Phylogenetic and Molecular Analysis of HTLV-1 Isolates From a Medium Sized Town in Northern of Brazil: Tracing a Common Origin of the Virus From the Most Endemic City in the Country

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Salvador-Bahia has the highest prevalence of HTLV-1 infection in Brazil; about 2% of the population is infected. In this city, the prevalence of HTLV in pregnant women is 1%. There is no data of the HTLV-1 prevalence in others cities of the Bahia’s Recôncavo, where the population has similar social and demographic characteristics to those from Salvador. Our aim was to evaluate the seroprevalence of HTLV in pregnant women in Cruz das Almas-Bahia, a medium-sized city from the Bahia’s Recôncavo. All individuals were tested for HTLV (ELISA) and the positive samples were confirmed by Western Blot. Phylogenetic analyses of the total LTR region were performed in all positive samples. We tested 408 samples (45.4% of the estimate pregnant women population) between June 1st and October 31, 2005. The prevalence of HTLV infection was 0.98%. In addition, all isolated virus were grouped in the subtype HTLV-1a, in the Latin American group. Our results suggest that the introduction of HTLV-1 occurred after the slave trade into Salvador. In addition, HTLV-1-infection should be screened during the pregnancy in women originating from HTLV-1 endemic areas. J. Med. Virol. 80:2040–2045, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: HTLV-1; pregnant women; prevalence and molecular epidemiology

INTRODUCTION

It is estimated that 20 million individuals are infected by human T-lymphotropic virus type 1 (HTLV-1) worldwide, mainly in the south of Japan, Sub-Saharan Africa, the Caribbean region and some regions of south and central America [Mueller, 1991]. The HTLV-1 prevalence increases with age and is higher among females [Murphy et al., 1989; Proietti et al., 2005]. In fact, the male-to-female sexual transmission of HTLV seems to be more efficient than the other way around. The probability of a woman becoming infected after 5 years of stable sexual exposure with an HTLV infected partner is 3.9 times higher than it is for the man when the woman is infected [Stuver et al., 1993].

Given its continental proportions, Brazil may harbor the highest absolute number of HTLV-1-infected individuals. Salvador, the capital of the State of Bahia, has the highest prevalence of HTLV-1-infection among blood donors 1.35%, while the global prevalence of infection in Brazil is 0.45% [Galvao-Castro et al., 1997]. A population based study in Salvador recently demonstrated a prevalence of HTLV-1 infection of 1.76% in the overall population. It is estimated that about 50,000 individuals are infected by the virus in this city. In females over 51 years of age infection rate reached 9.3%. In addition, lower income and poor education seem to be important factors determining the infection [Dourado et al., 2003]. Moreover, approximately 1% of pregnant women in this city are HTLV-1-infected [dos Santos et al., 1995; Bittencourt et al., 2001].

The authors disclose any financial or other conflict of interest that might be construed to influence the contents of the manuscript, including the results or interpretation of publication.

Grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Grant sponsor: Fundação de Amparo a Pesquisa da Bahia (FAPESB).

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Accepted 19 May 2008

DOI 10.1002/jmv.21278

Published online in Wiley InterScience (www.interscience.wiley.com)
Another epidemiological characteristic of HTLV-1-infection is the difference in seroprevalence rates according to the geographical area and the socio-demographic composition of the population. HTLV-1 infection trends to cluster among families and neighbors [Proietti et al., 2005]. In the state of Bahia, there is evidence that HTLV-1-infection occurs mostly in Salvador, but is rare in other cities [Moreira et al., 1993; Britto et al., 1998]. The main circulating HTLV-1 strain in Salvador belongs to the transcontinental subgroup of the Cosmopolitan subtype [Alcantara et al., 2006; Mota et al., 2007], which is the most frequent subgroup and subtype around the world [Yamashita et al., 1999]. However, the molecular characteristics of circulating HTLV-1 subtypes are unknown in other towns in the state of Bahia. The Bahia Recôncavo area is located around the Todos os Santos Bay. The economic apogee of this region occurred during the sugar-cane cycle in the 17th century. Its population, mostly black and mixed Western African and Portuguese descendents, has ethnic and socio-demographic characteristics similar to those of the population from Salvador [Azevedo et al., 1982]. In this study, we aimed to evaluate the seroprevalence and molecular epidemiological characteristics of HTLV-1 in pregnant women from Cruz das Almas, a medium-sized town in the Bahia Recôncavo region to support the hypothesis that HTLV-1 isolates circulating in Bahia have a closer relationship to South African strains than to West-African HTLV-1 strains.

**METHODS**

**Area and Subjects**

The study was carried out in the town of Cruz das Almas, located in the Bahia Recôncavo area. The town is 149 km west from Salvador, the capital of Bahia, in the Northeast of Brazil. According to the year 2000 census the population was estimated at 53,049 (52.4% of which were women). The population living in the urban area was 39,604 (74.7%) and in rural area 13,445 (25.3%) (IBGE, 2000). The total number of infants born alive was 888 and 877 in 2004 and 2005 respectively (www.saude.ba.gov.br). Four hundred and eight pregnant women over 10 weeks pregnant were included in the study. Pregnancy diagnosis was based on clinical, laboratorial and ultrasound examinations. The women were followed on an outpatient basis at Perinatology Institute (IPER) and by the seven teams of the Family Health Program (PSF), four from the town center (Sindicato Rural, Bairro Alberto Passos, Suzana, Escola de Agronomia) and three from the rural area (Pumba, Araçá e Sapucaia). These centers are responsible for more than 90% of the pregnant women care program. The patients were selected randomly between June and October 2005. The study protocol included obstetric-clinical examination, sample blood collection and questionnaire data, including the past medical history of the participants. The Oswaldo Cruz Foundation (FIOCRUZ) ethics committee approved this study, and informed consent was obtained from all enrolled patients.

All samples were screened for HTLV-I/II antibodies by enzyme-immune assay (ELISA) (Ab-Capture ELISA Test System—Ortho-Clinical Diagnostics, Inc., Raritan, NJ), and were confirmed by Western blot assay (HTLV Blot 2.4, Genelabs Technologies, Singapore).

Nested PCR was performed in four positive samples, and LTR region was amplified as two overlapping fragments: a LTR-gag segment of 473 bp and a tax-LTR segment of 479 bp, as previously described [Alcantara et al., 2006]. All amplification products were electrophoresed on 1% agarose gel stained with ethidium bromide, and visualized with ultraviolet light.

The LTR amplification products were purified using the QIAquick Gel Extraction kit (Qiagen) and directly sequenced on an automated 3100 genetic analyzer (Applied Biosystems, Inc., Foster City, CA). Sequencing reactions were carried out using the inner PCR primers, and a Taq FS Dye terminator cycle sequencing kit (Applied Biosystems).

The phylogenetic analysis, based on the total LTR region, of the new sequences, including reference strains from different geographic regions and distinct ethnic groups corresponding to all subtypes and subgroups described were selected from the NCBI/Nucleotide Sequence Database (GenBank). As previously described [Alcantara et al., 2006], these sequences were aligned using the ClustalX software and manually edited using the GeneDoc program. The Tamura Nei (TrN + G) evolutionary model (which takes into account different substitution rates for transversions and transitions, as well as inter-site substitution rate heterogeneity, using a γ-distribution) was selected using the Modeltest software as the best model. The Neighbor-Joining (NJ) and Maximum-Likelihood (ML) trees were generated using the PAUP* 4.0b10 software. The NJ tree was constructed with an optimized nucleotide substitution rate matrix and a γ-shape parameter (alpha parameter = 0.8215). The reliability of the NJ trees was assessed by analyzing 1,000 bootstrap replicates. For ML tree, a heuristic search was performed with a subtree-pruning-regrafting branch swapping algorithm using the NJ tree as the starting material, including its optimized parameters. The likelihood ratio test (RT) method was used to calculate statistical support for the branches: $P < 0.001$ (highly significant **) and $P < 0.005$ (significant *). Bootstrap and ML values were added to the NJ tree which was drawn using TreeView 1.4 software.

**Accession Numbers**

The new LTR sequences included in this study are: EU108721-EU108724. The reference sequences included in this study are listed below: Mel5, L02534; Pyg19, L76310; MOMJ, Z31659; ITIS, Z32527; MWMG, Z31662; StDen, L76306; PH236, L76307; T49, L76305; GAB7, L76311; 12503 A, L76309; H24, L76308; GH78, D23693; HS35, D13784; NM1626, AF063821; OD, U12805; F132, U12806; Bo, U12804; B11 Peru, Y16481;
Bl3Peru, Y16483; ATM, J02030; MT4LB, Z31661; ATK1, J02029; Ni3Peru, Y16485; TBH5, L76027; Ni1Peru, Y16484; H5, M37299; Ni2Peru, Y16487; TBH4, L76028; TBH6, L76030; CH26, D23690; HTLV24, DQ005565; FNN148, DQ005548; FNN159, QD005554; 73RM, M81248; TBH2, L76025; HKN, X88874; TBH5, L76027; AMA, X88871; HTLV20, DQ005564; 73RM, M81248; HTLV01, DQ005556; JCP, X88876; Bl2Peru, Y16482; TBH1, L76026; TBH3, L76034; Afs911, L77212; AINU, D23694; ESTMD, AF494241; Cam, AF063819; CMC, X88872; Nar, AF063820; Me3Peru, Y16480; FNN155, DQ005551; MT2, L03562; CR1, K02722; Boi, L36905; TBH7, L76029; TSP1, M86840; HTLV30, DQ005567; HTLV33, DQ005568; HTLV18, DQ005568; HTLV25, DQ005566; HTLV15, DQ005562; MAQS, X88876; FCR, X88873; FNN100, DQ005547; FNN155, DQ005550; FNN158, DQ005553; MASU, X88877; IDUSSA, DQ005555; Qu3 Peru, Y16477; Qu1Peru, Y16475; Qu2 Peru, Y16476; Me2Peru, HTLV06, DQ005558; Y16479; Me1, Peru, Y16478; FNN156, DQ005552.

The mean inter-patient genetic distances were measured using the Kimura 2-\(\alpha\)-parameter model with a distance matrix implemented in the MEGA 3.0 package. The pair wise genetic distance using the HTLV-1 LTR sequences, was estimated within and between the two different groups: Cruz das Almas and Salvador.

Statistical Analysis

Data are shown as median (range). The Graph Pad, Prisma 4.3, Graph Pad Software (San Diego, CA) were used in the analysis.

RESULTS

The pregnant women came mainly from the urban region (75%) of Cruz das Almas. The median age was 24 years old, ranging from 14 to 32. The levels of schooling were low (median 7 years of school attendance) and the income was one minimum wage (which corresponds to US $200 per month, at the time of the study) (Table I). The median ages at first menstrual cycle and at the first sexual intercourse were 13 and 17 years old, respectively. The women reported having had a median number of one sexual partner both for the 6 months prior to the interview and since they had initiated their sexual life. The median number of offspring was one.

Breast-feeding was reported by 214 pregnant women, with a median period of 8 months (range 0–48 months). There was no report of blood transfusion or injecting drug users among the enrolled patients.

The prevalence of HTLV-1 infection was 0.98% (4/408). No HTLV-2 infection was observed. The median age of HTLV-1-infected patients was 25 years old and they were from the rural area. The socio-demographic and gynecological characteristics of HTLV-1-infected patients were similar to the non-infected women, mainly as regards the variables age, education, number of offspring and abortion and age of first period and first sexual intercourse. The number of sexual partners to date of HTLV-1-infected pregnant women (4) was higher than in the non-infected group (1) however, this was not statistically significant (\(P = 0.07\)). Breast feeding was reported by only one of the HTLV-1-infected women (12 months).

The phylogenetic analysis of the total LTR of subtype of the four viral isolates (Fig. 1) demonstrated that all sequences belonged to the transcontinental (A) subgroup of the Cosmopolitan (a) subtype (bootstrap value of 68% and ML analyses (\(P < 0.001\)). Furthermore, these isolates clustered within the larger Latin American cluster (Latin American beta cluster) (bootstrap value (59%), ML analyses (\(P < 0.001\)). The sequence corresponding to the isolate 423 clustered in a monophyletic group with a South African isolate inside the Latin American cluster Beta, however, this finding was supported only by ML analysis (\(P < 0.005\)).

The average inter-sequence diversity into LTR sequences from Cruz das Almas was of the 0.8% while among these sequences and the Salvador sequences it was 1%. In addition, as previously demonstrated, the average inter-sequence diversity within LTR sequences from Salvador was 1.1% [de Queiroz et al., 2007].

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (min–max)</th>
<th>Median (min–max)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pregnant women (all), n = 408</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>24 (14–32)</td>
<td>25 (22–28)</td>
</tr>
<tr>
<td>Education (years)</td>
<td>7 (0–16)</td>
<td>10 (5–15)</td>
</tr>
<tr>
<td>Income (minimum wage)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 (0–1)</td>
<td>1 (0–1)</td>
</tr>
<tr>
<td>Parity</td>
<td>1 (0–10)</td>
<td>1 (0–10)</td>
</tr>
<tr>
<td>Age at first menstrual cycle</td>
<td>13 (9–20)</td>
<td>12 (11–14)</td>
</tr>
<tr>
<td>Age at first sexual intercourse</td>
<td>17 (7–30)</td>
<td>16 (15–18)</td>
</tr>
<tr>
<td>Number of sexual partners (all life)</td>
<td>1 (1–10)</td>
<td>4 (1–5)</td>
</tr>
<tr>
<td>Number of sexual partners (last 6 months)</td>
<td>1 (0–3)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Offsprings</td>
<td>1 (0–10)</td>
<td>1 (0–9)</td>
</tr>
<tr>
<td>Number of abortion</td>
<td>0 (0–4)</td>
<td>0.5 (0–2)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Prevalence of HTLV-1-infection 0.98%.

<sup>b</sup>US $200.

Fig. 1. Rooted NJ tree of HTLV-1 strains based on a 696-bp fragment of the LTR region. The bootstrap values (>50% and using 1000 bootstrap samples) on the branches represent the percentage of trees for which the sequences located at the right end of the branch form a monophyletic group. The LTR sequence of the isolate Mel5 from Melanesia was used as outgroup. Newly sequenced LTR included in this analysis are in bold. The ** means that the maximum likelihood method was highly statistically significant (p < 0.001) and the * means that it was significant (p < 0.05).
DISCUSSION

We demonstrated for the first time that the prevalence of HTLV-1 infection in pregnant women of a mediumsized town in Bahia was similar to the prevalence in Salvador. These populations have the same ethnic origin and share similar social and demographic characteristics. Our data corroborate the fact that HTLV-1-infection is not restricted to Salvador, the city with the highest prevalence of this infection in Brazil [Galvao-Castro et al., 1997]. Indeed, HTLV-1 spread to surrounding towns as well as other towns far from Salvador [Moreira et al., 1993; Britto et al., 1998]. It has been demonstrated that the introduction of HTLV-1-infection in Salvador occurred during the post-Colombian period, as a result of the slave trade to Brazil between the 16th and early 19th centuries [Alcantara et al., 2006]. The population of Cruz das Almas, mostly black and mixed African and Portuguese descendents, has ethnic and socio-demographic characteristics similar to the population from Salvador [Azevedo et al., 1982].

In two previous studies carried out in Salvador, the prevalence of HTLV-1-infection of pregnant women was 0.88% in 1995 and 0.84% in 2001 [dos Santos et al., 1995; Bittencourt et al., 2001]. However, there was no HTLV-1-infection in the pregnant women from Cruz das Almas, while the prevalence in Salvador was only 0.03% [Bittencourt et al., 2001]. The major occurrence of HTLV-1 has been described mainly among injecting drug users and Amerindians, which could explain the absence of HTLV-1 infection in the studied group [Andrade et al., 1998; Shindo et al., 2002]. The socio-demographic characteristics of the HTLV-1-infected pregnant women from Cruz das Almas were similar to those reported previously in Salvador, Bahia [dos Santos et al., 1995; Bittencourt et al., 2001]. As regards the possible routes of HTLV-1 transmission, in our study there was no report of blood transfusion or sexually transmitted diseases. The four HTLV-1 pregnant women reported a higher number of sexual partners compared to the non-infected group. However, this difference was not statistically significant. Recently, in a population-based study, Dourado and cols suggested that sexual transmission is the main HTLV-1 transmission route in this city. In fact, the HTLV-1-infection was absent in individuals younger than 13 years old [Dourado et al., 2003].

The fact that sequences from Cruz das Almas have grouped only into the Latin American cluster beta suggests a unique introduction of HTLV-1 in this population. The observation that the sequences clustered homogeneously into this cluster suggests that this introduction was relatively recent, most likely occurring after the slave trade to Salvador, Bahia, Brazil, as previously reported [Van Dooreen et al., 1998; Alcantara et al., 2006].

As HTLV-1 is a retrovirus with highly conservative genetic characteristics, we found very little genetic diversity among the HTLV-1 isolates from Cruz das Almas and Salvador. This low level of diversity among HTLV-1 LTR sequences from Cruz das Almas could support the hypothesis of a more recent introduction of this virus. Furthermore, when we compared the genetic distance between each group (Cruz das Almas or Salvador sequences) with a South African isolate (TBH-5/L76027), we observed the same genetic distance (1.8%), supporting the suggestion of the genetic neighborhood among viral isolates circulating in Cruz das Almas and Salvador.

In conclusion, the prevalence of HTLV-1-infected pregnant women found in this study in a medium sized town in Bahia points to a need for HTLV-1 screening during prenatal follow-up to prevent vertical transmission. However, further studies are necessary to determine HTLV-1 prevalence in the general population and to measure the real extension of this infection.

ACKNOWLEDGMENTS

This article is part of Themistocles Magalhães’s M.Sc. thesis, Bahia School of Medicine, Postgraduate Course in Medicine and Human Health. We thank Augusto Mota for his critical review of the manuscript. We also thank Noilson Lazaro for his technical assistance.

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