

## Molecular evidence of mother-to-child transmission of HTLV-IIc in the Kararao Village (Kayapo) in the Amazon Region of Brazil

Evidência molecular da transmissão do HTLV-IIc de mãe para filho, na aldeia Kararao (Kayapó), na região amazônica brasileira

Ricardo Ishak<sup>1</sup>, Antonio Carlos R. Vallinoto<sup>1</sup>, Vânia Nakauth Azevedo<sup>1</sup>, Martha Lewis<sup>2</sup>, William W. Hall<sup>2</sup> and Marluísa O. Guimarães Ishak<sup>1</sup>

**Abstract** Blood samples from native Indians in the Kararao village (Kayapo), were analysed using serological and molecular methods to characterize infection and analyse transmission of HTLV-II. Specific reactivity was observed in 3/26 individuals, of which two samples were from a mother and child. RFLP analysis of the pX and env regions confirmed HTLV-II infection. Nucleotide sequence of the 5' LTR segment and phylogenetic analysis showed a high similarity (98%) between the three samples and prototype HTLV-IIa (Mot), and confirmed the occurrence of the HTLV-IIc subtype. There was a high genetic similarity (99.9%) between the mother and child samples and the only difference was a deletion of two nucleotides (TC) in the mother sequence. Previous epidemiological studies among native Indians from Brazil have provided evidence of intrafamilial and vertical transmission of HTLV-IIc. The present study now provides molecular evidence of mother-to-child transmission of HTLV-IIc, a mechanism that is in large part responsible for the endemicity of HTLV in these relatively closed populations. Although the actual route of transmission is unknown, breast feeding would appear to be most likely.

**Key-words:** HTLV-II. Molecular epidemiology. Vertical transmission. Amerindian populations.

**Resumo** Amostras de sangue de índios nativos na aldeia Kararao (Kayapó) foram analisadas, usando-se métodos sorológico e molecular, para caracterizar a infecção e analisar a transmissão do HTLV-II. Observou-se reatividade específica em 3/26 indivíduos, dos quais duas amostras eram de uma mãe e de seu filho. A análise pela RFLP de regiões pX e env confirmou a infecção pelo HTLV-II. A seqüência de nucleotídios do segmento 5'LTR e a análise filogenética mostraram alta similaridade (98%) entre as três amostras e o protótipo HTLV-IIa (mot) e confirmaram a ocorrência do subtipo HTLV-IIc. Houve uma alta similaridade genética (99,9%) entre as amostras da mãe e do filho e a única diferença foi uma deleção de dois nucleotídios (TC) na seqüência materna. Estudos epidemiológicos anteriores entre índios nativos do Brasil forneceram prova da transmissão intrafamiliar e vertical do HTLV-IIc. O presente estudo fornece evidência molecular da transmissão do HTLV-IIc de mãe para filho, um mecanismo que em grande parte é responsável pela endemicidade do HTLV nessas populações epidemiologicamente fechadas. Embora a verdadeira via de transmissão seja desconhecida, a amamentação materna poderia ser a mais provável.

**Palavras-chaves:** HTLV-II. Epidemiologia molecular. Transmissão vertical. Populações ameríndias.

Human T-cell lymphotropic virus, types I and II (HTLV-I and HTLV-II), is endemic in several different geographical regions and its infection in the human host is associated to hematological and neurological disorders<sup>13 15 26</sup>. HTLV-II is endemic among intravenous drug users from North and South America, Europe, Southeast Asia and in a large number of native Indian groups throughout the Americas and certain pygmy groups in Africa<sup>1 2 4 12 13 16 17 25 27 29</sup>.

HTLV-I and HTLV-II infections among urban populations are maintained through blood transfusions, drug usage, and sexual intercourse as their most important routes of transmission<sup>5 11 21</sup>. However, in several Indian populations, mother-to-child transmission has been reported as the main route of transmission and maintenance of endemicity of HTLV-II<sup>21 17 30</sup>.

The present paper describes the occurrence of HTLV-IIc, in three individuals from the Kararao tribe (Kayapo) and the molecular evidence of vertical transmission of this virus.

1. Laboratório de Virologia do Centro de Ciências Biológicas da Universidade Federal do Pará, Belém, PA, Brasil; 2. Laboratory of Medical Virology, University College, Dublin, Ireland.

Address to: Dr. Ricardo Ishak. Caixa Postal 13005, 66040-970, Belém, PA, Brazil,

Tel.: 55 91 211-1587, Fax 55 91 249-0373

e-mail: rishak@canal13.com.br

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## MATERIAL AND METHODS

**Population Examined and Samples.** Blood samples were collected from twenty-four subjects residing in the Kararao tribe (Kayapo), a Je speaking community, located in the State of Para, Brazil. All the subjects had a sample of blood drawn and placed in tubes without anticoagulant to obtain serum and in other tubes containing HESPAN (Du Pont, USA) in order to separate peripheral blood mononuclear cells (PBMC). Serum and PBMC were stored at  $-20^{\circ}\text{C}$  before use.

**Serological Assays.** Serum samples were screened for the presence of antibodies to HTLV-I/II using an enzyme immunoassay (EIA, Ortho Diagnostic, USA) and the positive samples were further analyzed by Western blot (Genelab 2.4, Singapore) which permits differentiation of HTLV-I and HTLV-II seroreactivity.

**Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) Analysis.** DNA was extracted from PBMC from HTLV-II seroreactive samples, and was used in a nested PCR to amplify part of the *pX* region. The first round PCR was carried out using  $1\mu\text{g}$  of the extracted DNA,  $125\mu\text{M}$  of each dNTP (Perkin-Elmer, USA),  $20\text{ pmol}/\mu\text{L}$  of each of the two external primers and  $10\times$  PCR buffer -  $\text{MgCl}_2$  (Perkin-Elmer, USA), ending up with a final volume of  $50\mu\text{L}$ . The reaction was incubated in a thermocycler (Perkin-Elmer, USA) for five minutes at  $94^{\circ}\text{C}$ , followed by 35 cycles at  $94^{\circ}\text{C}$  (40 seconds),  $51.6^{\circ}\text{C}$  (30 seconds),  $72^{\circ}\text{C}$  (40 seconds) and extended for 10 minutes at  $72^{\circ}\text{C}$ .

Five microliters of the amplified product were used in the nested PCR using a set of internal primers, and maintaining the same mixture (with a final volume of  $100\mu\text{L}$ ), and the same temperature and incubation periods employed in the first reaction. The external primers sequences were 5'-TTCCCAGGGTTTGGACAGAG-3' (nucleotides 7219-7238) and 5'-GGGTAAGGACCTTGAGGGTC-3' (nucleotides 7483-7464). The internal primers sequences were 5'-CGGATACCCAGTCTACGTGTT-3' (nucleotides 7248-7268) and 5'-GAGCCGATAACGCGTCCATCG-3' (nucleotides 7406-7386). The two amplified products were analysed by electrophoresis in 2% agarose gels.

RFLP typing of this product (159 bp for both HTLV-I and HTLV-II) was performed by incubating the reaction product ( $10\mu\text{L}$ ) with  $0.4\mu\text{L}$  of the restriction endonuclease enzyme *TaqI* and incubating at  $65^{\circ}\text{C}$  for at least 5 hours. The restriction site for the enzyme (T/CGA) is present in the amplified product of HTLV-II, generating two bands (85bp and 53bp) that are visualized following an electrophoresis in a 3% agarose gel. HTLV-I lacks this particular site.

The subtyping of HTLV-II was obtained using a nested PCR, with two sets of primers that match a conservative nucleotide sequence flanking the *env* region, and coding for the envelope protein, as previously described<sup>7,14</sup>. The PCR product (631 bp) was digested enzymatically by *XhoI*. The RFLP pattern obtained discriminates between HTLV-IIa and HTLV-IIb subtypes by the presence of the

C/TCGAG site in the HTLV-IIa. This is evidenced by the presence of two fragments (179 and 452bp) that are visualized on a 3% agarose gel electrophoresis.

Following the subtyping, all samples initially characterized as HTLV-IIa were submitted to a nested PCR to 5'*LTR* region, as previously described<sup>7</sup> with two sets of primers, 5'-TCGCGATGACAATGGCGACTAGCCTC-3' and 5'-GGGGGCTTTGGGTATTGGAGTTGGG-3', corresponding to nucleotides 1-26 and 855-831 to the first step and 5'-GCCTCCCAAGCCAGCCAC-3' and 5'-GGGAAAGCCCGTGGATTTGCCCAT-3' in a second step, corresponding to nucleotides 16-33 and 831-807 from HTLV-II MoT strain. Following the amplification, the *LTR* fragment was electrophoresed on a 0.8% agarose gel and purified by QIA Quick Purification Kit (Quiagene, USA). The *LTR* PCR fragments were directly cloned into pCR<sup>TM</sup>II (Introgen Original TA Cloning Kit) following the manufacturer's instructions. Plasmid DNA was prepared from recombinant clones using Wizard Miniprep kit (Promega) prior to sequencing of the product.

**Nucleotide Sequence Analysis.** Sequencing of 5'*LTR* was carried out using the vector-based primers M13R and T7 and internal sequence primers designed from the published HTLV-IIa MoT sequence. The samples were sequenced automatically with a Perkin-Elmer/ABI Prism 377 DNA Stretch Sequencer using *Taq* FS dye terminator cycle sequencing (Perkin-Elmer Cetus, Norwalk, California).

**Phylogenetic Analysis.** The nucleotide sequences originated from the 5'*LTR* region (630 bp), obtained in the present study (Genbank Accession Number: KAA8878, Kararao, AF306731; KAA8879, Kararao, AF306732; KAA8883, Kararao, AF306733), were used to establish the phylogenetic relationship together with the twenty-six following HTLV-II strains described in the Genbank: MO, USA, M10060; NRA, USA, L20734; PYGCAM-1, Cameroon, Pygmy, Z46888; PH230PCAM, Cameroon, Z46838; ATL18, Georgia, U10252, BRAZ.A21, Brazil, U10253, LA8A, California, U10256, NOR2N, Norway, U10258, PUEB.AG and PUEB.RB, New Mexico, Pueblo, U10261, U10262, ITA47A and ITA50A, Italy, U10254, U10255, NY185, New York, U10259, PENN7A, Pennsylvania, U10260, SEM1050 and SEM1051, Florida, Seminole, U10263, U10264, SPAN129 and SPAN130, Spain, U10265, U10266; WYU2, Colombia, Wayu, U12794; GHKT, Ghana, L42507, KAY73 and KAY139, Kayapo, Amerindian, L42509, L42508; Pygmy-2, Efe Bambuti Pygmy tribe, Congo, Y14365. The sequence alignments were performed using the EyeBall Sequence Editor<sup>3</sup>. The phylogenetic relationship was performed by the Phylogenetic Inference Package - PHYLIP 3.5v<sup>8</sup>. The Neighbor-joining (NJ) method was used for the tree construction considering the standard Kimura two-parameter model. The statistical reliance of the tree was evaluated using 2000 bootstrap samples.

## RESULTS

**Serology.** Specific seroreactivity to HTLV-II was observed in three individuals from the Kararao tribe. Two samples were mother and child (KAA8878 and KAA8879, respectively), as observed in Figure 1. Seroreactivity to HTLV-II was confirmed by Western blot.

**Molecular Characterization.** PCR amplification of the *pX* genomic region, followed by enzymatic digestion using *TaqI* endonuclease confirmed HTLV-II infection among the Kararao indians. The amplification of *env* and subsequent restriction analysis with *XhoI* exhibited the

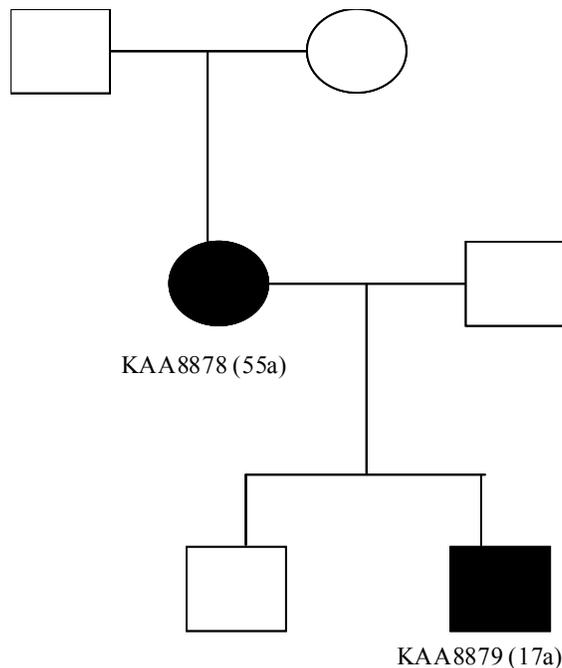


Figure 1 - Dendogram showing evidence of vertical transmission of HTLV-II in the Kararao village

presence of two fragments (179bp and 492bp) as it is currently described as HTLV-II, subtype a.

Nucleotide sequence analysis (Figure 2) of the 5' LTR segment showed a high similarity (98%) between the Kararao samples and the prototype IIa (HTLV-II-Mot). However, a consistent divergence (95%) in nucleotide sequences was observed from the prototype IIb (HTLV-II-NRA). The differences found between Kararao virus and the prototype Mot sequences were characterized mainly by point mutation without large deletion or insertion. Significant changes in regulatory elements of

the viral replication such as TATA box, Poli-A site and Cap site were not observed. Furthermore, the phylogenetic analysis using the 630bp amplified from the 5' LTR segment confirmed the occurrence of HTLV-IIc (Figure 3).

The sequence analysis of the Kararao samples exhibited a high genetic similarity (99.9%) between the mother and child samples (KAA8878 and KAA8879, respectively). The only difference was a deletion of two nucleotides (TC) in the mother sequence (positions 236 and 237). This result shows a strong evidence of vertical transmission.

## DISCUSSION

The geographical distribution of HTLV-II in the Amazon region of Brazil exhibits the largest area worldwide, it is mainly distributed among native Indian groups and it is in a current epidemiological process of dissemination into urban areas<sup>17 18 24 31</sup>. Transmission of the virus is primarily through blood transfusion, drug usage, sexual intercourse and breast feeding<sup>5 9 19 20 28</sup>.

Seroprevalence of HTLV-II among urban communities is commonly low with the exception of some groups with specific behavior that contribute for the transmission of the virus<sup>5 9</sup>. A high prevalence of HTLV-II infection has been reported among IVDU from USA<sup>6 22</sup>. Similarly, HTLV-II infection was found in 14.6% of IVDU from Dublin, Ireland<sup>5</sup>.

<b>U3</b>	<b>21 pb (repetitions)</b>						
MO	AAGTAAAGGC	TCTGACGTCT	CCCCCTTTTT	TTAGGAACTG	AAACCACGGC	CCTGACGTCC	60
KAA8878	.....	.....	.....	--.....	.....	.....	
KAA8879	.....	.....	.....	--.....	.....	.....	
KAA8883	.....	.....	.....	--.....	.....	.....	
MO	CTCCCCCT-	AGGAAC-AGG	AACAGCTCTC	CAGAAAAAAA	TAGACCTCAC	CCTTACCCAC	120
KAA8878	.....CC	.....C..	.....	.....--	.....	.....	
KAA8879	.....CC	.....C..	.....	.....--	.....	.....	
KAA8883	.....C.	.....C..	.....	.....--	.....	.....	
	<b>21 pb (repetitions)</b>						
MO	TTCCCTAGC	GCTGAAAAAC	AAGGCTCTGA	CGATTACCCC	CTGCCCATAA	AATTTGCCTA	180
KAA8878	.....	A.....	.....	.....	.....	.....	
KAA8879	.....	A.....	.....	.....	.....	.....	
KAA8883	.....	A.....	.....	.....	.....	.....	
	<b>Poli-A</b>		<b>TATA box</b>		<b>Cap</b>	<b>R</b>	
MO	GTCAAATAA	AAGATGCCGA	GTCTATAAAA	GCGCAAGGAC	AGTTCAGGAG	GTGGCTCGCT	240
KAA8878	.C.....	.....	.....	.....	.....	.GCT.---	
KAA8879	.C.....	.....	.....	.....	.....	.GCT.....	
KAA8883	.C.....	.....	.....	.....	.....	.GCT.....	
MO	CCCTCACCGA	CCCTCTGGTC	ACGGAGACTC	ACCTTGGGGA	TCCATCCTCT	CCAAGCGGCC	300
KAA8878	.....	.....	.....	.....	.....	.....	
KAA8879	.....	.....	.....	.....	.....	.....	
KAA8883	.....	.....	.....	.....	.....	.....	
MO	TCGGTTGAGA	CGCCTTCCGT	GGGA-CCGTC	TCCCGGCCTC	GGCACCTCCT	GAAGTGTCTC	360
KAA8878	.....	.....T..	.....	.....	.....	.....	
KAA8879	.....	.....T..	.....	.....	.....	.....	
KAA8883	.....	.....	.....	.....	.....	.....	
MO	TCCAAGGTA	AGTCTCCTCT	CAGGTCGAGC	TCGGCTGCCC	CTTAGGTAGT	CGCTCCCCGA	420
KAA8878	.....G...	.....	.....	.....	.....	.....	
KAA8879	.....G...	.....	.....	.....	.....	.....	
KAA8883	.....G...	.....	.....	.....	.....	.....	
MO	GGGTCTTTAG	AGACACCCGG	GTTTCCGCT	GCGCTCGGCT	AGACTCTGCC	TTAAACTTCA	480
KAA8878	.....	.....	.....	.....	.....G.	.....	
KAA8879	.....	.....	.....	.....	.....G.	.....	
KAA8883	.....	.....	.....	.....	.....G.	.....	
	<b>U5</b>						
MO	CTTCCGCGTT	CTTGTCTCGT	TCTTTCCTCT	TCGCCGTCAC	TGAAAACGAA	ACCTCAACGC	540
KAA8878	.....	.....	.....	.....	.....	.....	
KAA8879	.....	.....	.....	.....	.....	.....	
KAA8883	.....	.....	.....	.....	.....	.....	
MO	CGCCCTCTTG	GCAGGCGT--	CCCCGGGCCA	ACATAC-GCC	GTGGAG-CGC	AGCAAGGGCT	600
KAA8878	.....C..	.....T.GG	.....	.....T..	.....G..	.....	
KAA8879	.....C..	.....T.GG	.....	.....T..	.....G..	.....	
KAA8883	.....C..	.....T.GG	.....	.....T..	.....G..	.....	
MO	AGGGCTTCCT	GAACCTCTCC	GGGAGAGGTC				630
KAA8878	.....	.....	.....				
KAA8879	.....	.....	.....				
KAA8883	.....	.....	.....				

Figure 2 - Nucleotide sequences of the 5' LTR region obtained from Kararao samples of the Amazon region of Brazil. The sequences are compared with HTLV-IIa prototype (Mo).

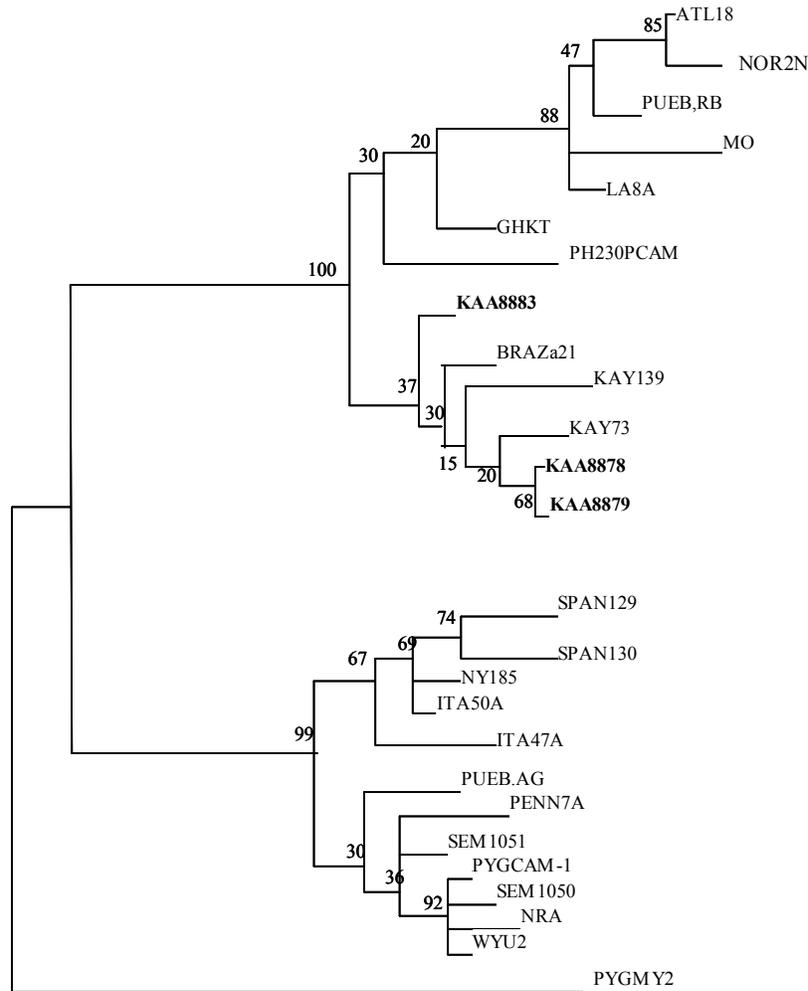


Figure 3 - Unrooted phylogenetic tree showing the evolutionary relationship of HTLV-II reported so far including newly sequenced isolates from the present study (Amerindian subjects: KAA8883, KAA8878, KAA8879). The tree was constructed by N-J method after alignment of 630 nucleotide of the 5' LTR region. The statistical support was applied using 2000 bootstrap replicate.

HTLV-II infection is endemic in other Amerindian populations from North, Central and South America<sup>29</sup>. In Colombia, 29/92 (31,5%) Guahibo Indians were found with specific antibodies to HTLV-II<sup>11</sup>. The Yaruro and Guahibo indians of Venezuela showed a high prevalence of 61% of antibodies to HTLV-IIb<sup>23</sup>. On the other hand, a low prevalence of 2% was observed among the Mapuche tribe from Argentina, but, the infection ranged from 4% to 44% in different villages of the Gran Chaco, Paraguai<sup>10</sup>.

RFLP analysis of *pX* and *env* genes and the nucleotide sequence analysis of 5' LTR, followed by phylogenetic inference, exhibited the presence and dissemination of a new molecular subtype named HTLV-IIc<sup>7, 17</sup>. Previous epidemiological studies among native Indians from Brazil have provided evidence of intrafamilial and vertical transmission of HTLV-IIc among several

families of the Kubenkokre and the Xicrin, both Kayapo, Je speaking community groups<sup>2, 17</sup>. The present paper reports the serological and molecular evidences that confirm vertical transmission of HTLV-IIc between mother and child within the Kararao village, another Kayapo group.

A strong molecular evidence of mother-to-child transmission of HTLV-IIc is provided when comparing the 5' LTR nucleotide sequence of the HTLV-II virus of the mother and the child. There was a high genetic similarity of 99.9% between the two samples with a single difference of a deletion of two nucleotides in the maternal sequence (Figure 2). Considering that the two nucleotides were present in two of the three virus sequences of the Kararao tribe (including the child), it is possible that the deletion could have occurred after the vertical transmission from mother-to-child.

Furthermore, it is also possible that the HTLV-IIc selected from individual KAA8878 was one of a pool of naturally infecting virus, and the cloning procedures might have had the chance to preferentially select clones exhibiting the deletion instead.

Endemicity of HTLV-II, particularly among small communities is maintained by a specific process of virus latency alternating with a productive period, a mechanism that is in large part responsible for long lasting infections. Although the actual route of transmission of HTLV-IIc is unknown, breast feeding would appear the most likely. Breast feeding is a common practice in several native populations and the main

source of nutrition for the infants. Furthermore, it is a common procedure that women breast-feed other children, contributing to the dissemination of HTLV-II. The source of the mother infection is unknown. It is probable that it occurred by intrafamilial transmission, but it could not be successfully proven since her parents were already dead and her husband did not exhibit seroreactivity to HTLV-II.

The present paper confirms the widespread distribution of HTLV-IIc in the Amerindian tribes of Brazil and emphasizes the need to better understand the routes of transmission and maintenance of HTLV in small communities.

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