

## Sequence Note

# Brazilian HTLV Type 2a Strains from Intravenous Drug Users (IDUs) Appear to Have Originated from Two Sources: Brazilian Amerindians and European/North American IDUs

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### ABSTRACT

In Brazil, HTLV-2 has been detected in blood donors, in intravenous drug users (IDUs) from urban areas, and in Amerindians living in the Amazon basin. Of the three main HTLV-2 subtypes (2a, 2b, and 2d) only subtype 2a has been detected in Brazil. However, a molecular variant of subtype 2a (also called HTLV-2c) characterized by an extended Tax protein has been isolated from Brazilian blood donors, IDUs, and Indians. Here, we analyzed HTLV-2 isolates from 10 IDUs and a Chilean woman living in Salvador, Bahia, Brazil. Sequencing of *env*, pX, and long terminal repeat (LTR) genes demonstrated that 10 of the isolates are related to the Brazilian subtype 2a molecular variant described previously. We show that most HTLV-2a Brazilian strains comprise a phylogenetic group harboring a considerable degree of diversity within the *env* region but not within the LTR region. Interestingly, we demonstrated for the first time in Brazil the presence of a subtype 2a in IDUs that is closely related to the prototype Mo but distinct from the Brazilian 2a molecular variant.

**I**NTRAVENOUS DRUG USERS (IDUs) residing in Salvador (north-east Brazil) present the highest HTLV-2 seroprevalence rate (8.8%) observed so far in a Brazilian population.<sup>1</sup> We demonstrated through phylogenetic analysis that HTLV-2 *env* sequences from Tiriyo Indians are closely related to isolates from IDUs living in urban areas of southern Brazil.<sup>2</sup> In the present study, analysis of three different regions of the proviral genome (*env*, pX, and long terminal repeat [LTR]) demonstrated that most of the studied isolates are related to the subtype 2a molecular variant previously reported for isolates from other Brazilian populations,<sup>3-6</sup> which is distinct from the North American and European subtypes 2a and 2b.<sup>7,8</sup>

As part of two different cross-sectional studies,<sup>1,9</sup> intravenous blood samples were collected between 1994 and 1996

from 10 HIV-1- and HTLV-2-coinfected IDUs (BAIDU2, BAIDU25, BAIDU28, BAIDU70, BAIDU72, BAIDU81, BAIDU86, BAIDU148, BAIDU187, and BAIDU211) and one HTLV-2-seropositive Chilean woman living in Salvador, Bahia (NS291) who did not report the use of intravenous drugs. Genomic DNA was extracted from whole blood, using a GFX genomic blood DNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). Because of the limited availability of DNA, not all genomic regions were analyzed for all samples (Table 1). For amplification of the 630-nucleotide *env* gene fragment, nested polymerase chain reaction (PCR) was performed on samples NS291, BAIDU2, BAIDU72, BAIDU81, BAIDU86, BAIDU187, and BAIDU211, using outer primers BSEF4/FLENVR1 and inner primers GP21F1/GP21R1.<sup>5</sup> For

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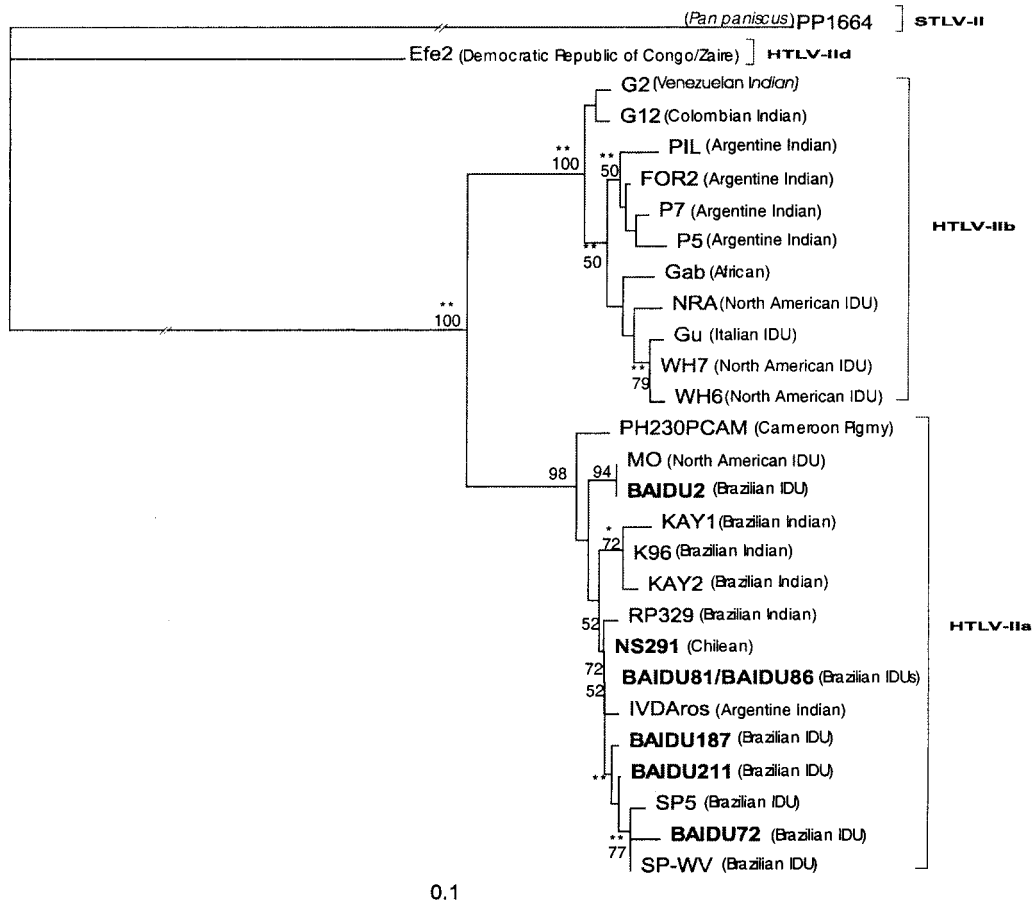
TABLE 1. GENOMIC ANALYSIS OF SAMPLES

Sample	PCR/sequenced genomic region		
	env	LTR	pX
BAIDU2	Yes	Yes	Yes
BAIDU25		Yes	
BAIDU28		Yes	
BAIDU70		Yes	
BAIDU72	Yes		
BAIDU81	Yes		
BAIDU86	Yes	Yes	
BAIDU148		Yes	
BAIDU187	Yes		
BAIDU211	Yes		
NS291	Yes		

amplification of the HTLV-2 LTR gene, samples BAIDU2, BAIDU25, BAIDU28, BAIDU70, BAIDU86, and BAIDU148 were subjected to nested PCR with outer primers BSQF6/BSDR3 and inner primers BSQF2/BSDR4, which amplify a 672-bp

fragment of HTLV-2.<sup>10</sup> For amplification of the pX gene, sample BAIDU2 was analyzed by nested PCR, using primers and conditions as described elsewhere.<sup>4</sup> All amplified products were electrophoresed on a 2% agarose gel stained with ethidium bromide.

All PCR products amplified from the *env*, LTR, and pX regions were purified with a Promega (Madison, WI) Wizard PCR prep system, and sequenced in a PerkinElmer/ABI PRISM 377 DNA stretch sequencer using Taq FS Dye terminator cycle sequencing and the same PCR inner primers as described above. Multiple sequence alignment of the *env*, LTR, and pX regions of the samples studied, together with related sequences in the GenBank/EMBL database, was conducted with the Dambe program (CLUSTAL algorithm),<sup>11</sup> and further edited in the GeneDoc program.<sup>12</sup> The empirical transition:transversion ratios were determined with the program Puzzle 4.0.2. Aligned sequences were analyzed with Phylip version 3.572<sup>13</sup> and neighbor-joining (NJ) and maximum-likelihood (ML) trees were obtained with the F84 substitution model. The reliability of the NJ trees was evaluated by analyzing 1000 bootstrap replicates. The trees were drawn with the TreeView 1.4 program.



**FIG. 1.** Rooted NJ tree of seven new HTLV-2 strains along with a representative set of HTLV-2 strains based on a 586-bp fragment of the *env* region. The bootstrap values (above 50% and using 1000 bootstrap samples) on the branches represent the percentage of trees for which the sequences at one end of the branch form a monophyletic group. Efe2 and PP1664 strains are used as outgroups. Geographical origin and ethnic origin are given in parentheses. Newly sequenced *env* included in this analysis are in boldfaces. Statistical evaluation of branch lengths, using ML, results in some clades being better supported than others (\*\* $p < 0.01$ ; \* $p < 0.05$ ).

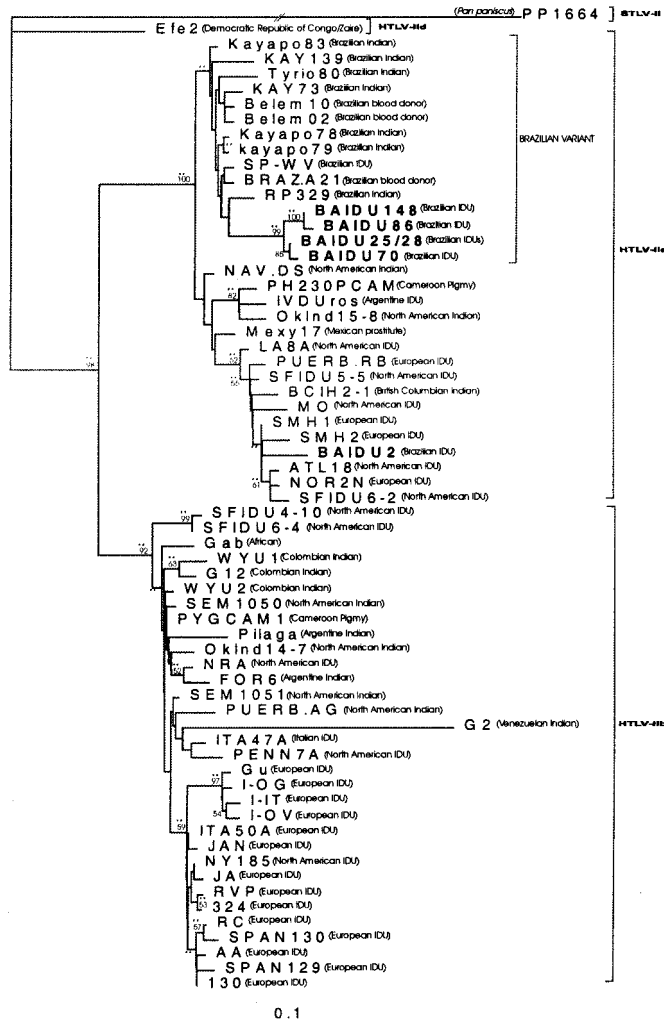
Phylogenetic analysis of a 586-bp *env* fragment corresponding to the coding region of the viral gp21 envelope protein was conducted on seven isolates (Fig. 1) and demonstrated that all of them belong to subtype 2a (98% of bootstraps). However, these sequences formed two separate clusters, both well supported by bootstrap analysis: six (NS291, BAIDU72, BAIDU81, BAIDU86, BAIDU187, and BAIDU211) formed a cluster inside the subtype 2a cluster (72% bootstraps support), whereas one (BAIDU2) clustered together with North American isolate Mo (94% bootstrap support).

Phylogenetic analysis of a 626-bp fragment composed of part of the LTR region was conducted on six isolates (BAIDU2, BAIDU25, BAIDU28, BAIDU70, BAIDU86, and BAIDU148) and showed that they belonged to subtype 2a (100% bootstrap support) (Fig. 2). Whereas five of these sequences formed a unique cluster inside subtype 2a ( $p < 0.01$ , ML method; 99% bootstrap support), sample BAIDU2 clustered together with North American and European sequences ( $p < 0.01$  in the ML

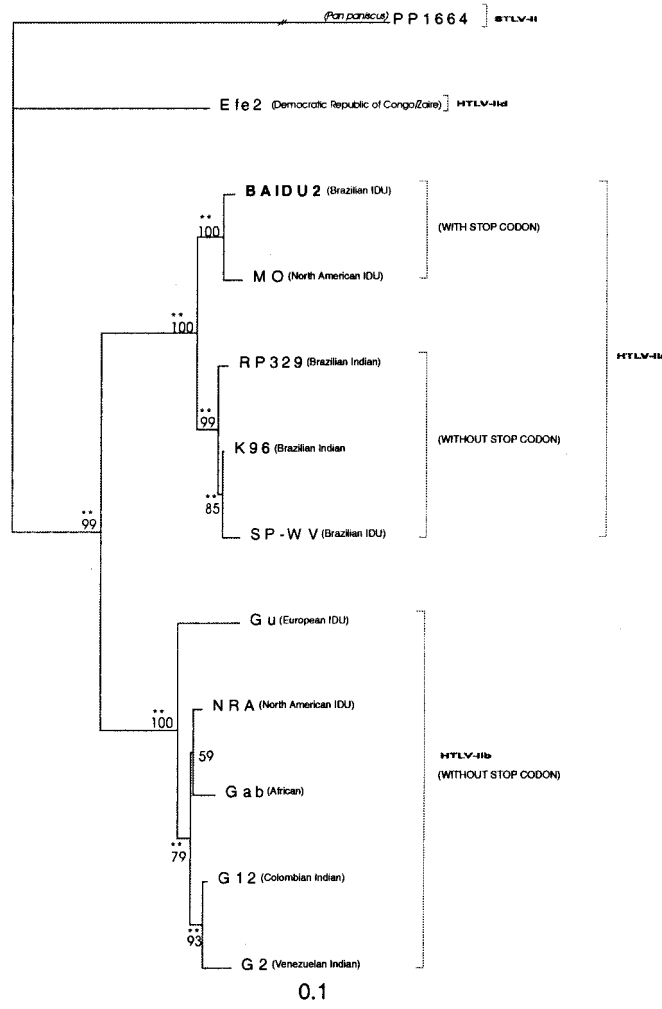
analysis; 62% bootstrap support). Because of the limited availability of genomic DNA, BAIDU2 and BAIDU86 were the only isolates for which we could study LTR sequences besides *env* (Table 1).

A 1643-bp fragment covering almost the entire pX gene and corresponding to bp 6055–8896 in the HTLV-2a Mo prototype was studied in sample BAIDU2, because this was the only sample with sufficient available genomic DNA (Table 1 and Fig. 3). In this analysis BAIDU2 is displayed outside the Brazilian subtype 2a cluster in the phylogenetic tree, but together with the North American IDU HTLV-2a prototype sequence Mo. This result was significantly supported by both bootstrap (100%) and ML analysis ( $p < 0.01$ ).

The isolate NS291, obtained from a Chilean woman residing in Salvador, was characterized as subtype 2a in the *env* phylogenetic analysis. Up to now, only subtype IIb has been identified in Chile.<sup>14</sup> Because this sequence clustered inside the Brazilian 2a clade (72% of bootstrap), it is possible that she



**FIG. 2.** Rooted NJ tree of six HTLV-2 strains based on a 626-bp fragment of the LTR region. The bootstrap values (above 50% and using 1000 bootstrap samples) on the branches represent the percentage of trees for which the sequences at one end of the branch form a monophyletic group. Efe2 and PP1664 strains are used as outgroups. Geographical origin and ethnic origin are given in parentheses. Newly sequenced LTR included in this analysis are in boldfaces. Statistical evaluation of branch lengths results in some clades being better supported than others (\*\* $p < 0.01$ ; \* $p < 0.05$ ).



**FIG. 3.** Rooted NJ tree of one HTLV-2 strain based on a 1643-bp fragment of the pX region. The bootstrap values (above 50% and using 1000 bootstrap samples) on the branches represent the percentage of trees for which the sequences at one end of the branch form a monophyletic group. Efe2 and PP1664 strains are used as outgroups. Geographical origin and ethnic origin are given in parentheses. New pX sequences included in this analysis are in boldface. Statistical evaluation of branch lengths results in some clades being better supported than others (\*\* $p < 0.01$ ).

was infected in Brazil. Unfortunately, the lack of genomic DNA made it impossible to analyze the *env*, LTR, and *tax* genes in most of the samples, thereby limiting the phylogenetic information available for these strains. Interestingly, the sequence analysis of the *tax* gene in this isolate identified a stop codon at position 8203, which is similar to that found in North American and European HTLV-2a sequences, and different from the other IDU strains belonging to the Brazilian HTLV-2a molecular variant. Previous studies of the *tax* gene sequence demonstrated that the Brazilian HTLV-2 isolates phylogenetically classified as subtype 2a show a nucleotide substitution at position 8203 (T→C), in comparison with the subtype 2a prototype Mo. This substitution leads to the generation in these isolates of a Tax protein that is 25 amino acids longer<sup>4,6</sup> than that produced by the subtype 2a prototypes. This extended Tax protein is characteristic of the HTLV-2b subtype. BAIDU2 represents the first description of Brazilian HTLV-2a, which is phylogenetically related to North American and European strains. The similarity of this strain with the prototype HTLV-2a strain Mo is 99% in the *env* gene and 95% in the LTR region. It is pos-

sible that this sample represents a new introduction of North American/European HTLV-2a in Salvador, Bahia, Brazil, because it was obtained during a cross-sectional study in the historical district of the city (Pelourinho), which is the most important tourist destination in northeast Brazil, and is surrounded by areas where drug users are prevalent.<sup>1</sup>

In the present study, we demonstrated that HTLV-2 isolates from infected patients living in Salvador belong to subtype 2a. Our results confirm previous data describing subtype 2a as the only HTLV-2 subtype circulating in Brazil.<sup>4,5,6,15</sup> In addition, the majority of the isolates studied here clustered together, generating the so-called Brazilian cluster inside the subtype 2a clade (also called 2c). Because we did not observe a single Brazilian clade within subtype 2a in the analysis of *env* sequences, our results are compatible with previous studies indicating that the existence of this unique Brazilian 2a clade was poorly supported on the basis of *env* analysis.<sup>6</sup> However, when LTR sequences were analyzed, a clear Brazilian clade was defined on the basis of the ML method ( $p < 0.01$ ), in agreement with previously published results.<sup>6</sup> Our phylogenetic results

demonstrate that different genomic regions of HTLV-2 proviruses must be analyzed in order to genetically characterize Brazilian strains and to infer the possible origins of this virus in the South American continent.

### ACKNOWLEDGMENTS

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### SEQUENCE DATA

The GenBank accession numbers for the sequences included in the phylogenetic study are as follows: *env analysis*: PP1664 (Y14570), Efe2 (Y14365), G2 (AF074965), G12 (S67034), PIL (AF058086), FOR2 (AF058079), P7 (AF058085), P5 (AF058083), Gab (Y13051), NRA (L20734), Gu (X89270), WH7 (U32900), WH6 (M85226), PH230PCAM (Z46837), MO (M10060), KAY1 (U19110), KAY2 (U19109), K96 (AF326584), RP329 (AF326583), IVDARos (AF058089), SP5 (U32895), SP-WV (AF139382); *LTR analysis*: PP1664 (Y14570), Efe2 (Y14365), NAV.DS (U10257), PH230PCAM (Z46838), LA8A (U10256), Mo (M10060), PUERB.RB (U10262), NOR2N (U10258), ATL18 (U10252), Mexy17 (L42510), BRAZ.A21 (U10253), KAY73 (L42509), KAY139 (L42508), Kayapo83 (AF139390), Kayapo78 (AF139388), Kayapo79 (AF139389), RP329 (AF326583), SP-WV (AF139382), Tyrio80 (AF139391), Belem02 (AF139392), Belem10 (AF139393), G12 (L11456), WYU1 (U12792), PUERB.AG (U10261), SEM1051 (U10264), PENN7A (U10260), WYU2 (U12794), PYGCAM1 (Z46888), SEM1050 (U10263), NRA (L20734), ITA47A (U10254), NY185 (U10259), AA (L77238), JA (L77239), 324 (L77243), RVP (L77244), SPAN129 (U10265), SPAN130 (U10266), 130 (L77242) RC (L77235), ITA50A (U10255), Gu (X89270), JAN (L77241), SMH1 (Y09147), SMH2 (Y09148), I-OV (Y09155), I-OG (Y09154), I-IT (Y09151), SFIDU 5-5 (U73010), SFIDU 6-2 (U73022), OkInd 15-8 (U73015), IVDUros (AF054272), SFIDU 4-10 (U73016), SFIDU 6-4 (U73018), Pilaga (AF054271), OkInd 14-7 (U73009), FOR6 (AF054273), Gab (Y13051), G2 (AF074965), BCIH2-1 (AF185282); *px analysis*: PP1664 (Y14570), Efe2 (Y14365), Mo (M10060), RP329 (AF326583), K96 (AF326584), SP-WV (AF139382), Gu (X89270), NRA (L20734), Gab (Y13051), G2 (AF074965), G12 (S678304).

The GenBank accession numbers of the HTLV-2 fragments sequenced in our laboratory and included in the phylogenetic analysis are as follows: *env*: NS291 (AF197284), BAIDU86 (AF197283), BAIDU81 (AF197282), BAIDU72 (AF197281), BAIDU211 (AF197280), BAIDU187 (AF197279), BAIDU2 (AF401496); *LTR*: BAIDU70 (AF401495), BAIDU148 (AF401491), BAIDU86 (AF401492), BAIDU25 (AF401493), BAIDU28 (AF401494), BAIDU2 (AF401496), *pX*: BAIDU2 (AF401496).

### REFERENCES

- Andrade TM, Dourado I, and Galvao-Castro B: Associations among HTLV-I, HTLV-II, and HIV in injecting drug users in Salvador, Brazil. *J AIDS Hum Retrovirol* 1998;18:186-187.
- Shindo N, Alcantara LCJ, Van Dooren S, *et al.*: Human retroviruses (HIV and HTLV) in Brazilian Indians: Seroepidemiological study and molecular epidemiology of HTLV-II isolates. *AIDS Res Hum Retroviruses* 2002;18:71-77.
- Biggar RJ, Taylor ME, Neel JV, *et al.*: Genetic variants of human T-lymphotropic virus type II in American Indian groups. *Virology* 1996;216:165-173.
- Eiraku N, Novoa P, da Costa Ferreira M, *et al.*: Identification and characterization of a new and distinct molecular subtype of human T-cell lymphotropic virus type 2. *J Virol* 1996;70:1481-1492.
- Ishak R, Harrington WJ Jr, Azevedo VN, *et al.*: Identification of human T cell lymphotropic virus type IIa infection in the Kayapo, an indigenous population of Brazil. *AIDS Res Hum Retroviruses* 1995;11:813-821.
- Lewis MJ, Novoa P, Ishak R, *et al.*: Isolation, cloning, and complete nucleotide sequence of a phenotypically distinct Brazilian isolate of human T-lymphotropic virus type II (HTLV-II). *Virology* 2000;271:142-154.
- Shimotohno K, Takahashi Y, Shimizu N, *et al.*: Complete nucleotide sequence of an infectious clone of human T-cell leukemia virus type II: An open reading frame for the protease gene. *Proc Natl Acad Sci USA* 1985;82:3101-3105.
- Lee H, Idler KB, Swanson P, *et al.*: Complete nucleotide sequence of HTLV-II isolate NRA: Comparison of envelope sequence variation of HTLV-II isolates from U.S. blood donors and U.S. and Italian i.v. drug users. *Virology* 1993;196:57-69.
- Gomes I, Melo A, Proietti FA, *et al.*: Human T lymphotropic virus type I (HTLV-I) infection in neurological patients in Salvador, Bahia, Brazil. *J Neurol Sci* 1999;165:84-89.
- Switzer WM, Pieniazek D, Swanson P, *et al.*: Phylogenetic relationship and geographic distribution of multiple human T-cell lymphotropic virus type II subtypes. *J Virol* 1995;69:621-632.
- Xia X: *DAMBE: Data Analysis in Molecular Biology and Evolution*. Department of Ecology and Biodiversity, University of Hong Kong, 2000.
- Nicholas KB, Nicholas HBJ, and Deerfield DW: GeneDoc: Analysis and visualization of genetic variation; 1997; EMBNEW. NEWS 4.
- Felsenstein J: *PHYLIP (Phylogeny Inference Package)*, version 3.572 [distributed by the author]. Department of Genetics, University of Washington, Seattle, Washington, 1989.
- Miura T, Yamashita M, Zaninovic V, *et al.*: Molecular phylogeny of human T-cell leukemia virus type I and II of Amerindians in Colombia and Chile. *J Mol Evol* 1997;44 (Suppl 1):S76-S82.
- Switzer WM, Black FL, Pieniazek D, Biggar RJ, Lal RB, and Heneine W: Endemicity and phylogeny of the human T cell lymphotropic virus type II subtype A from the Kayapo Indians of Brazil: Evidence for limited regional dissemination. *AIDS Res Hum Retroviruses* 1996;12:635-640.

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