical and prevention purposes. *JAMA* 1998;280(1):42–48.
4. Guy R, *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(12):747–759.
5. Murphy G and Parry JV: Assays for the detection of recent infections with human immunodeficiency virus type 1. *Euro Surveill* 2008;13(36):18966.
6. Dobbs T, *et al.*: Performance characteristics of the immunoglobulin G-capture BED-enzyme immunoassay, an assay to detect recent human immunodeficiency virus type 1 seroconversion. *J Clin Microbiol* 2004;42(6):2623–2628.
7. Masciotra S, *et al.*: Antibody Avidity-Based Assay for Identifying Recent HIV-1 Infections Based on Genetic Systems TM 1/2 plus O EIA. In 17th Conference on Retroviruses and Opportunistic Infections, Abstract 937. San Francisco, CA, February 16–19, 2010.
8. Barnighausen T, *et al.*: HIV incidence in rural South Africa: Comparison of estimates from longitudinal surveillance and cross-sectional cBED assay testing. *PLoS One* 2008;3(11):e3640.
9. Karita E, *et al.*: Investigating the utility of the HIV-1 BED capture enzyme immunoassay using cross-sectional and longitudinal seroconverter specimens from Africa. *AIDS* 2007;21(4):403–408.
10. Marinda ET, *et al.*: Significantly diminished long-term specificity of the BED capture enzyme immunoassay among patients with HIV-1 with very low CD4 counts and those on antiretroviral therapy. *J Acquir Immune Defic Syndr* 2010; 53(4):496–499.
11. Hladik W, *et al.*: Effect of CD4⁺ T cell count and antiretroviral treatment on two serological HIV incidence assays. *AIDS Res Hum Retroviruses* 2011;28(1):95–99.
12. Hayashida T, *et al.*: Effects of low HIV type 1 load and antiretroviral treatment on IgG-capture BED-enzyme immunoassay. *AIDS Res Hum Retroviruses* 2008;24(3):495–498.

13. Barnighausen T, *et al.*: HIV incidence estimation using the BED capture enzyme immunoassay: Systematic review and sensitivity analysis. *Epidemiology* 2010;21(5):685–697.
14. Hallett TB, *et al.*: Errors in 'BED'-derived estimates of HIV incidence will vary by place, time and age. *PLoS One* 2009;4(5):e5720.
15. Kim AA, *et al.*: Estimating HIV incidence among adults in Kenya and Uganda: A systematic comparison of multiple methods. *PLoS One* 2011;6(3):e17535.
16. Laeyendecker O, *et al.*: Specificity of four laboratory approaches for cross-sectional HIV incidence determination: Analysis of samples from adults with known non-recent HIV infection from five African countries. *AIDS Res Human Retroviruses* 2012;28(10):1177–1183.
17. Masciotra S, *et al.*: Antibody Avidity-Based Assay for Identifying Recent HIV-1 Infections Based on Genetic Systems TM ½ plus O EIA. In 17th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, 2010.
18. Conroy SA, *et al.*: Changes in the distribution of HIV type 1 subtypes D and A in Rakai District, Uganda between 1994 and 2002. *AIDS Res Hum Retroviruses* 2010;26(10):1087–1091.
19. Parekh BS, *et al.*: Determination of mean recency period for estimation of HIV type 1 incidence with the BED-capture EIA in persons infected with diverse subtypes. *AIDS Res Hum Retroviruses* 2011;27:265–273.
20. Silvina Masciotra TD, *et al.*: Antibody avidity-based assay for identifying recent HIV-1 infections based on GeneticSystems TM ½ plus O EIA. In 17th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, 2010.
21. Hall T: BioEdit: A user-friendly biological sequence alignment editor analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;41:95–98.
22. Parekh B, *et al.*: Quantitative detection of increasing HIV type 1 antibodies after seroconversion: A simple assay for detecting recent HIV infection and estimating incidence. *AIDS Res Human Retroviruses* 2002;18(4):295–307.
23. Tamura K, *et al.*: MEGA5: Molecular Evolutionary Genetic Analysis maximizing likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;28:2731–2739.
24. Kiwanuka N, *et al.*: Effect of human immunodeficiency virus type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. *J Infect Dis* 2008;197:707–713.
25. Kiwanuka N, *et al.*: HIV-1 viral subtype differences in the rate of CD4+ T-cell decline among HIV seroincident anti-retroviral naive persons in Rakai district, Uganda. *J Acquir Immune Defic Syndr* 2010;54(2):180–184.
26. Baeten J, *et al.*: HIV-1 subtype D infection is associated with faster disease progression than subtype A in spite of similar plasma HIV-1 loads. *J Infect Dis* 2007;195(8):1177–1180.

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