Relation Between Chemokine Receptor Use, Disease Stage, and HIV-1 Subtypes A and D

Results From a Rural Ugandan Cohort

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Objectives: To determine whether there are differences in coreceptor use in subjects infected with HIV-1 envelope subtypes A and D that could explain the differences in progression rates between these subtypes in a rural Ugandan cohort.

Methods: HIV-1 was subtyped in *env* by V3 sequencing or heteroduplex mobility assay. Coreceptor use was determined by the ability of the isolates to replicate in U87 $CD4^+$ cells expressing different coreceptors. The Fisher exact test was used to examine the relation between coreceptor use and subtype, clinical stage, and V3 charge. The Kruskall-Wallis nonparametric test was used to examine the association between median CD4 cell counts, coreceptor use, and subtype. Logistic regression was used to examine predicted coreceptor use at different CD4 groupings.

Results: Isolates from 66 participants were analyzed. Thirty-one were infected with subtype A, and 35 were infected with subtype D. Although this work was based on a small sample size, we found statistically significant differences. The probability of having an X4 virus was higher in subtype D infections than in subtype A infections among those with a non-AIDS clinical status (Fisher exact test, $P = 0.040$). Logistic regression analysis, in which we predicted X4 use by subtype and stratified by CD4 group, confirmed these findings among those with a CD4 count >200 cells/μL (likelihood ratio test, $P = 0.003$). R5 viruses were associated with higher median CD4 cell counts than X4 or X4/R5 (Kruskall-Wallis test, $P = 0.0045$). A V3 charge of +5 and greater was highly associated with X4 virus (Fisher exact test, $P = 0.006$).

Conclusions: These subtype differences in coreceptor use may partially explain the faster progression rates we have previously reported in individuals infected with subtype D compared with subtype A. Our observations may have implications for the future use of coreceptor inhibitors in this population.

Key Words: coreceptor use, HIV-1, subtypes A and D

(*J Acquir Immune Defic Syndr* 2007;45:28–33)

One of the major characteristics of HIV is its extremely high genetic variability.[1] HIV-1 strains can be subdivided into 3 main groups: major (M), outlier (O), and new (N).[2] Most HIV-1 strains responsible for the pandemic belong to group M. In addition, circulating recombinant forms (CRFs) and unique recombinants have been identified.[2] In Uganda, the predominant subtypes are A and D;[3–5] however, unique recombinants are also being increasingly identified.[4,6]

Although many studies have been carried out to determine whether these different genetic subtypes have biologic relevance in terms of transmission, disease progression, and vaccine development,[1,7] the broad view is that genetic subtypes do not have major biologic significance. Presently, one of the most widely studied biologic characteristics is coreceptor use. Virus isolates are distinguishable by their ability to utilize different chemokine receptors for entry into target cells.[8] Slow/low non–syncytium-inducing (NSI) viruses tend to use CCR5 (fifth receptor for CC chemokines) for viral entry, whereas rapid/high syncytium-inducing (SI) viruses are generally able to use CXCR4 (fourth receptor for CXC chemokines) exclusively or additionally.[9–11] As a result, it has been suggested that the designation of HIV-1 phenotype be revised to indicate coreceptor use rather than the less biochemically defined characteristics of target cell tropism or SI properties.[12] Thus, the corresponding phenotypes of the viruses are R5, X4, or R5X4 if they use CCR5, CXCR4, or both coreceptors, respectively.

For subtypes A, B, D, and E (CRF01_A/E), R5 viruses predominate during primary HIV-1 infection, whereas X4 or R5X4 viruses often emerge in late stages of HIV disease.[11,13,14] The appearance of SI or X4 HIV-1 variants in the course of HIV infection is a strong predictive marker for $CD4^+$ T-cell depletion and progression to AIDS.[15–17] Approximately half of infected individuals progress to AIDS without detectable SI or X4 viruses, however. Subtype C seems to be the exception, with an underrepresentation of X4 viruses, and thus a low

Received for publication April 4, 2006; accepted January 17, 2007.

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Funded by the Medical Research Council and the Department for International Development of the United Kingdom.

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frequency of SI phenotype among patients with AIDS.[11,18] In addition, dual tropism is not common with subtype D.[18] It is unclear whether patterns of coreceptor use translate into differences in disease progression or transmission.

We have previously reported that individuals infected with subtype D viruses progressed faster to death and had significantly higher viral loads than those infected with subtype A.[5,19] In this population, antiretroviral (ARV) drugs have recently been initiated and it is not yet clear how coreceptor use influences ARV efficacy. Because the presence of X4 viruses is associated with an increase in CD4 T-cell destruction, their presence may warrant initiation of therapy at higher CD4 cell counts than is the case with R5 viruses. R5 and X4 viruses seem to be sensitive to conventional highly active antiretroviral therapy (HAART), although there have been a few reports implying some differences in susceptibility to HAART by the 2 phenotypes.[20]

Currently, however, there is a great need for new ARV drugs with reduced toxicity and increased activity against drug-resistant viruses. To meet this need, there are a number of coreceptor antagonists in early clinical trials; however, there is some evidence to suggest that CCR5 antagonists may not be as effective in individuals harboring R5X4 or X4 strains.[21] Hence, screening may be required before use of such drugs, whereas, at the same time, there is a need to understand coreceptor use better.

The main purpose of the current study was to determine whether differences in coreceptor use could explain the differences in progression between subtypes A and D. Although our findings are based on a comparatively small sample size, it is worthy of note that this is the largest cross-sectional study relating phenotype to disease stage in an African cohort in which antiretroviral therapy (ART) is still not commonly used.

SUBJECTS, MATERIALS, AND METHODS

The Science and Ethics Committee of the Uganda Virus Research Institute granted approval to the study in 1999. This has been followed by annual reviews and progress reports. All participants provided written informed consent for their participation. ART has been available in Uganda since 2004.

Study Population

The study participants were adults (aged ≥13 years) from a cohort established by the Medical Research Council Program on AIDS in southwestern Uganda. The cohort is composed of seropositive-prevalent patients who were already infected by the time of the start of the cohort study in 1990, seropositive incident cases with known dates of seroconversion, and HIV-1–seronegative controls. Participants are seen routinely at 3-month intervals and when ill. At each visit, a clinical examination is carried out, followed by laboratory investigations and treatment for those who are unwell. World Health Organization (WHO) clinical staging of HIV disease[22] is performed at each routine visit using a computer algorithm, and a CD4/CD8 T-cell count by FACSCount (Becton Dickinson International, Erembodegem-Aalst) is performed every 6 months.[23] WHO stage 4 is defined as AIDS. For this study, only HIV-1–positive individuals with *env* subtype A or D were studied. This study was completed in November 2003, after which participants with CD4 counts ≤200 cells/μL with no contraindications began ART.

Genetic Subtype and V3 Sequencing

An aliquot of whole blood was used for DNA extraction utilizing a Puregene DNA isolation kit (Gentra Systems, NC). The *env* gene subtype was determined using a heteroduplex mobility assay (HMA) or direct sequencing of the V3 region.[6,24]

In addition, DNA was extracted from culture-infected peripheral blood mononuclear cells (PBMCs), and a nested polymerase chain reaction (PCR) assay was performed. For this, we used the same viral supernatants to infect PBMCs as for the coreceptor phenotype studies. The net V3 loop charge was calculated by assigning each amino acid residue a value of +1 for those with a positively charged R group (K, R, and H), a value of −1 for those with a negatively charged R group (D and E), or a value of 0 for those with an uncharged R group.[25]

HIV Isolation

Blood samples were received from the field station in ethylenediaminetetraacetic acid (EDTA) tubes, and on the next day, PBMCs were separated on Ficoll gradient and resuspended in 10% dimethyl sulfoxide (DMSO) and 50% fetal bovine serum (FBS) before storage at −80°C and later in liquid nitrogen. Virus isolates were obtained by coculture of thawed patient PBMCs with phytohemagglutinin (PHA)-stimulated healthy HIV-1–negative donor cells.[26] The donor cells were grown on RPMI 1640 medium containing PHA, 15% FBS, L-glutamine, and antibiotics for 3 to 4 days, after which approximately 2×10^6 donor cells were cocultured with approximately 1×10^6 patient cells in 2 mL of RPMI containing interleukin-2 (IL-2), antibiotics, FBS, and L-glutamine on a 24-well plate for 28 days. Supernatants were removed for p24 assay every 3 to 4 days, and fresh IL-2–containing medium was added, with donor cells being added every 7 days. Positive isolates were expanded to increase the virus stock and later titrated to obtain a 50% tissue culture infectious dose ($TCID_{50}$).[26] Isolates with a $TCID_{50} > 100$ were used for further assays.

Assays for Coreceptor Use of HIV-1

The biologic phenotype was determined in vitro by the ability of the isolates to replicate in U87 $CD4^+$ cells expressing the chemokine receptors CCR1, CCR2b, CCR3, CCR5, and CXCR4.[27] U87 $CD4^+$ cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco BRL) with high glucose in the presence of selective antibiotics (geneticin [G418] and puromycin) and 10% FBS. These cells were grown at 37°C in 5% carbon dioxide for 2 to 3 days until they reached 50% confluence. They were then seeded overnight in a 48-well plate at a concentration of 4×40^4 cells in 400 μL of DMEM containing penicillin and streptomycin. The medium was aspirated, and virus supernatant was added to a total volume of 400 μL with DMEM. Coreceptor use for each isolate was determined by scoring areas of syncytia using a focal assay after 5 to 7 days as previously described.[28] Briefly, media were

removed, the cells were fixed with methanol for 5 minutes, and the fixative was poured off and rinsed with PBS containing 1% FBS. A primary antibody, which was pooled sera from HIV-1–positive individuals (HIV immunoglobulin [Ig]), was added (100 μL per well), followed by a 45-minute incubation at room temperature. Two washes of phosphate-buffered saline (PBS) were performed, followed by addition of antihuman Ig diluted at a ratio of 1:500 and incubation for 45 minutes. A peroxidase substrate was then added and left to incubate for 30 minutes. This was rinsed off with water, after which the plate was left to dry. The foci were counted under a microscope and scored as follows: −, <3; +, 3 to 10; ++, 10 to 100; and +++, >100 foci per well. Those with a negative score had low replicative capacity not attaining high infections in cells; therefore, only +, ++, and +++ were considered positive.

Ex Vivo Characterization of Virus Replication Rates

Three cell types were used in replication kinetic experiments: (1) CD8 cell-depleted PBMCs from 2 HIV-1–seronegative healthy blood donors, (2) PBMCs derived from a healthy donor who was δ32 homozygous in CCR5, and (3) MT-2 cell lines. A total of 150 μL of RPMI medium containing 50,000 cells, 15% FBS, penicillin, streptomycin, and IL-2 was added to a 96-well plate in triplicate. A total of 100 $TCID_{50}$ of virus was added to each well. Supernatants were harvested on days 2, 5, 7, 9, and 11 for p24 antigen capture enzyme-linked immunosorbent assay (ELISA), and fresh media containing 10% FBS, IL-2, and penicillin and streptomycin were added back. On day 7, cultures were supplemented with their respective cells.

Statistical Analysis

We conducted a Fisher exact test to examine the relation between coreceptor use and envelope subtype, the relation between coreceptor use and clinical stage, and the relation between coreceptor use and grouped CD4 counts (<200, 200–500, and >500 cells/μL). A Fisher exact test was also used to relate V3 charge to coreceptor use. In addition to grouping CD4 cell counts, we conducted Kruskall-Wallis nonparametric tests to examine the association between median CD4 counts and coreceptor use as well as median CD4 cell counts and subtype. A logistic regression analysis was used to predict coreceptor use for the 2 envelope subtypes at different CD4 groupings. The logistic regression analysis allowed us to assess the combined relation between envelope subtype (A or D), CD4 count (<200, 200–500, and >500 cells/μL), and coreceptor use. For example, it allowed an assessment of the relation between envelope coreceptor use and subtype at different levels of CD4 counts (<200, 200–500, and >500 cells/μL).

Because SI isolates can be X4 or R5X4 (dual tropic), for some of our analyses, we combined X4 and R5X4 viruses.

RESULTS

We successfully characterized isolates from 66 of 93 participants whose viruses had previously been subtyped in the envelope region: 19 by HMA and 47 by gene sequencing. Participants were selected according to their latest CD4 cell counts so as to have representation from categories <200, 200 to 500, and >500 cells/μL for the 2 subtypes A and D (Table 1). There were 33 male participants (17 infected with subtype A and 16 with subtype D) and 33 female participants (14 infected with subtype A and 19 with subtype D). The median age of the subjects was 33.3 (95% confidence interval [CI]: 30.3 to 37.4) years for subtype A and 35.2 (95% CI: 31.6 to 39.9) years for subtype D. Twenty-seven samples failed to be characterized, because the viruses failed to grow (n = 10) or grew to extremely low titers (n = 17). These comprised 19 subtype A and 8 subtype D samples. Of those for which V3 sequence data were available, 59% (10 of 17 samples) had a V3 charge associated with R5 isolates.

Coreceptor Use and Envelope Subtype

Although X4 and dual-tropic isolates were more common in subtype D compared with subtype A, there were no statistically significant differences in coreceptor use according to envelope viral subtype for the 3 (R5, X4, and R5X4) coreceptor categories ($P = 0.22$) or after combining X4 with dual-tropic isolates ($P = 0.197$) (data not shown).

Coreceptor Use, Envelope Subtype, and Clinical Stage

Participants' clinical status is shown in Table 2. Among non-AIDS participants, those with subtype D were less likely to have R5 viruses than those with subtype A (56% vs. 83%; Fisher exact test, $P = 0.040$). In our group of participants, among those with AIDS, those with subtype A were more likely to have X4 viruses. Four (50%) of the 8 participants with AIDS infected with subtype A had X4 viruses. In contrast, 3 (38%) of the 5 participants with AIDS infected with subtype D had X4 viruses. This is based on small numbers, however, and the difference between the groups was not statistically significant ($P = 0.500$; see Table 2).

We observed a significant association between CD4 cell counts and coreceptor use, with the median CD4 cell count being significantly higher for participants with R5 virus as opposed to X4 or R5X4 virus (410 participants with R5 virus vs. 209 participants with X4 or R5X4 virus; Kruskall-Wallis test, $P = 0.0045$). When CD4 counts were grouped (<200, 200–500, and >500 cells/μL), R5 viruses became less common and X4 and dual tropism more common as CD4 cell counts declined (Fisher exact test, $P = 0.014$). This association was significant for subtype A (Fisher exact test,

TABLE 1. Infecting Subtype and CD4 Cell Count in the Participants From Whom Isolates Were Obtained

Subtype	CD4 Range (Cells/μL)	No. Isolates
A	<200	7
	200–500	12
	>500	12
D	<200	11
	200–500	13
	>500	11

TABLE 2. Comparison of Coreceptor Use and Subtype by Clinical Stage

Clinical Status	Subtype	No. Isolates	R5	X4/R5X4	*P*
Non-AIDS	A	23	19 (83%)	4 (17%)	0.040
	D	27	15 (56%)	12 (44%)	
AIDS	A	8	4 (50%)	4 (50%)	0.500
	D	8	5 (62%)	3 (38%)	

$P = 0.007$) but failed to reach significance for subtype D (Fisher exact test, $P = 0.099$).

The logistic regression model showed associations between coreceptor use (R5 vs. X4/R5X4), the use of subtype (A or D), and the CD4 count groups (<200, 200–500, and <500 cells/μL). All predictors in the model (ie, the CD4 count groups and subtypes) were significantly associated with coreceptor use (Wald test *P* values all <0.05; see appendix). At higher CD4 cell counts, participants with subtype D were more likely to harbor X4 viruses than those with subtype A, whereas at CD4 counts <200 cells/μL, this was no longer the case (Fig. 1).

Replication Kinetics

Six viruses from our cohort (2 isolates using R5, 1 using R5X4, and 3 using X4), were characterized by their replication rates in different cells to check the phenotype of these viruses. These were compared with 3 known viruses: 92/UG/031 (subtype A and R5), 89.6 (subtype B and R5X4), and 92/UG/024 (subtype D and X4). Although there were differences in growth kinetics, all X4-using viruses were able to grow in the 4 cell types, including the homozygous δ32 CCR5 cells (ie, all reached p24 antigen of >10,000 pg/mL on different days [days 5, 9, and 12] and two thirds reached p24 antigen >1000 pg/mL in the δ32 cells by day 12). In contrast, R5 viruses could not grow in the homozygous δ32 CCR5 cells or in MT-2 cells, and p24 antigen >1000 pg/mL was reached in PBMCs later at 7 to 9 days, with only 1 reaching >10,000 pg/mL by 12 days. Finally, the R5X4 virus grew in MT-2 and X4 cell lines and both wild-type PBMCs, producing p24 antigen >1000 pg/mL between 5 and 7 days. This virus did not grow in the homozygous δ32 cells, however (data not shown).

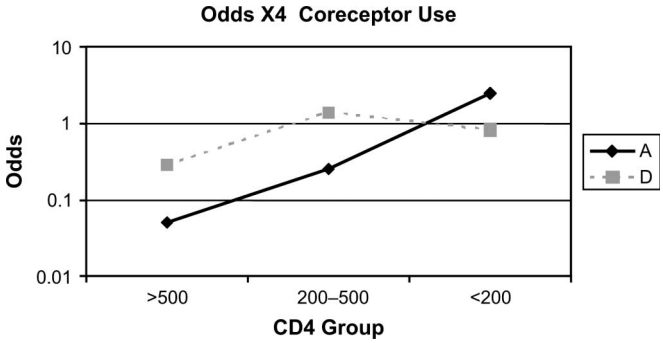

FIGURE 1. Illustration of the coreceptor use probability of subtypes A and D at different CD4 cell counts.

Association Between Biologic Phenotype and V3 Genotype

We compared V3 charge and coreceptor use in 57 samples (Table 3). A charge of +5 or +6 or greater was associated with CXCR4 use (Fisher exact test, $P = 0.006$), whereas with a charge of +7 and greater, the association was even higher (Fisher exact test, $P = 0.002$).

DISCUSSION

In this cross-sectional study, we investigated the relation between coreceptor use and HIV-1 disease stage in 66 individuals infected with HIV-1 subtype A or D in a rural Ugandan population. In addition, replication kinetics and V3 loop charge were related to HIV phenotype. We restricted this study to CCR5 and CXR4, which are the major coreceptors implicated in viral entry[29] and exhibit different pathogenicity in vivo.[30]

The main purpose of the study was to establish if there are differences in patterns of coreceptor use between these 2 subtypes that may be partially responsible for the differences in virulence[5] and higher viral load[19] reported previously in this cohort. We have shown a higher probability of X4 or R5X4 viruses in participants who have not yet developed AIDS and at higher CD4 cell counts among subtype D–infected patients than in subtype A–infected patients. This difference may suggest an earlier switch to X4 or dual tropism among subtype D–infected patients and could explain the faster disease progression seen in those infected with subtype D compared with subtype A in our cohorts.

There are 2 other studies indicating that subtype D is associated more with SI (X4 or R5X4) phenotype.[31,32] V3 loop heterogeneity, length polymorphism, and a large number of positively charged amino acid substitutions associated with SI phenotype have been found more frequently among subtype D variants than among subtype B, C, and E variants.[31] The other study conducted among HIV-infected children[32] found that the child with the greatest number of X4 viruses over the greatest time span carried HIV-1 subtype D.

TABLE 3. Correlation Between V3 Charge and Coreceptor Use: +5 Charge, +6 Charge, and +7 Charge as Cutoff

Charge	R5	X4/R5X4
A. +5 Charge		
V3 ≤5	27	7
V3 >5	11	14
P = 0.006, 1-sided Fisher exact test.		
B. +6 Charge		
V3 ≤6	30	9
V3 >6	8	12
P = 0.006, 1-sided Fisher exact test.		
C. +7 Charge		
V3 ≤7	36	13
V3 >7	2	8
P = 0.002, 1-sided Fisher exact test.		

One study has reported a low frequency of dual tropism among subtype D–infected patients;[11] however, in our study, there was no difference in the frequency of dual tropism between A and D subtypes. Our observation that coreceptor use is associated with disease stage is in agreement those of with other studies using other subtypes of HIV-1.[11] At high CD4 cell counts, R5 viruses predominate, and X4 or dual-tropic viruses dominate at lower CD4 counts. In line with other studies,[33,34] however, we also noted a few X4 or dual-tropic viruses at relatively high CD4 cell counts. R5 viruses are predominant in non-AIDS participants, and X4 viruses increase in frequency in those with AIDS. As reported by others,[35,36] however, even among participants with AIDS, some do not have the SI/X4 isolates. This was especially noted in those infected with subtype D.

It has been reported that the shift to an X4 virus is associated with a dramatic increase in the rate of T-cell destruction by a factor of 3- or 4-fold.[16,17] The higher cytopathogenicity of X4 strains is considered to be attributable to direct cytolysis in susceptible targets[14] and to greater induction of apoptosis (programmed cell death).[37]

Surprisingly, we noted a greater tendency for participants with subtype A to harbor X4 viruses than for participants with subtype D among those with CD4 counts <200 cells/μL. It may be hypothesized that those with subtype D switch to X4 earlier, and hence die earlier, and that by the time the CD4 count reaches levels of <200 cells/μL, the few remaining subtype D–infected individuals are those who have not switched to X4 and are the ones still alive. At this stage, there is no statistically significant difference between subtypes A and D in terms of coreceptor use.

In our study of subtype A and D viruses, the X4 viruses were more replication competent in vitro than R5, as has also been reported by others using subtype B viruses.[35,38] In vivo extrapolation would suggest that higher replication rates of X4 viruses would be a factor in accelerated CD4 decline, and thus disease progression, among these participants. Consistent with other reports,[39,40] we noted that R5X4 viruses failed to grow in homozygous δ32 cells, although we did not determine the extent of coreceptor expression by flow cytometry.

Although V3 charge is not always a dependable predictor of coreceptor use,[41] our findings concur with others in that there was a high correlation between V3 charge and coreceptor use,[25,31] with viruses with a high V3 charge being more likely but not exclusively to be SI (X4 or R5X4).

Viral entry inhibitors have recently raised great interest, and there are major trials underway to study the effect of these drugs. Early data suggest that CCR5/CXR4 tropism of a virus population may influence the efficacy of HIV entry inhibitors.[21] Agents that inhibit the interaction between HIV and CCR5 are likely to be most effective in individuals with higher CD4 T-cell counts. Results from our study may imply that these CCR5 inhibitors may be less effective in individuals infected with HIV subtype D, who generally already harbor X4 viruses at higher CD4 counts. The lower frequency of R5 using viruses among subtype D in participants without AIDS may therefore have implications for the efficacy of these drugs in subtype D–infected patients compared with subtype A–infected patients.

This study was based on only 66 patients; hence, our results should be confirmed by a larger study. Unfortunately, there are insufficient published data or in the Los Alamos database to support or refute our observation that X4 viruses are more prevalent in asymptomatic patients infected with subtype D virus compared with subtype A virus. In another recent study in Uganda,[42] however, differences in disease progression and coreceptor use were reported among those individuals infected with HIV subtypes A and D. Those infected with subtype D viruses progressed faster to death, and subtype A viruses were reported to utilize only CCR5 as a coreceptor, whereas 25% of subtype D viruses bound to both CCR5 and CXCR4.

Our study has 2 potential methodologic shortcomings. First, the use of cocultivation of patient PBMCs with donor PBMCs could have led to the selection of CCR5-using viruses, because PHA and IL-2 can upregulate CCR5 expression;[43] however, 59% of the samples in which we failed to characterize the isolates had a V3 charge associated with R5 use. Second, we studied only PBMC-derived virus isolates rather than plasma-derived isolates, but a good correlation between PBMC and plasma virus phenotypes has been shown previously.[32]

In conclusion, this study has further confirmed the relation between coreceptor use and disease stage for subtypes A and D. We have also shown that there seems to be an earlier switch from R5 to X4 viruses among subjects infected with subtype D compared with subtype A, and this may be one of the reasons for differences in pathogenicity between these subtypes. Because of the small numbers in our study, however, this needs to be confirmed by a larger study. Finally, one of the main reasons not to delay starting ART for too long is that once the shift to X4 virus begins, it may be difficult to recover lost immune competence. If, indeed, there are differences in the time of the switch, this may have implications regarding the optimum time to start therapy in this population.

We are currently undertaking a longitudinal study in this cohort to understand better the timing and determinants of the change in coreceptor use in our population of predominantly subtype A– and D–infected patients.

ACKNOWLEDGMENTS

The authors thank the study participants of the rural cohort. They acknowledge the contribution of technical Medical Research Council staff, especially Brian Magambo, for performing the viral culture, and Dr. Heiner Grosskurth for reviewing the draft.

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