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# The Complexity of Circulating HIV Type 1 Strains in Oyo State, Nigeria

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## ABSTRACT

**Multiple HIV-1 subtypes and circulating recombinant forms (CRFs) are known to circulate in West Africa. We undertook a survey of HIVs in Oyo state, in southwestern Nigeria. We analyzed 71 samples from Ibadan, the capital city, and 33 samples from Saki, 100 miles west of Ibadan. We sequenced part of the *gag* gene and the envelope C2V3 region from 102 and 89 samples, respectively. In the 87 samples for which both genes were sequenced, subtype G and CRF02_AG were found in equal proportions (32.2% each). Other samples included CRF06_cpx (8.0%), subtype A (2.3%), C (1.1%), unclassified (1.1%), or discordant sequences suggesting the presence of a large number of recombinants involving CRF02_AG and/or subtype G (20.7%) or other subtypes (2.3%). The subtype/CRF designation was concordant in two gene fragments in the majority of samples evaluated. However, we observed differences in subtype distribution between the two locations with a predominance of subtype G in Ibadan and CRF02 in Saki. This is the first in-depth analysis of HIV variability at a state level in Nigeria. Our analysis revealed a significant level of viral heterogeneity and a geographical difference in subtype distribution, and demonstrated that CRF02_AG does not account for the majority of circulating strains.**

## INTRODUCTION

To date, the remarkable viral diversity of HIV-1 has resulted in the classification of the virus into 9 subtypes and over 30 circulating recombinant forms (CRFs). Although the epidemic in the western world is primarily due to subtype B, most of the other subtypes and CRFs are found in Africa.[1] In addition to well-characterized CRFs, there are a large number of recombinant forms that are not established as stable circulating recombinants or not yet fully characterized[1–4] termed URFs for unique recombinant forms.

The phenotypic consequences of this genetic diversity are not well understood, but studies suggest differences in transmission, pathogenesis, and response to antiretroviral therapy.[1,5–14] Nonetheless, characterization of circulating subtypes in populations is invaluable to further our understanding of HIV molecular epidemiology and its pathogenic consequences and also inform the design of candidate vaccines.[7] The changing distribution of circulating strains and the emergence of new recombinants or subtypes might represent a serious challenge for the development of a broadly effective HIV vaccine.

In West Africa, most of the epidemic is believed to be due to subtype A and recombinant viruses including subtype A, in particular CRF02_AG.[15–17] Studies conducted in Senegal and Ivory Coast in the mid-1990s have demonstrated that CRF02_AG and subtype A account for up to 70% of the circulating viruses.[17,18] In Nigeria, situated further south, CRF02_AG and subtype A, together with subtype G, were shown to account for 98–99% of all infections.[2,19–22]

Two large surveys of HIV diversity have been conducted in Nigeria.[20,21] While one survey covered 4 states and the other 34 of the 36 states of Nigeria, none has focused on one defined geographical area for an in-depth study of viral diversity. In addition, both surveys used primarily heteroduplex mobility assay, and not sequence data, for subtype assignment.[20,21]

We analyzed 104 samples from subjects infected by HIV in Oyo state, in southwestern Nigeria. Partial *gag* and *env* sequences were analyzed for 102 and 89 of these samples, re-

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spectively. A phylogenetic and molecular analysis revealed a much more complex distribution of subtypes and recombinant viruses than previously recognized.

## MATERIALS AND METHODS

### *Study subjects*

A total of 104 samples were analyzed: 71 samples from Ibadan, the capital city of Oyo state, and 33 samples from Saki, a town 100 miles west of Ibadan, toward the border with the republic of Benin. The samples were collected in 2002–2003 from a study of HIV prevalence that was conducted from various socioprofessional groups in Oyo state. The study received ethical approval from the Institutional Review Boards of both the Harvard School of Public Health and the University of Ibadan/University College Hospital, Ibadan in accordance with 45 CFR 46.

### *Amplification and sequencing*

DNA was extracted from peripheral blood mononuclear cells or whole blood by adsorption purification column (Qiagen). The DNA was resuspended in Tris-EDTA buffer and the concentration was determined by measuring the optical density at 260 nm. One microgram was used for each polymerase chain reaction (PCR) amplification.

To amplify the HIV-1 envelope V3 region (337 bp), a nested PCR was performed using two sets of primers, WT1-WT2 for the first round and KK30-KK40 for the second round, as previously described.[11] For some samples in which the PCR was not successful, alternative primer pairs were used. In the first round, we used WT1 and WT3 (5′-ATGGGAGGGGCATACATTGCT-3′), and for the second round KK40 and WT2a (5′-CAATAGAAAAATTCYCCTCYACAAT-3′) .

To amplify the HIV-1 *gag* 3′-p24/5′-p7 region (451 bp), we performed a nested PCR using two primer sets: p108–p109 for the first round and p91–p92 for the second round, as previously described.[17]

### *Cloning and sequencing*

The PCR product was purified by agarose gel electrophoresis and purification columns (Qiaquick gel extraction kit, Qiagen Inc., Chatsworth, CA) and directly sequenced using the second round primers. When necessary, the purified product was cloned in pCR2.1 vector (T/A cloning, Invitrogen, Carlsbad,

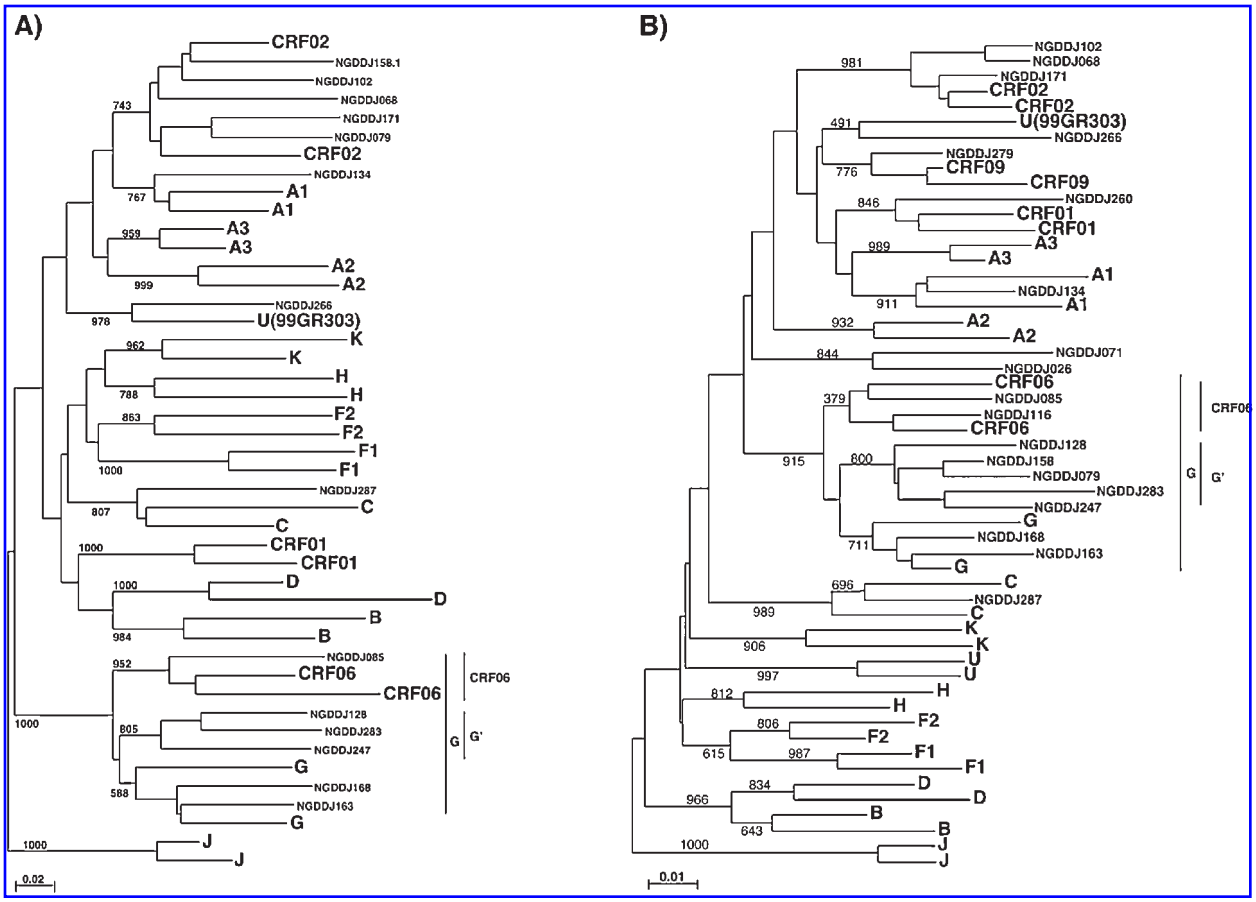


**FIG. 1.** Phylogenetic analysis of representative sequences from Ibadan and Saki. (**A**) gag sequences; (**B**) *env* sequences. In both **A** and **B** the cluster including subtype G is outlined with a vertical bar on the right of the figure and the subclusters CRF06 and G′ are similarly indicated. Reference sequences are in larger fonts and are denoted by a letter (subtype), a letter and a number (subsubtype), or CRF followed by a double-digit number (CRF). An unclassified reference sequence is denoted U (99GR303).

CA). Plasmid preparation for double-stranded DNA sequencing was performed by alkaline lysis, using silica (SNAP miniprep kit, Invitrogen, Carlsbad, CA) columns. The sequence was determined by dye terminator cycle sequencing using Taq polymerase (Perkin-Elmer, Applied Biosystem Division, Foster City, CA) and an automatic sequencer ABI 377 (Perkin-Elmer, Applied Biosystem Division, Foster City, CA).

### *Sequence analysis*

For the *env* C2V3 and the *gag* 3′-p24/5′-p7 regions, multiple alignments of generated sequence and reference sequences from the HIV database were performed with the Clustal package of multiple alignment programs (Clustal-X 1.83) with minor manual adjustment when necessary.[23] Phylogenetic analyses were performed by the neighbor-joining method using the Clustal package and correcting for multiple substitutions, complete removal of positions that contained gaps, and reliability estimated by 1000 bootstrap resamplings. Sequences of uncertain classification were reanalyzed using an FY84 substitution model, a transition/transversion ratio of 1.4, complete removal of positions that contained gaps, and reliability estimated by 1000 bootstrap resamplings using PAUP* (Sinauer Associates, Sunderland, MA). Reference sequences used were obtained from the Los Alamos sequence database and at least two representatives of subtypes and subsubtypes A1, A2, A3, A4, B, C, D, F1, F2, G, K, and of CRF01 to CRF23 and CRF28, 29, 31, and 32. We also included three unclassified sequences from the database that exist as full-length sequences (AF286236, AF457101, and AY046058). For phylogenetic trees in Fig. 1, for the sake of clarity of the figure, a subset of sequences was selected to depict the overall diversity in our study and to highlight a few sequences of interest. Reference sequences were limited to major subtypes and subsubtypes and to CRFs represented in the population.

## RESULTS

We obtained *gag* and *env* sequences in 102 and in 89 samples out of 104, respectively (Table 1). Two samples in the *gag* and 15 samples in the *env* could not be amplified and sequenced successfully.

The samples for this study were obtained from various sociodemographic groups in Oyo state in 2002–2003.[24] The study was conducted in Ibadan, the capital city of the state, and Saki, a semiurban community in the state. In the year preceding the collection of samples, a national sentinel survey of antenatal clinic attendees reported a prevalence of 3.3% and 4.7% in the two communities, respectively. A survey conducted since showed similar results. The population in our study was part of a large community-based survey seeking to identify subpopulations engaging in high-risk behaviors in both localities. The characteristics of the population will be published elsewhere. The subjects from whom our samples were derived included members of various artisan and trade groups, families of individuals deemed to be at high risk, and self-identified commercial sex workers. Accordingly, the prevalence in these groups was higher than in the general population. We did not observe group-specific repartition of strains within the same locality, suggesting that socioprofessional activities or risk behavior were not a primary determinant of infection by a particular subtype in this study (data not shown).

Nearly half the *gag* sequences were subtype G (46.1%), whereas 43 were CRF02_AG (42.1%). We also found at least one of the following subtypes, subsubtypes, and CRFs: A1, A3, C, CRF01, and CRF09_cpx. Six sequences could not be classified, suggesting the presence of divergent strains or recombinants not resolvable due to the small length of the sequenced fragment. Of note, sequences from one individual (DDJ266) clustered in the *gag* and *env* with the unclassified strain GR303 (AY046058) from Greece. The GR303 strain has been shown to be a complex recombinant virus with regions that are yet unclassified.[25] Incidentally, the same study reports that a partial sequence from a subject from Nigeria clusters with the GR303 strain. This suggests the existence of yet another CRF. Within the subtype G cluster, 8 sequences formed a tight subcluster with CRF06_cpx and were further classified as such, whereas 30 sequences formed a unique monophyletic subcluster referred to as G′ and likely represent a previously described subcluster

Table 1. Sequence Results for 104 Samples from Oyo State

| *GAG/ENV subtype* | *Ibadan* | *(%)* | *Saki* | *(%)* |
|---|---|---|---|---|
| G′/G′[a] | 16 | (27.6) | 3 | (10.3) |
| CRF02[a] | 15 | (25.9) | 13 | (44.8) |
| G/G[a] | 8 | (13.8) | 1 | (3.4) |
| CRF06[a] | 4 | (6.9) | 3 | (10.3) |
| C[a] | 1 | (1.7) | 0 | (0) |
| A1/A1[a] | 0 | (0) | 1 | (3.4) |
| A3/A[a] | 1 | (1.7) | 0 | (0) |
| G′/CRF02[a] | 3 | (5.2) | 3 | (10.3) |
| CRF02/A | 3[a] | (5.2) | 0 | (0) |
| CRF02/A3[a] | 2 | (3.4) | 2 | (6.9) |
| CRF02/G′[a] | 1 | (1.7) | 0 | (0) |
| CRF06/G[a] | 1 | (1.7) | 0 | (0) |
| U/U[a] | 1 | (1.7) | 0 | (0) |
| U/G[a] | 1 | (1.7) | 0 | (0) |
| U/CRF02[a] | 1 | (1.7) | 0 | (0) |
| U/CRF06[a] | 0 | (0) | 1 | (3.4) |
| U/CRF11[a] | 0 | (0) | 1 | (3.4) |
| CRF02/U[a] | 0 | (0) | 1 | (3.4) |
| Subtotal | 58 | (100) | 29 | (100) |
| G′/NA[b] | 5 | — | 0 | — |
| CRF02/NA[b] | 4 | — | 2 | — |
| CRF09/NA[b] | 2 | — | 0 | — |
| CRF01/NA[b] | 1 | — | 0 | — |
| CRF06/NA[b] | 1 | — | 0 | — |
| NA/A3[b] | 0 | — | 1 | — |
| NA/G′[b] | 0 | — | 1 | — |
| Subtotal | 13 | — | 4 | — |
| Total samples ($n$ = 104) | 71 | — | 33 | — |

[a]Samples with both gene fragments sequenced. U, unclassified; NA, not available.

[b]Samples with one gene fragment sequenced. NA, not available.

of subtype G viruses in Nigeria (Fig. 1). The remaining nine sequences were classified as prototypical G.

Similarly, the subtype G cluster in the envelope was the most represented (42.7%) and was further subdivided into three subclusters: G′ (23.6%), prototypical G (11.2%), and CRF06_cpx (7.9%). CRF02_AG accounted for 39.3% of all the envelope sequences. A significant number (11.2%) of subtype A sequences, which did not cluster with CRF02_AG, were also found and classified as A1, A3, or simply A when we had a lack of resolution of the subsubtypes.

In evaluating the concordance between the *gag* and the *env* in subtype assignment, we noted that 25.3% of the samples for which both genes were sequenced had discordant subtype assignments or were unclassifiable. These results indicate that the viruses in these samples were likely URFs. The addition of these 22 URFs to the 35 concordant CRFs (28 CRF02_AG and 7 CRF06_cpx) suggests that at least 65.5% (57 out of 87) of the circulating viruses in this region of Nigeria are recombinant viruses.

Ibadan and Saki are about 100 miles apart; despite this close proximity, the proportion of HIV subtypes varied significantly between the two. Strikingly, based on being either CRFs in one of the gene fragments or having discordant subtype assignments, up to 78.9% of the sequences in Saki could be recombinant viruses, whereas only 56.3% of the sequences in Ibadan were recombinant. This is due in part to the fact that nearly half (44.8%) of the samples from Saki were concordant CRF02_AG strains, whereas this virus represented only 25.9% of the strains sequenced from Ibadan. In contrast, concordant subtype G (G and G′) accounted for 41.4% of the strains in Ibadan, but only 13.8% of the ones in Saki. Generally, the overall subtype distribution differed between Ibadan and Saki, localities that are nearly 100 miles apart (Fig. 2).

## DISCUSSION

We obtained *gag* and *env* sequences in 102 and 89 samples, respectively, and were unable to amplify 2 strains in the *gag* and 15 in the *env*. The failure of amplification could be attributed to viral diversity at primer binding sites. For these amplifications, we employed primers that we had previously used extensively in other studies in West Africa, where viral diversity is also very high.[11,17,26,27] Some samples were successfully amplified only after the use of alternative amplification primers, as described. We tested all the samples that failed to amplify in the envelope with group O-specific primers at equivalent positions, but these also failed to amplify (data not shown), suggesting that the lack of amplification is unlikely to be due to group M/group O recombinant viruses.[28,29] Further studies of strains circulating in this geographical area, in particular full-length sequences, will provide a more complete description of the viral diversity in this region.

Two previous studies conducted in Nigeria reported a predominance of subtype A and subtype G virus with very few strains belonging to other subtypes.[20,21] These studies were done primarily using HMA, but some samples were sequenced. Analysis of the few sequenced strains indicated that most subtype A viruses clustered with CRF02_AG. As our study is based solely on sequence data, we have shown that the majority of viruses do indeed cluster with subtypes A and G in the *gag* and *env*. However, two significant differences emerged. First, al-

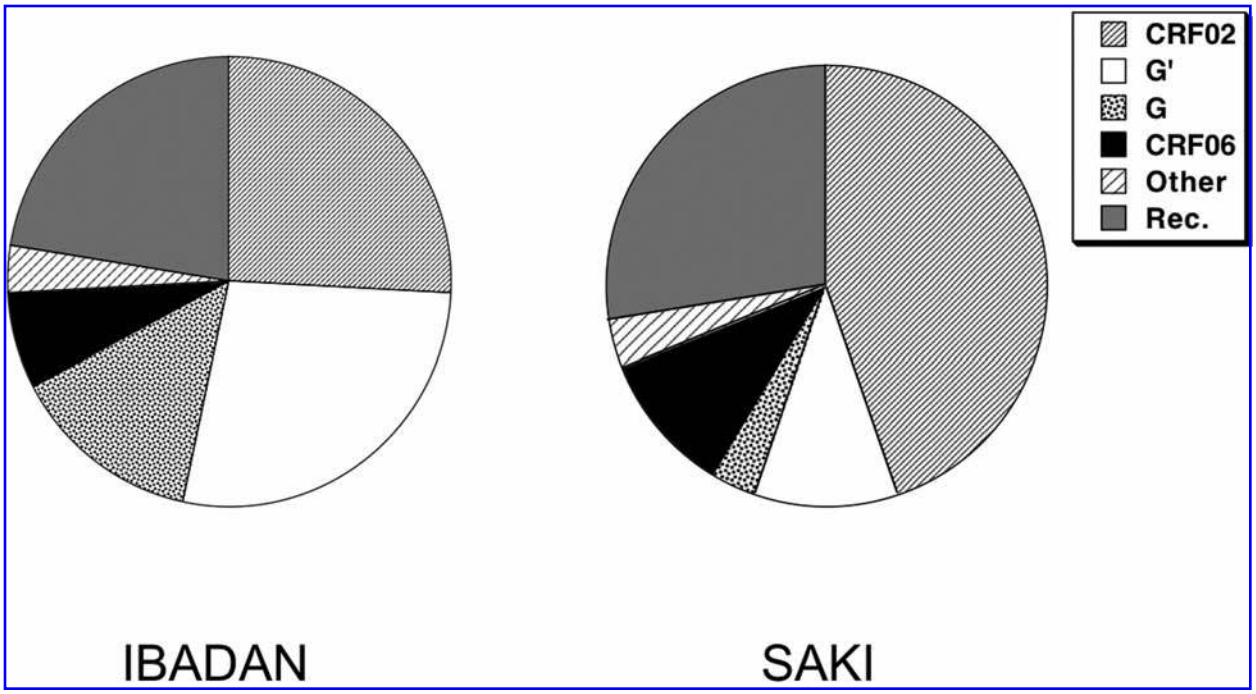


**FIG. 2.** Proportion of various subtypes and CRFs in Ibadan and Saki. Other: clearly assigned concordant subtypes or CRFs other than CRF02_AG, CRF06, G, and G′. Rec.: putative recombinant viruses with discordant subtype/CRF assignment between the two genes or unclassifiable.

though the subtype A viruses were predominantly CRF02_AG, we also found other subtype A sequences, including subsubtypes A1 and A3. Second, we found that although subtypes A and G represented the majority of sequences, other subtypes accounted for 6.8% and 9.8% of the *env* and *gag* sequences, respectively, unlike previous studies where they accounted for only 1–2% of the sequences. Our findings suggest that either the proportion of other subtypes is increasing or that sequencing of multiple regions provides a more complete discrimination of subtypes than does HMA.

Our data indicate that the level of viral diversity in Nigeria is more complex than previously described. In addition to CRF02_AG, we demonstrated the presence of at least four other CRFs (CRF01_AE, CRF06_cpx, CRF09_cpx, and CRF11_cpx) as well as subtype C. Six (5.9%) of the *gag* sequences and 3 (3.4%) of the *env* sequences were unclassified. These sequences would either cluster loosely with known subtypes or represent lone branches in phylogenetic tree analysis. In one case, sequences from one subject clustered with a complex URF sequence, strain 99GR303 (AY046058), in the *gag* and *env*.[25] Two of the *gag* unclassified sequences (DDJ026 and DDJ071) clustered with one another, suggesting the presence of yet another CRF or subtype. It is not clear what these unclassified sequences really represent; they could correspond to new subtypes or subsubtypes, but are more likely to be recombinant viruses for which the recombination pattern was not resolvable in the absence of a full-length sequence. These unclassified sequences and the discordance between gene fragments from the same samples suggest the presence of new recombinant viruses not assigned to previously reported CRFs. These could represent URFs or emerging CRFs, suggesting the need for further viral characterization in the region. Out of the 22 unclassified or putative recombinants, 19 of them (86.4%) included portions of CRFs, mostly CRF02, suggesting that CRFs are prone to further recombination (Table 1). Our study included relatively small gene fragments from *gag* and *env*; we therefore could not do a full assessment of the recombinant viruses. It is thus possible that even strains assigned to one subtype or CRF might be recombinant viruses. This limitation in our study suggests that additional work is needed to fully characterize the recombinant viruses circulating in this region.

We also report here the presence of CRF09_cpx sequences in Nigeria for the first time. This recently described CRF has also been found in other West African countries such as Senegal, Côte d'Ivoire, and Cameroon.[30–33]

The complexity of the circulating viral strains is further emphasized by the presence of three subclusters in the subtype G radiation. One of the subclusters corresponds to the prototypic subtype G and another one to CRF06_cpx. The third subcluster, representing the majority of the subtype G sequences, was classified as G′.[2,20,24] The G′ subcluster had been previously described in the *env* but was not found to exist in the *gag*.[20] Here we show that both *env* and *gag* sequences exhibit this clustering pattern. Full-length sequencing is underway to fully assess the relationship of these sequences to the prototypical subtype G for future immunological and vaccine studies in Nigeria.

It has been postulated that the proportion of subtype A and G viruses differs in Nigeria on a north–south axis of the country.[20] Data from previous studies suggested a dominance of subtype A viruses in southwestern Nigeria, and more subtype G virus in the north.[20,21] Our data on two localities situated 100 miles from each other in a southwestern state of Nigeria suggest that the situation is likely to be more complex; significant differences in subtype distribution, probably due to founder effects, may occur within the same region. In addition, we found that subtype G viruses (G, G′, and CRF06_cpx) contribute to an equally important proportion of infections than CRF02_AG. Given the lag time between sample collection and the publication of this study, it is possible that the epidemiology of the proportion of virus populations might have changed since. This is an inherent limitation of most molecular epidemiology studies.

Nigeria is the most populated country in Africa contributing to 20% of the overall population of the continent. Our data suggest a complex and possibly changing proportion of HIV subtypes contributing to the HIV epidemic in the country. The proportion of HIV subtypes varied significantly between two centers even in the same southwestern state. The large number of recombinant and unclassified viruses in both locales suggests that HIV diversification may be ongoing. This may represent a significant obstacle to vaccine development, should efficacy be subtype specific. It is important to conduct extensive surveys covering all regions of the country, with a sufficient number of samples, to fully assess the molecular epidemiology of HIV in the country, to understand the immune response to and the pathogenesis of the diverse viral variants, and to guide decisions about candidate vaccine development.

## SEQUENCE DATA

All sequences have been submitted to Genbank. Accession numbers are pending.

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