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Abstract: *Background*: The epidemiologic utility of STARHS hinges not only on producing accurate estimates of HIV incidence, but also on identifying risk factors for recent HIV infection.

Methods: As part of an HIV seroincidence study, 800 Rwandan female sex workers (FSW) were HIV tested, with those testing positive further tested by BED-CEIA (BED) and AxSYM Avidity Index (Ax-AI) assays. A sample of HIV-negative (N=397) FSW were followed prospectively for HIV seroconversion. We compared estimates of risk factors for: 1) prevalent HIV infection; 2) recently acquired HIV infection (RI) based on three different STARHS classifications (BED alone, Ax-AI alone, BED/Ax-AI combined); and 3) prospectively observed seroconversion.

Results: There was mixed agreement in risk factors between methods. HSV-2 coinfection and recent STI treatment were associated with both prevalent HIV infection and all three measures of recent infection. A number of risk factors were associated only with prevalent infection, including widowhood, history of forced sex, regular alcohol consumption, prior imprisonment, and current breastfeeding. Number of sex partners in the last 3 months was associated with recent infection based on BED/Ax-AI combined, but not other STARHS-based recent infection outcomes or prevalent infection. Risk factor estimates for prospectively observed seroconversion differed in magnitude and direction from those for recent infection *via* STARHS.

Conclusions: Differences in risk factor estimates by each method could reflect true differences in risk factors between the prevalent, recently, or newly infected populations, the effect of study interventions (among those followed prospectively), or assay misclassification. Similar investigations in other populations/settings are needed to further establish the epidemiologic utility of STARHS for identifying risk factors, in addition to incidence rate estimation.

Keywords: HIV/AIDS, incidence, cross-sectional surveys, prospective studies, risk factors, Rwanda.

INTRODUCTION

Reliable information on HIV incidence is critical to public health practitioners and policymakers working in HIV prevention in order to identify target populations, evaluate the impact of HIV prevention interventions, and identify important trends in dynamic epidemics [1]. Data on risk factors for incident HIV infection help identify at-risk sub-groups in immediate need of primary prevention interventions [2, 3]. Despite these important applications, data on HIV incidence rates and risk factors are scant,

especially in sub-Saharan Africa where the majority of new HIV infections are occurring [4, 5], and where most information on risk factors is derived from HIV prevalence data, such as from the Demographic and Health Surveys (DHS) [6]. Key public health agencies, including the World Health Organization and US Centers for Disease Control and Prevention, have begun recommending strengthening of HIV incidence surveillance systems at the country and regional levels [7-11].

The Serologic Testing Algorithm for Recent HIV Seroconversion (STARHS) is a laboratory test-based approach that offers a promising alternative to prospective incidence measurement for incidence surveillance. Laboratory assays used under STARHS, such as the BED-CEIA (BED) [12] and AxSYM Avidity Index method (Ax-AI) [13], exploit immunologic properties of early HIV infection, such as development of HIV antibodies, to distinguish recent infections (RI) from long-term infections

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(LTI) in HIV-positive persons, enabling estimation of incidence in a cross-sectional sample of HIV-positive and negative individuals [9, 10, 14-24]. Further, if demographic and behavioral data are collected alongside biologic samples for STARHS testing, risk factors for RI, as distinguished from prevalent infection, can be identified.

The potential public health impact of the STARHS method lies in its epidemiologic utility, which we define as the ability of an assay or algorithm to: 1) accurately estimate HIV incidence rates, and 2) distinguish risk factors for RI from LTI when such differences exist, within a population sample of individuals tested for HIV and interviewed for behavioral and other risk factor information. The latter is particularly useful when factors associated with HIV transmission and acquisition shift over time, such that groups currently at risk for infection (eventual "incident cases") have different behavioral, demographic or other characteristics from those with LTI [25, 26]. To date, few studies have evaluated STARHS from the perspective of epidemiologic utility. Instead, studies have focused on assessing assay validity, concluding that STARHS assays tend to misclassify certain individuals with LTI as RI [12, 17, 27-36]. At the population level, this often results in an overestimation of HIV incidence rates relative to prospectively observed seroconversion rates [18]. However, from an epidemiologic standpoint, a certain degree of assay/outcome misclassification may be less problematic. For example, an assay or algorithm that misclassifies certain individuals and overestimates HIV incidence can still be considered epidemiologically useful if associations and inference related to risk factors are not appreciably altered.

We conducted a combined cross-sectional survey (with STARHS testing) and prospective cohort study of female sex workers (FSW) at Projet Ubuzima in Kigali, Rwanda. An in-depth analysis of the validity of STARHS-based incidence estimates in this population was reported separately, and found that HIV incidence rate estimates derived *via* the BED assay alone and BED combined with Ax-AI were similar to those based on prospective observation of HIV seroconversion [24]. However, incidence rate estimates derived *via* Ax-AI alone were substantially higher than those based on observed HIV seroconversion. The present analysis focused on risk factor estimation using STARHS plus behavioral interview data. Risk factor estimates (adjusted odds ratios) were compared for: 1) prevalent HIV infection; 2) RI based on three different STARHS RI classifications (BED alone, Ax-AI alone, BED/Ax-AI combined) with CD4 correction (<200 cells/µl excluded from RI); and 3) observed HIV seroconversion in the prospective study.

MATERIALS AND METHODOLOGY

Study Design and Procedures

The study was conducted by Projet Ubuzima, a non-governmental medical research organization in Kigali, Rwanda. Between October 2006 and August 2007, 800 women participated in the cross-sectional survey with HIV, HSV-2 and pregnancy testing. Individuals testing HIV positive were further tested with the BED and Ax-AI assays, and CD4 cytometry. All women underwent a face-to-face interview for demographic and behavioral risk factor information. Women were eligible for the cross-sectional

study if they were: ≥18 years; at high risk for sexual exposure to HIV, defined as having exchanged sex for money at least once in the last month and/or currently having sex with multiple partners plus having sex at least twice per week (all enrolled women self-reported sex work); HIV serostatus unknown or last test negative; and willing and able to provide written informed consent.

Of 608 women identified as HIV-uninfected during the cross-sectional survey, 397 (65%) who consented and were not pregnant were enrolled consecutively into a prospective cohort study. Cohort participants returned for five visits over two years for HIV counseling and testing (including condom provision), pregnancy and HSV-2 testing, and face-to-face interviewing. Twelve-month retention was 96%, and median follow-up was 689 days (range: 0-836). Specimens from women who became HIV infected during follow-up were tested by CD4 cytometry. Women who tested positive for HIV, HSV-2 or pregnancy were referred for care and treatment. All HIV-positive study participants were ART-naïve.

The study was approved by the National Ethics Committee and National HIV/AIDS Committee (CNLS) in Rwanda, and by Columbia University Medical Center's Institutional Review Board in the United States.

Laboratory Assessments

Blood specimens were tested for HIV by First Response (Premier Medical Corporation, India) and Uni-Gold (Trinity Biotech Plc, Ireland) rapid tests, with Capillus HIV-1/HIV-2 Rapid Test (Trinity Biotech Plc, Ireland) as a tie-breaker. HIV rapid test-positive results were confirmed by Murex HIV Ag/Ab Combination ELISA (Abbott Laboratories, Germany), and then tested further by CD4 cytometry at Rwanda's national reference laboratory in Kigali. HSV-2 infection was assessed by HerpeSelect 2 ELISA (Focus Technologies, USA) and pregnancy by the Fortress hCG test (Fortress Diagnostics, UK). HSV-2, pregnancy, HIV rapid and ELISA testing was done onsite at Projet Ubuzima.

Blood specimens from HIV-positive survey participants were tested by the BED and Ax-AI assays. BED testing was performed onsite following standard procedures as described in the literature [23, 24] and manufacturer's package insert (Calypte® Biomedical Corporation, Oregon, USA), using a cutoff of OD-n ≤0.8 to indicate RI. Ax-AI testing was performed by the Pediatric HIV Research Unit in South Africa using an unmodified AxSYM HIV-1/2gO ELISA (Abbott, USA), and following procedures described in the literature [13], with an avidity index ≤0.85 indicating RI (*Ax-AI cutoff based on personal communication with B. Suligoi*).

STATISTICAL METHODS

Outcome Definitions

In the cross-sectional sample, a prevalent HIV case was a participant who tested positive on HIV rapid tests (with ELISA confirmation), irrespective of STARHS results. Prevalent cases represent undiagnosed HIV infections only in the sampled population, given the eligibility criterion of having an unknown HIV serostatus or a negative last HIV test. In analyses of BED and Ax-AI separately, RI cases

were HIV-positive participants who were classified as RI by the BED or Ax-AI, respectively. In analyses using BED and Ax-AI combined, we counted as RI cases only those survey participants who tested HIV positive and were then classified as RI by <u>both</u> BED and Ax-AI assays. Additionally, individuals with CD4<200 cells/µl were considered probable LTI cases regardless of STARHS results, and so individuals classified as RI but with CD4<200 cells/µl were removed from analyses of recent infection [37], but examined in sensitivity analyses. In the prospective sample, HIV seroconversion date was estimated as the midpoint between the last negative and first positive HIV test (±3-month interval).

Analysis

For the cross-sectional sample, odds ratios (OR) and 95% confidence intervals (CI) for prevalent HIV (based on HIV rapid testing), and for RI (based on STARHS classifications) were derived from logistic regression models, with HIV-negative participants as the comparison group for each. All OR are age-adjusted, and factors with $P<0.05$ were considered statistically significant.

For the prospective sample, Cox proportional hazards regression was used to estimate hazard ratios (HR) and 95% CI for HIV seroconversion. Only results for baseline covariates are presented to enable comparison with logistic models for the cross-sectional sample. All HR are age-adjusted, and factors with $P<0.05$ were considered statistically significant.

We qualitatively compared the direction, magnitude, and statistical significance of putative risk factors for: 1) prevalent HIV; 2) RI based on three different STARHS classifications (BED alone, Ax-AI alone, and BED/Ax-AI combined); and 3) prospectively observed seroconversion in the cohort study. All statistical tests are two-sided. Data were analyzed using SAS, version 9.2 (SAS Institute, Inc., Cary, NC).

RESULTS

Sociodemographics

HIV-positive survey participants (N=192), HIV-negative, non-enrolled survey participants (N=211), and prospective cohort participants (N=397) were similar on most sociodemographic characteristics (Table **1**). Median age in the three groups was 27 (IQR: 23-32), 25 (23-31), and 24 (22-28), respectively. Approximately one-fifth of participants in all groups (23%, 17%, and 22%) had no formal schooling. Most participants (93%, 91%, and 91%) reported currently using at least one contraceptive method, with about three-quarters (72%, 72%, and 75%) reporting condom use by their last sex partner. The majority of women in the three groups had 1-2 pregnancies during their lifetimes. The proportion currently breastfeeding varied across the groups (21%, 45% and 55%).

Prevalent, Recent, and Incident HIV Infections

In the cross-sectional survey, 192 women tested HIV positive for a prevalence of 24.0% (95% CI, 21.0-27.0). Among 190 prevalent HIV cases with BED/Ax-AI results, 36 (19%) were classified as RI by BED, and 56 (30%) as RI by Ax-AI. Twenty-three participants (12%) were classified

as RI by both assays; 121 (64%) as LTI by both assays; and 46 (24%) were classified discordantly on the assays (i.e., recent on BED and long-term on Ax-AI, or vice versa). Two individuals concordant for RI on the assays (9%) had CD4<200 cells/µl, indicating possible LTI. Nineteen of the 397 cohort participants (5%) seroconverted for HIV during 686.5 person-years of follow-up (2.8 per 100 person-years).

Risk Factors for Prevalent and Recent HIV Infection in the Cross-Sectional Sample

Table **2** presents age-adjusted odds ratios (aOR) for putative risk factors in the cross-sectional sample for prevalent HIV infection (by HIV rapid testing and ELISA), and recent infection (by STARHS assays, alone or in combination), compared to HIV-negative participants. Only number of sex partners in the prior 3 months (60-75 versus 76-120, aOR 5.1, 95% CI (1.0, 25.1)) was associated with RI (by BED/Ax-AI combined), but not prevalent HIV infection. Two factors—HSV-2 co-infection and having recently sought treatment for a sexually transmitted infection (STI)—were positively associated with both prevalent infection (aOR 8.4 (4.8, 14.7) and 2.1 (1.3, 3.3), respectively) and RI by BED/Ax-AI combined (aOR 17.9 (2.4, 134.7), and 3.9 (1.4, 10.4), respectively). Several factors were associated (either positively or negatively) with prevalent HIV, but not RI (by BED/Ax-AI combined), including: history of forced sex (aOR 2.2 (1.5, 3.1)); current breastfeeding (aOR 0.3 (0.2, 0.4)); being widowed (aOR 1.7 (1.0, 2.8)); lifetime HIV testing history (≥2 tests versus none, aOR 0.3 (0.2, 0.5); 1 test versus none, aOR 0.6 (0.4, 0.9)); regular alcohol consumption (aOR 1.5 (1.1, 2.2)); and history of imprisonment (aOR 1.8 (1.3, 2.6)). Education level, number of lifetime pregnancies, condom use, frequency of vaginal sex, and marital status were not significantly associated with prevalent or recent HIV infection.

Table **2** also presents risk factors for recent HIV infection on the BED and Ax-AI assays analyzed separately. HSV-2 seropositivity and recent STI treatment were consistently identified as risk factors in both models. However, the Ax-AI method identified two additional factors—district of residence and HIV testing history (≥2 times versus never)—that were not significant in the BED model. Risk factor associations for LTI, which excludes recent infections, and prevalent HIV, which includes RI, were nearly identical to one another (data not shown).

Comparison of Risk Factors Associated with STARHS Results to those Identified *via* Prospectively Observed Seroconversion

In the prospective cohort, having been HIV tested ≥2 times versus never in one's lifetime and HIV testing within the past 6 months were positively associated with HIV seroconversion (Table **2**). Having had two versus ≥4 lifetime pregnancies was borderline statistically significant (aOR 8.0 (1.0, 65.4), $P=0.05$). None of the risk factors for prospective seroconversion was also identified as a risk factor for RI (by BED/Ax-AI combined) in the cross-sectional sample.

DISCUSSION

In these samples of urban Rwandan FSW, we found mixed agreement between three different methods of

Table 1.   Demographic and Behavioral Characteristics[a] of Female Sex Workers in Kigali, Rwanda

	HIV-Positive Cross-Sectional Survey Participants (N=192) N (%)	HIV-Negative, Non-Enrolled Survey Participants (N=211) N (%)	Prospective Cohort Participants (N=397) N (%)
Median age, in years (IQR)***	27.0 (23-32)	25.0 (23-31)	24.0 (22-28)
Age groups: *			
18-24	66 (34)	86 (41)	199 (50)
25-29	52 (27)	63 (30)	116 (29)
30-34	41 (21)	29 (14)	48 (12)
≥35	33 (17)	33 (16)	32 (8)
Education level: *			
No formal schooling	45 (23)	35 (17)	87 (22)
Some primary school	77 (40)	97 (46)	158 (40)
Completed primary school	48 (25)	46 (22)	111 (28)
Secondary school (partial or completed)	22 (12)	31 (15)	41 (10)
Median weekly income in Rwandan francs[b] (IQR)***	15,000 (8,000-23,000)	20,000 (12,000-33,000)	12,000 (7,000-20,000)
Currently using family planning method	179 (93)	191 (91)	362 (91)
Lifetime no. pregnancies			
None	14 (7)	14 (7)	28 (7)
1	52 (27)	60 (29)	119 (30)
2	52 (27)	57 (27)	113 (29)
3	34 (18)	36 (17)	77 (19)
≥4	39 (20)	43 (21)	60 (15)
Currently breastfeeding*	40 (21)	95 (45)	219 (55)
Marital status[c]:			
Married (legal or common-law marriage)	0	0	4 (1)
Divorced/separated*	24 (13)	40 (19)	55 (14)
Widowed	37 (19)	22 (10)	34 (9)
Never married*	130 (68)	148 (70)	303 (76)
Currently have steady partner[d]***	62 (32)	92 (44)	122 (31)
At last sex[c]:			
Received money or gift from partner**	179 (93)	191 (91)	374 (94)
Partner used a condom	138 (72)	151 (72)	296 (75)
Used vaginal lubricant**	8 (4)	23 (11)	26 (7)
Vaginal cleansing beforehand	100 (52)	76 (36)	172 (43)
No. vaginal sex acts in last month			
<20	31 (16)	32 (15)	73 (18)
20-39	57 (30)	52 (25)	120 (30)
40-59	42 (22)	54 (26)	81 (20)
60-89	45 (23)	52 (25)	78 (20)
≥90	17 (9)	21 (10)	45 (11)
No. sex partners in last 3 months			
3-30	22 (12)	8 (4)	41 (10)
31-59	25 (13)	28 (13)	68 (17)
60-75	37 (19)	25 (12)	59 (15)
76-120	34 (18)	48 (23)	81 (21)
≥121	74 (39)	102 (48)	146 (37)
No. clients per week in last month			
<5	32 (17)	31 (15)	87 (22)
5-9	59 (31)	53 (25)	104 (26)
10-15	52 (27)	66 (31)	110 (28)
16-25	39 (20)	42 (20)	66 (17)
>25	10 (5)	19 (9)	30 (8)

(Table 1) contd…..

	HIV-Positive Cross-Sectional Survey Participants (N=192) N (%)	HIV-Negative, Non-Enrolled Survey Participants (N=211) N (%)	Prospective Cohort Participants (N=397) N (%)
No. years working as sex worker*			
≤1	43 (22)	45 (21)	89 (22)
2	16 (8)	30 (14)	86 (22)
3	44 (23)	36 (17)	77 (19)
4-5	35 (18)	48 (23)	70 (18)
≥6	54 (28)	52 (25)	75 (19)
Ever had forced sex**	72 (38)	55 (26)	76 (19)
No. lifetime HIV tests (IQR)			
Never tested	97 (51)	72 (34)	119 (30)
Once	64 (33)	81 (39)	137 (35)
Twice	20 (10)	57 (27)	67 (17)
3-5 times	9 (5)	0	60 (15)
≥6 times	2 (1)	0	13 (3)
HIV testing in the last 6 months	4 (2)	22 (11)	57 (15)
≥ 1 Genital symptom[e] in past month	50 (26)	50 (24)	78 (20)
Sought treatment for STI symptom in last 3 months	34 (18)	22 (10)	32 (8)
Drink alcohol regularly	120 (63)	119 (56)	203 (51)
Have sex with clients who have consumed alcohol	185 (97)	196 (93)	371 (94)
Ever imprisoned	89 (47)	77 (37)	150 (38)

Abbreviations: IQR = Inter-quartile range ; HIV = Human Immunodeficiency Virus; STI = Sexually transmitted infections.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ for comparisons between cohort participants and HIV-negative, non-enrolled survey participants.
[a]Sample sizes for different questions may vary slightly from N=800 based on missing responses.
[b]1 US Dollar=approximately 555 Rwandan francs.
[c]Multiple responses allowed.
[d]A steady partner is a regular sex partner with whom the woman has sex more often than with other partner(s), but does not live with and is not married to.
[e]Includes: genital itching, burning, rash, pain; abnormal vaginal discharge, odor, or bleeding (non-menstrual); pain or difficulty urinating; genital ulcers, sores or blisters; pain during sex; acute lower abdominal pain; other genital symptoms.

assessing risk factors for HIV infection, even when the HIV incidence rates based on BED alone, BED/Ax-AI combined, and observed seroconversion were relatively similar [24]. We identified a number of putative and protective risk factors for prevalent HIV infection using conventional serologic testing. We identified only a few risk factors for recent infection using two STARHS assays (BED and Ax-AI) with three different classifications. Although two of the three factors (HSV-2 coinfection and recent STI treatment) were also risk factors for prevalent infection, the third factor (number of sex partners in the past 3 months) was only identified by the STARHS classification that best classified recent HIV infection in this sample [24], the BED/Ax-AI combined. Finally, risk factors for recent infection (by single or combined STARHS assays) were different from those associated with HIV seroconversions in the prospective cohort.

Decisions by ministries of health to institutionalize the use of STARHS for incidence surveillance—specifically, to generate HIV incidence estimates and identify risk factors for recently acquired infection from cross-sectional samples in settings such as Kigali, Rwanda—hinge on the epidemiologic utility of the approach. We defined epidemiologic utility, in part, as the ability of an assay or algorithm to distinguish risk factors for recent from longer-term infection when such differences exist. From the

perspective of epidemiologic utility, factors associated with prevalent infection differed substantially from those associated with recent infection in this sample.

Our findings are consistent with at least two plausible scenarios in this specific population of Rwandan female sex workers. First, in reality, there may be differences between factors associated with incident versus prevalent HIV infection in this population, for example if HIV transmission dynamics in this group have recently begun to evolve. Alternatively, true similarities in the risk factor profile for incident and prevalent infection in this population could have been masked in our analyses by misclassification on the assays or low statistical power to identify risk factors as statistically significant. However, prevalent infections, recent infections by STARHS, and prospectively observed seroconversions measure varying stages of infection in different time periods, and so risk factors could be expected to be different when the epidemic is evolving. Nonetheless, the use of epidemiologic utility, including assay sensitivity and specificity, as a metric of STARHS is conceptually and methodologically important, particularly given the intent to use STARHS as an epidemiological tool at the population level, not at the individual level [38]. Future studies, ideally with more statistical power, are needed to further examine the epidemiologic utility among at-risk sub-Saharan African populations.

Table 2. Risk Factors for Prevalent HIV Infection, Recent HIV Infection[a] (by BED-CEIA Assay and Ax-AI, Separately and Combined) and HIV Seroconversion in a Sample of ART-Naïve, Rwandan Female Sex Workers

Risk Factor[b]	Prevalent HIV (N=192) Age-Adjusted OR (95% CI)	RI by BED Alone (N, RI=31) Age-Adjusted OR (95% CI)	RI by Ax-AI Alone (N, RI=50) Age-Adjusted OR (95% CI)	RI by BED and Ax-AI Combined (N, RI=21) Age-Adjusted OR (95% CI)	HIV Seroconversion (Cohort N=397) Age-Adjusted HR (95% CI)
Age group:					
18-24	0.8 (0.5, 1.2)	1.3 (0.5, 3.5)	1.1 (0.5, 2.2)	1.1 (0.4, 3.1)	1.0 (0.3, 3.1)
25-29	--REF--	--REF--	--REF--	--REF--	--REF--
30-34	**1.9 (1.2, 3.1)**	2.0 (0.6, 6.9)	1.8 (0.7, 4.5)	0.8 (0.2, 4.1)	1.9 (0.6, 6.2)
≥35	**1.8 (1.1, 3.0)**	**3.3 (1.1, 10.2)**	1.9 (0.7, 4.8)	0.9 (0.2, 4.8)	*undefined*
District of residence:					
Nyarugenge	--REF--	-- REF --	-- REF --	-- REF --	-- REF --
Gasabo	**2.0 (1.3, 3.0)**	2.2 (0.9, 5.4)	**2.4 (1.1, 5.3)**	2.1 (0.7, 6.2)	0.7 (0.2, 2.1)
Kicukiro	**3.1 (2.0, 4.8)**	1.8 (0.6, 4.9)	**3.2 (1.4, 7.2)**	1.8 (0.5, 6.0)	1.1 (0.4, 3.4)
Education level:					
No formal schooling	--REF--	--REF--	--REF--	--REF--	--REF--
Some primary school	0.9 (0.6, 1.4)	1.5 (0.5, 4.4)	1.1 (0.5, 2.4)	1.8 (0.5, 6.5)	0.9 (0.3, 2.9)
Completed primary school	0.8 (0.5, 1.3)	1.1 (0.3, 3.5)	1.1 (0.5, 2.7)	1.3 (0.3, 5.4)	2.5 (0.6, 10.2)
Secondary school (some/completed)	0.7 (0.4, 1.3)	1.6 (0.4, 5.6)	1.4 (0.5, 3.8)	1.1 (0.2, 6.4)	*undefined*
Lifetime no. pregnancies:					
None	1.9 (0.9, 4.2)	0.9 (0.2, 4.8)	1.9 (0.5, 6.7)	0.9 (0.1, 5.7)	*undefined*
1	1.5 (0.9, 2.7)	0.3 (0.1, 1.3)	0.9 (0.3, 2.7)	0.2 (0.1, 1.3)	1.2 (0.1, 14.7)
2	1.5 (0.8, 2.5)	1.2 (0.4, 3.4)	1.4 (0.5, 3.5)	1.0 (0.3, 3.9)	8.0 (1.0, 65.4)
3	1.1 (0.6, 1.9)	0.8 (0.3, 2.4)	0.9 (0.3, 2.4)	0.8 (0.2, 3.3)	4.7 (0.5, 41.1)
≥4	--REF--	--REF--	--REF--	--REF--	--REF--
Currently breastfeeding	**0.3 (0.2, 0.4)**	0.8 (0.4, 1.8)	0.7 (0.3, 1.5)	0.9 (0.4, 2.1)	1.6 (0.6, 4.3)
Marital status[c]:					
Currently have steady partner[d]	0.9 (0.7, 1.3)	0.8 (0.4, 1.7)	1.1 (0.6, 1.9)	1.1 (0.5, 2.8)	0.6 (0.2, 1.8)
Divorced/separated	0.7 (0.4, 1.1)	0.9 (0.3, 2.5)	0.7 (0.3, 1.7)	1.0 (0.3, 3.4)	0.5 (0.1, 4.2)
Widowed	**1.7 (1.0, 2.8)**	1.3 (0.4, 4.0)	1.5 (0.6, 3.7)	1.2 (0.2, 5.8)	*undefined*
Never married	1.0 (0.7, 1.4)	0.9 (0.4, 2.2)	1.1 (0.5, 2.2)	1.0 (0.3, 3.0)	2.1 (0.6, 7.5)
At last sex[c]:					
Received money/gift from partner	1.1 (0.6, 2.1)	1.1 (0.3, 4.8)	1.1 (0.3, 4.8)	0.7 (0.2, 3.2)	1.1 (0.2, 8.1)
Partner used a condom	0.9 (0.6, 1.3)	0.6 (0.3, 1.4)	1.2 (0.6, 2.5)	0.4 (0.2, 1.1)	1.0 (0.9, 1.1)
Vaginal cleansing beforehand	1.6 (1.2, 2.2)	1.5 (0.7, 3.1)	1.6 (0.9, 2.8)	1.9 (0.8, 4.6)	0.8 (0.3, 1.9)
No. vaginal sex acts in past month:					
<20	0.7 (0.4, 1.3)	1.7 (0.5, 6.1)	0.8 (0.3, 1.9)	2.3 (0.4, 13.0)	0.4 (0.1, 1.4)
20-39	1.0 (0.6, 1.5)	2.7 (0.9, 8.4)	1.1 (0.5, 2.3)	3.6 (0.8, 16.6)	0.4 (0.1, 1.6)
40-59	1.0 (0.6, 1.6)	1.5 (0.4, 5.6)	0.8 (0.4, 2.0)	1.9 (0.4, 10.7)	0.2 (0.02, 1.4)
60-89	--REF--	--REF--	--REF--	--REF--	--REF--
≥90	0.8 (0.4, 1.5)	0.5 (0.1, 4.6)	0.5 (0.1, 1.9)	1.0 (0.1, 11.1)	1.1 (0.2, 6.2)
No. sex partners in last 3 months[e]:					
3-30	1.3 (0.7, 2.4)	3.2 (0.9, 11.7)	1.1 (0.3, 3.7)	3.5 (0.6, 21.5)	2.6 (0.8, 8.7)
31-59	0.9 (0.5, 1.6)	1.5 (0.4, 5.9)	1.2 (0.5, 3.4)	2.6 (0.5, 14.5)	1.3 (0.4, 3.5)
60-75	1.5 (0.9, 2.5)	2.7 (0.8, 9.4)	2.2 (0.9, 5.6)	**5.1 (1.0, 25.1)**	
76-120	--REF--	--REF--	--REF--	--REF--	--REF--
≥121	1.0 (0.6, 1.6)	1.0 (0.3, 3.3)	1.1 (0.4, 2.5)	0.9 (0.6, 1.4)	*undefined*
No. clients per week in last month:					
<5	0.7 (0.4, 1.2)	1.7 (0.5, 5.9)	0.8 (0.3, 2.0)	2.1 (0.4, 11.2)	0.5 (0.1, 1.6)
5-9	1.1 (0.7, 1.8)	2.2 (0.7, 7.1)	1.3 (0.6, 2.9)	3.1 (0.7, 14.7)	0.6 (0.2, 2.2)
10-15	0.9 (0.6, 1.5)	1.0 (0.3, 3.7)	0.7 (0.3, 1.7)	1.2 (0.2, 6.9)	0.5 (0.1, 2.3)
16-25	--REF--	--REF--	--REF--	--REF--	--REF--
>25	0.6 (0.3, 1.4)	0.6 (0.1, 5.7)	0.7 (0.2, 2.8)	1.1 (0.1, 12.8)	*undefined*

(Table 2) contd…..

Risk Factor[b]	Prevalent HIV (N=192) Age-Adjusted OR (95% CI)	RI by BED Alone (N, RI=31) Age-Adjusted OR (95% CI)	RI by Ax-AI Alone (N, RI=50) Age-Adjusted OR (95% CI)	RI by BED and Ax-AI Combined (N, RI=21) Age-Adjusted OR (95% CI)	HIV Seroconversion (Cohort N=397) Age-Adjusted HR (95% CI)
No. years working as sex worker[f]:					
≤1	1.0 (0.6, 1.7)	1.9 (0.7, 5.6)	1.5 (0.7, 3.6)	4.2 (0.9, 19.4)	1.8 (0.6, 5.3)
2	**0.4 (0.2, 0.8)**	0.8 (0.2, 2.8)	0.3 (0.1, 1.1)	1.0 (0.1, 6.9)	0.3 (0.1, 1.3)
3	1.2 (0.7, 1.9)	0.6 (0.1, 2.3)	1.0 (0.4, 2.6)	1.9 (0.3, 10.7)	
4-5	0.8 (0.5, 1.4)	0.8 (0.3, 2.6)	0.7 (0.2, 1.7)	1.4 (0.2, 8.8)	--REF--
≥6	--REF--	--REF--	--REF--	--REF--	*undefined*
Ever had forced sex	**2.2 (1.5, 3.1)**	1.7 (0.8, 3.7)	1.8 (1.0, 3.4)	2.3 (0.9, 5.7)	0.8 (0.2, 2.6)
Lifetime no. HIV tests					
None	--REF--	--REF--	--REF--	--REF--	--REF--
1	**0.6 (0.4, 0.9)**	0.6 (0.3, 1.5)	0.6 (0.3, 1.2)	0.6 (0.2, 1.9)	4.8 (0.6, 41.2)
≥2	**0.3 (0.2, 0.5)**	0.6 (0.3, 1.5)	**0.4 (0.2, 0.9)**	1.1 (0.4, 3.0)	**12.9 (1.7, 98.9)**
HIV tested in last 6 months	**0.2 (0.1, 0.4)**	0.2 (0.1, 1.8)	0.1 (0.01, 1.0)	0.3 (0.04, 2.2)	**3.4 (1.2, 9.1)**
Sought STI treatment in last 3 months	**2.1 (1.3, 3.3)**	**2.8 (1.2, 6.9)**	**3.1 (1.5, 6.2)**	**3.9 (1.4, 10.4)**	0.6 (0.1, 4.3)
Drink alcohol regularly	**1.5 (1.1, 2.2)**	1.1 (0.5, 2.3)	1.3 (0.7, 2.3)	0.7 (0.3, 1.7)	1.3 (0.5, 3.3)
Ever imprisoned	**1.8 (1.3, 2.6)**	0.9 (0.4, 2.0)	1.2 (0.6, 2.2)	0.6 (0.2, 1.7)	1.4 (0.5, 3.5)
HSV-2 seropositive (baseline)	**8.4 (4.8, 14.7)**	**3.9 (1.5, 10.5)**	**9.1 (3.2, 25.9)**	**17.9 (2.4, 134.7)**	1.4 (0.5, 3.8)
Pregnant (baseline)	0.7 (0.3, 1.3)	0.8 (0.2, 3.5)	0.7 (0.2, 2.5)	1.1 (0.3, 5.0)	NA

Abbreviations: OR=odds ratio; HR=hazard ratio; CI=confidence interval; HIV=Human Immunodeficiency Virus; RI=recent infection; STI=sexually transmitted infection; NA=not applicable.
Bolded effect estimates have *P*< 0.05.
[a] Participants with CD4<200 removed from RI classification, considered probable long-term HIV infection.
[b] Sample sizes for different questions may vary slightly from the total N based on missing responses.
[c] Multiple responses allowed.
[d] A steady partner is a regular sex partner with whom the woman has sex more often than with other sex partner(s), but does not live with and is not married to.
[e] Categories for age-adjusted HR's for prospective cohort are: 3-30; 31-75; and ≥76 partners (referent group is ≥76).
[f] Categories for age-adjusted HR's for prospective cohort are: ≤1; 2-3; and ≥4 years (referent group is ≥4).

We conducted a number of sensitivity analyses of our data. Specifically, adjustment of STARHS results from bivariable models with CD4 count data (with individuals with CD4<200 cells/µl excluded as probable LTI) did not change inference regarding the existence of associations for specific risk factors. Additional sensitivity analyses (data not shown) showed that excluding persons classified as RI with CD4 counts up to 500 cells/µl did not alter risk factor findings. Misclassification rates may change with different study populations, for example a less healthy population with a different CD4 count distribution; misclassification of individuals with LTI and high CD4 counts is also possible. In our study, it is possible that greater specificity in the risk factor analysis from use of the BED and Ax-AI assays combined reduced the potential impact of CD4 adjustment. Indeed, the combined testing algorithm appeared to perform better for risk factor identification in the cross-sectional sample than the assays alone, particularly compared with the Ax-AI method alone. For example, the two additional risk factors for RI identified by the Ax-AI method alone were identified as risk factors for prevalent infection, which reflects the substantial misclassification of individuals with longer-term infection as RI by the Ax-AI method in this population [24]. CD4 adjustment may have a greater impact in studies using only a single assay versus a combined algorithm, and such studies should therefore conduct CD4 testing if possible. Finally, conducting STARHS studies among treatment-naïve individuals will help avoid misinterpretation of STARHS results with CD4 adjustment.

Comparison of the risk factors identified using the cross-sectional methods against those identified using the gold-standard prospective method may aid the interpretation of cross-sectional risk factor data, however caution when making such a comparison is warranted. In this study, there was no overlap between risk factors for recent infection in the cross-sectional sample and predictors of HIV seroconversion in the prospective sub-sample. Limited statistical power for identifying significant factors could have affected agreement between the methods, as there were relatively few events in both the RI and seroconversion models. However, important differences between the sub-sample of women who were eligible and agreed to participate in the prospective cohort study and HIV-negative survey participants not enrolled in the cohort may also contribute to the discordance. For example, HIV-negative cohort participants were younger, more likely to be breastfeeding, less likely to have a history of forced sex, and had more recently initiated sex work than HIV-negative non-enrolled women, all factors associated with HIV risk. Furthermore, changes over time in cohort participants' risk level (for example, because of the effect of prevention interventions or the Hawthorne effect), or unknown factors

Table 3. **Comparison of Demographic and Risk Characteristics of HIV Seroconverters (N=19) and HIV-Negative Participants (N=378), Prospective Cohort, Kigali, Rwanda**

	Seroconverters (N=19) N (%)	HIV-Negative Cohort Participants (N=378) N (%)
Median age (IQR)	25.0 (22-31)	24.0 (22-28)
Age group, years:		
18-24	8 (42)	191 (51)
25-29	5 (26)	111 (30)
≥30	6 (32)	74 (20)
Education level:		
No school	4 (21)	83 (22)
Primary (some/completed)	11 (58)	258 (68)
Some secondary	4 (21)	37 (10)
Currently breastfeeding	12 (63)	207 (55)
Lifetime no. pregnancies:		
None or 1	2 (11)	145 (38)
2	11 (58)	102 (27)
3	5 (26)	72 (19)
≥4	1 (5)	59 (16)
Male condom use at last sex	16 (84)	280 (74)
Current hormonal contraception use	5 (26)	67 (18)
≥1 Genital symptom in past month[+]	6 (32)	72 (19)
No. vaginal sex acts in past month:		
<20	6 (32)	67 (18)
20-39	4 (21)	116 (31)
40-59	1 (5)	80 (21)
60-89	5 (26)	73 (19)
≥90	3 (16)	42 (11)
No. sex partners in last 3 months:		
3-30	4 (21)	37 (10)
31-75	6 (32)	121 (32)
≥76	9 (47)	218 (58)
No. clients per week in past month:		
<5	6 (32)	81 (21)
5-9	4 (21)	100 (27)
10-15	2 (11)	108 (29)
≥16	7 (36)	89 (23)
No. years working as sex worker[¥]:		
≤1	6 (35)	61 (17)
2-3	3 (18)	160 (45)
≥4	8 (47)	137 (38)
No. lifetime HIV tests:		
Never tested	1 (5)	118 (31)
Once	5 (26)	132 (35)
≥Twice	13 (69)	127 (34)
HIV tested in prior 6 months	6 (32)	51 (14)
HSV-2 seropositive at baseline	12 (67)	202 (56)
Median days from enrollment to seroconversion (range)	269 (86-768)	NA

Abbreviations: IQR = Inter-quartile range; HIV = Human Immunodeficiency Virus; NA = Not applicable.
[+]Includes: genital itching, burning, rash, pain; abnormal vaginal discharge, odor, or bleeding (non-menstrual); pain or difficulty urinating; genital ulcers, sores or blisters; pain during sex; acute lower abdominal pain; other genital symptoms.
[¥]2 participants missing response, N=17.

causing misclassification on the assays, could also have contributed to discordance between cross-sectional and prospective risk factor findings. Most HIV seroconversions in the cohort occurred early during follow-up, and women who seroconverted differed from women who remained HIV-negative during follow-up on several potentially important factors. For example, seroconverters had spent fewer years in sex work, reported more frequent sex, had higher HSV-2 seroprevalence at baseline, and reported more genital symptoms in the month prior to enrollment (Table **3**). Additional study limitations are noted. Due to sample size (specifically, too few outcomes), we could only adjust for age and thus were unable to conduct a full multivariate analysis. This could have resulted in uncontrolled confounding, complicating comparisons between prospective and cross-sectional samples. Measurement error in covariates and bias due to unmeasured confounding could have affected risk factor findings.

This study also has several strengths and provides new insights. The use of two STARHS assays contributed important information about the assays' combined and individual performance in a high-prevalence population. The combined cross-sectional and prospective design, and extensive interview data, enabled in-depth exploration, triangulation, and comparison of risk factors for prevalent and recently acquired HIV infection in multiple samples. This in turn allowed us to explore an important dimension of the epidemiologic utility of HIV incidence assays, namely risk factor detection. Finally, availability of CD4 count data aided interpretation and enabled important sensitivity analyses.

CONCLUSION

In this selected high-risk group, factors associated with incident HIV infection appeared to differ from those associated with prevalent infection. More studies evaluating the ability of STARHS to detect risk factors when combined with survey data, alongside the validity of STARHS-based incidence estimates, are needed to further establish the epidemiologic utility of the approach for incidence surveillance. Such studies are especially needed in sub-Saharan Africa where there is very limited experience with STARHS, and where the potential impact of an alternative to prospective measurement of HIV incidence is greatest [5]. Specifically, such investigations should be conducted in populations in which risk factors for HIV incidence and prevalence are known or expected to differ, as well as populations with more diversity in risk factors (e.g., population-based samples). Despite persisting concerns about the validity of particular STARHS assays, the results of this study underscore the potential value of the approach in identifying and distinguishing groups in need of HIV prevention services when combined with complementary risk group and behavioral information.

Public health officials in Rwanda might consider incorporating STARHS into the next Demographic and Health Survey-Plus to conduct similar analyses on the national level and across population groups, as has been done successfully in Uganda [37] and South Africa [18]. Further evaluations of STARHS data in Rwanda and across diverse settings, as well as development of assays with

improved validity, will strengthen the argument to incorporate HIV incidence surveillance into routine HIV surveillance.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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