Molecular detection of human T cell lymphotropic virus type 1 in pregnant women from Maranhão state, Brazil

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Molecular detection of human T cell lymphotropic virus type 1 in pregnant women from Maranhão state, Brazil

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Abstract

The human T cell lymphotropic virus (HTLV) has a worldwide distribution. HTLV is endemic in some states in the northeastern region of Brazil. This study investigated the prevalence of HTLV-1/2 in 713 pregnant women attended at the Central Laboratory of Public Health of Maranhão (LACEN-MA) between February 2015 and May 2017. Serological screening was performed by chemiluminescent microparticle immunoassay (CMIA), and reactive sera were subsequently confirmed by Western blot (WB) analysis. Five samples were determined to be HTLV-1/2-reactive by CMIA analysis, while in the WB analysis, three sera were positive for HTLV-1, and two were indeterminate. The polymerase chain reaction (PCR) analysis used to detect HTLV-1 proviral DNA showed a specific 336 base pair fragment for HTLV-1 in all CMIA-reactive serum samples. PCR products were purified and sequenced. We observed a 0.7% molecular prevalence of HTLV-1 infection. The average age of the HTLV-1-positive pregnant women was 25.6 ± 8.2 years, and the average age of the HTLV-1-negative pregnant women was 24.3 ± 6.2 (p = 0.60). We observed that there was no association of HTLV-1 infection with age, ethnicity, marital status, educational level, family income, age of first sexual intercourse, previous pregnancy, breastfeeding, intravenous drug use by partner, history of blood transfusions, or use of condoms. The prevalence of HTLV-1 observed in pregnant women demonstrated the need to implement public health policies for the screening of HTLV-1/2 in prenatal care and counseling to avoid breastfeeding by infected women; this approach could control vertical transmission and reduce the spread of this virus in the population.

Keywords HTLV-1/2 · Pregnant woman · Prevalence · Seroprevalence · PCR

Introduction

The human T cell lymphotropic virus (HTLV) belongs to the genus *Deltaretrovirus* type C, family Retroviridae and subfamily *Orthoretrovirinae* [1], including four known serotypes, ranging from HTLV-1 to HTLV-4 [2]. Types 1 and 2 are more prevalent and are responsible for human infections. Types 3 and 4 have not been associated with disease in humans, although they have been detected and isolated from Central African populations [3, 4].

Transmission of HTLV-1/2 can occur through infected T lymphocytes via blood transfusion and/or hemoderivatives, transplantation of organs and tissues, sharing of needles and syringes, sexual intercourse, and vertical transmission (mother to child) [5].

Breastfeeding is the most common mode of vertical transmission of HTLV-1. It has been reported that some factors may be associated with the vertical transfer route, including

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prolonged breastfeeding, high proviral load in breast milk and blood cells, and elevated serum antibody titers [6, 7].

HTLV-1 infections are lifelong, and most infected individuals remain asymptomatic. Approximately 10% of carriers can develop severe clinical complications, such as adult T cell leukemia/lymphoma (ATLL) or HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [2, 8]. Other clinical manifestations related to HTLV-1 infections are uveitis, arthropathies, infective dermatitis, and polymyositis [8], as well as symptoms resulting from coinfection with other infectious agents [9, 10].

Brazil is considered to be the country with the most significant number of individuals affected by HTLV-1 [11], having approximately 800,000 carriers [5], and this virus is present in the populations of all Brazilian regions with varying prevalence, with the highest prevalence being observed in the North and Northeast of Brazil [12].

The prevalence of HTLV-1/2 may vary according to geographical region, ethnicity, and individual risk behavior [13]. Most HTLV-1 endemic areas may be affected by age, socioeconomic, and genetic conditions, with a higher incidence being observed in women [5]. HTLV-1 is endemic among individuals of African origin, and HTLV-2 is endemic among intravenous drug users and native Amazonian populations [14].

Serological tests are the most commonly used method for the laboratory diagnosis of HTLV-1/2 infections, and they are related to the detection of specific components of the viral core and the viral envelope [15]. The two most commonly used techniques are the enzyme-linked immunosorbent assay (ELISA) and chemiluminescent microparticle immunoassay (CMIA), but these assays may present false-positive results and require further confirmation [16]. The confirmatory analysis commonly used is the Western blot (WB) analysis, which can differentiate HTLV-1 from HTLV-2. However, the WB analysis frequently yields indeterminate results and is costly [17].

Polymerase chain reaction (PCR) is an alternative approach for the diagnostic confirmation of HTLV-1/2 infections and has been used qualitatively and quantitatively [15, 16]. PCR is sensitive, specific, and less costly than WB analysis and may be used for the early diagnosis of HTLV infection, especially in cases where serological tests are unable to detect the virus [15].

To date, the Brazilian Ministry of Health has not established a standard protocol for the detection of HTLV-1/2 in pregnant women during prenatal counseling. Therefore, the absence of routine procedures for maternal screening may enable vertical transmission and increase the prevalence of infection with these viruses. The present study employed serological and molecular methods to investigate the prevalence of HTLV-1/2 in pregnant women attended at the Central Laboratory of Public Health of Maranhão (LACEN-MA, Brazil).

Materials and methods

Ethics statement

This study was approved by the Research Ethics Committee of the CEUMA University (CEP-UNICEUMA, n° 372.289/2013) and was conducted in keeping with the ethical precepts specified by the Resolution of the National Health Council of Brazil n° 466/2012. All women who agreed to participate in the study signed an informed consent form (ICF). The anonymity and confidentiality of the data were guaranteed.

Study population, blood sampling collection, and serological screening

A cross-sectional study was conducted to determine the prevalence of HTLV-1 in pregnant women. The sample consisted of 713 pregnant volunteers who were selected by free choice during the prenatal period at the LACEN-MA between February 2015 and May 2017. The pregnant women were aged 15 to 45 years, and all agreed to participate in this study.

Two tubes of venous blood from each pregnant woman were collected by technicians of LACEN-MA according to the standard protocol for the collection of clinical samples. To isolate peripheral blood mononuclear cells (PBMCs), 5 mL of whole blood was collected in a Vacutainer® tube containing ethylenediamine tetraacetic acid (EDTA), and to obtain blood serum, a tube with separator gel was used. Serum samples were screened for HTLV-1/2 by CMIA (Abbott Diagnostics, Germany), and the reactive samples were confirmed by WB analysis (HTLV BLOT 2.4, MP Diagnostics, Singapore). All tests were performed and interpreted according to the manufacturers' instructions.

Extraction of proviral DNA from PBMCs and PCR reaction conditions

The isolation of PBMCs was performed by Ficoll density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Life Sciences, USA) according to the manufacturer's instructions. Proviral DNA extraction from PBMCs was performed using the commercial QIAamp DNA blood kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was quantified using a NanoDrop 2000/2000c spectrophotometer (Wilmington, USA).

The PCR analyses were performed in a final volume of 25 μL containing 10 pmol of each specific primer HTLV-1 sense, 5′-CTCCTTCCCCACCCARCGAACYTC-3′, and HTLV-1 antisense, 5′-TAGGGAACATTGGTGAGGAAG-3′, which amplifies a fragment with 336 base pairs (bp). This set of primers was designed specifically for this study from the HTLV-1 *tax* sequence. Also, 12.5 μL of PCR Master Mix (Promega, USA) (Taq DNA polymerase, dNTPs, MgCl2,

PCR buffer [pH 8.5]), 1.5 μL of nuclease-free water, and 300 ng of DNA template were added to each reaction tube. The MyCycler thermal cycler system (Bio-Rad, California, USA) was used for PCR assays under the following conditions: 1 cycle at 95 °C for 5 min (initial denaturation) followed by 35 cycles of 95 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. Positive controls for HTLV-1 (DNA of infected MT2 cells) and negative controls (nuclease-free water) were also used.

The PCR products were loaded into a 2% agarose gel and subjected to electrophoresis at a voltage of 85 V for 1 h in 1X Tris Borate EDTA (TBE) buffer containing (45 mM Tris Borate, 1-mM EDTA) pH 8.0. After electrophoretic running, the gels were stained with ethidium bromide (0.5 μg/mL) for 15 min [18].

Sensitivity and specificity of the primers evaluated in this study

To analyze the limit of detection of primers designed for this study, our template was the DNA extracted from the PBMCs of the 708 serologically negative pregnant donors by CMIA for HTLV-1. Serial dilutions were performed from 300 ng of proviral DNA as follows: 1:2 (150); 1:4 (75); 1:8 (37.5); and 1:16 (18.75) ng. PCR assays performed in duplicate on alternate days verified the specificity of the primers.

Sequencing of the PCR products and computational analysis

To confirm the identity of the profile obtained by PCR for HTLV-1, the amplified products were purified using the Wizard® SV Gel and PCR Clean-Up System kits (Promega, USA) according to the manufacturer's instructions. The sequencing of each purified product was performed by the dideoxynucleotide chain termination method [19] in both directions on both strands (sense and antisense) using the ABI Prism BigDye Kit on the ABI 3130 Genetic Analyzer (Applied Biosystems). All sequencing was performed at Myleus Biotecnologia Ltda. (Belo Horizonte, Minas Gerais, Brazil).

The similarity between the sequences was verified by the BLASTn program of the BLAST 2.0 package (Basic Alignment Search Tool – http://www.ncbi.nlm.nih.gov/BLAST/) [20] with HTLV-1 sequences from GenBank selected for comparative analyses by the MEGA program version 6.0 [21].

Statistical analysis

The data were evaluated by the software IBM SPSS Statistics 20.0 for Windows [22]. Quantitative variables were described by means and standard deviation, and the qualitative variables were described by absolute and relative frequencies. Fisher's exact test was used to analyze the association of the classificatory variables concerning the PCR-determined HTLV-1 groups (positive and negative). The variable "age" was evaluated in two HTLV-1 groups via Student's *t* test. The level of significance for the analysis was set at 5%.

Results

Sociodemographic data of pregnant women evaluated

The analysis of the sociodemographic data of the 713 pregnant women showed that the average age was 24.3 ± 6.2 years (15–43), 63.8% were mulatto, 95.2% lived in the metropolitan area of São Luis (São Luis, São José de Ribamar, Paço do Lumiar and, Raposa), 71.8% were either married or in a stable relationship, 61.0% had an education equal to or higher than a high school education, 53.7% were housewives, and 72.0% had a family income of 1 to 3 minimum wages. The other data, such as age, race, residence, marital status, level of education, occupation, previous pregnancies, family income, and number of residents per household, are summarized in Table 1.

Serological and molecular assays

Of the 713 clinical samples screened by CMIA, 5 were reactive to HTLV-1/2. After confirmation by WB analysis, only 3 samples were indicative of an HTLV-1 infection, which is equivalent to a seroprevalence of 0.42%.

The results of the PCR analyses, for which the template was the proviral DNA obtained from the 713 PBMC samples, showed that all 5 samples determined to be CMIA-positive for HTLV-1 were also PCR-positive and corresponded to a molecular prevalence of infection of 0.7%. The electrophoretic profile of the 336 bp fragment amplified by PCR for each HTLV-1-positive sample is shown in Fig. 1.

The demographic and epidemiological data were compared between the PCR-determined HTLV-1-positive and HTLV-1-negative pregnant women groups. The average age of virus-positive women was 25.6 ± 8,2 years (16–38), and the average age of virus-negative women was 24.4 ± 6.2 years with no significant difference between the ages ($p = 0.60$). Furthermore, no significant differences were found in any of the other analyzed variables (age, ethnicity, marital status, educational level, family income, first sexual intercourse, prior pregnancy, breastfeeding, use of intravenous drugs by partner, or use of condoms) (Table 2).

In the PCR-determined HTLV-1-positive group, all women were residents of the metropolitan area of São Luis (4 of São Luís and 1 of Paço do Lumiar). Sociodemographic data showed that most of these women were mulattos (3/5–60.0%) were married or in a stable relationship (3/5–60.0%),

Table 1 Sociodemographic characteristics of 713 pregnant women analyzed in this study

Characteristics	Number	%
Age (years)		
15–30	595	83.5
31–45	118	16.5
Ethnicity	116	16.3
White	142	19.9
Black	455	63.8
Mulatto	679	95.2
Place of residence	34	4.8
Metropolitan area	191	26.8
Other municipalities	512	71.8
Marital status	10	1.4
Single	273	38.3
Married/stable union	440	61.7
Divorced/separated	140	19.6
Educational level	186	26.1
< Full high school	4	0.6
≥ Full high school	383	53.7
Occupation	406	56,9
Unemployed	307	43,1
Paid employment	185	25.9
Retired/pensioner	513	72.0
Works at home	15	2.1
Previous pregnancy	354	49.6
Yes	307	43.1
No	52	7.3
Family income (minimum wages)		
< 1		
1–3		
> 3		
Residents per household		
1–3 people		
4–6 people		
> 6 people		
Total	713	100.0

possessed formal education equal to or higher than high school (3/5–60.0%), and had a monthly family income of less than 1 minimum wage (3/5–60.0%). We also observed that the women started sexual activities at 16 years of age or younger (3/5–60.0%) and were in their first pregnancy (3/5–60.0%), and they all had a fixed sexual partner. One pregnant woman reported receiving a blood transfusion. Among the HTLV-1-positive pregnant women who had had previous pregnancies, two breastfed their children, and one breastfed for more than 6 months (Table 2).

Sensitivity and specificity tests

The results of the sensitivity test with primers evaluated in the present study showed a detection limit of 37.5 ng of HTLV-1 proviral DNA (data not shown). No viral genetic material was amplified in 708 samples that were determined to be serologically negative by CMIA.

Sequencing of the amplified HTLV-1 fragment

The sequencing of the purified PCR products used to confirm the results showed that all amplified samples were positive for HTLV-1. The analysis of the alignment of the sequences of the 336 bp fragments of the *tax* gene region assessed in the present study showed 97 to 98% similarity with different nucleotide sequences of HTLV-1 in GenBank databases. These sequences were deposited in GenBank under accession numbers MN309698, MN309699, MN309700, MN309701, and MN309702.

Discussion

The infections caused by HTLV-1/2 remain a largely overlooked public health problem in Brazil, as these infections are usually asymptomatic and may go unnoticed by the carriers of the virus and by some health professionals; thus, HTLV-1/2 may be silently disseminated in the population [23].

Most studies that have been carried out in Brazil employed blood donors as the target population, of which men represent the majority [24]. A seroepidemiological survey carried out in blood banks of the 27 Brazilian capitals showed a regional

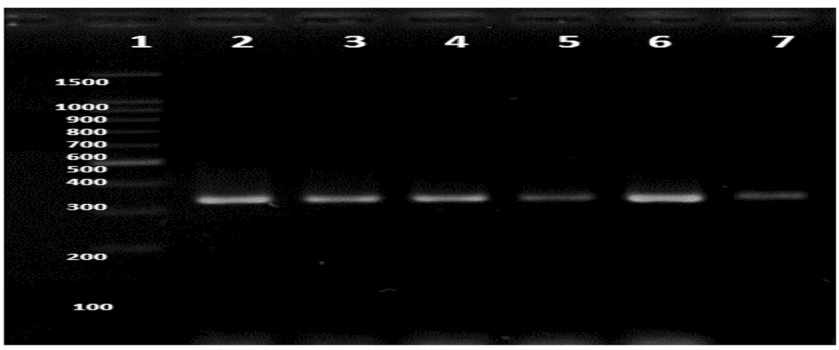

Fig. 1 HTLV-1 profiles obtained by PCR amplification of the proviral DNA extracted from peripheral blood mononuclear cells. Profiles of HTLV-1 in 2% agarose gel stained with ethidium bromide. Line 1, Molecular standard 100 bp DNA ladder (Promega, USA); line 2, HTLV-1 positive control (isolate profile in MT2);Lines 3–7, HTLV-1 profile of clinical samples

Table 2 Sociodemographic and epidemiological variables of HTLV-1 positive and negative pregnant women in serological and molecular methods

Variables	HTLV-1 positive $n = 5$/n (%)	HTLV-1 negative $n = 708$/n (%)	p value**
Age (years)			0.33
15–30	3 (60.0)	592 (83.6)	
31–45	2 (40.0)	116 (16.4)	
Ethnicity			0.23
White	0 (0.0)	116 (16.4)	
Black	2 (40.0)	140 (19.8)	
Mulatto	3 (60.0)	452 (63.8)	
Marital status			0.45
Single	2 (40.0)	189 (26.7)	
Married/stable union	3 (60.0)	509 (71.9)	
Divorced/separated	0 (0.0)	10 (1.4)	
Educational level			0.71
< Full high school	2 (40.0)	275 (38.8)	
≥ Full high school	3 (60.0)	433 (61.2)	
Family income (MW)			0.21
< 1	3 (60.0)	182 (25.7)	
1–3	1 (20.0)	512 (72.3)	
> 3	1 (20.0)	14 (2.0)	
First intercourse (years)			1.00
≤ 16	3 (60.0)	394 (55.6)	
> 16	2 (40.0)	314 (44.4)	
Previous pregnancy			0.47
Yes	2 (40.0)	404 (57.0)	
No	3 (60.0)	304 (43.0)	
Breastfeeding (months)*			0.75
≤ 6	1 (50.0)	134 (33.2)	
> 6	1 (50.0)	270 (66.8)	
Partner's intravenous drug use			0.76
Yes	0 (0.0)	26 (3.7)	
No	5 (100.0)	682 (96.3)	
Condom use			0.14
Never	2 (40.0)	198 (28.0)	
Occasionally	2 (40.0)	390 (55.1)	
Always	1 (20.0)	120 (16.9)	

MW minimum wages. * In the analysis of the variable breastfeeding, only volunteers with previous pregnancies were considered. ** Fischer's exact test. The significance level adopted was 5%

prevalence of HTLV-1/2 infection ranging from 0.04 to 1.0%; the prevalence was higher in the North and Northeast regions and lower in the South region. Among the capitals analyzed, São Luis (Maranhão) had the highest incidence of HTLV-1/2 (1.0%) followed by Salvador (Bahia) (0.94%), both of which are capitals in the Northeast region [12]. São Luis and Salvador have high numbers of HTLV carriers [12, 25]. The regional variation in the prevalence of HTLV-1/2 in Brazil can be explained by the different ethnic characteristics of its population [14, 26]. The North and Northeast regions have a higher percentage of African and Amerindian descendants than the South region [24].

The prevalence of HTLV-1/2 infection is higher among women [8, 27], with pregnant women being the group that best represents the general population, unlike blood donors [5]. Women have an important role in the transmission chain because they can transmit the virus to their children, mainly through breastfeeding [6]. However, studies in Brazil that assess the infection by HTLV-1/2 in pregnant women are still scarce and isolated [24], and notably few such studies have been conducted in Maranhão.

As with blood donors, there is a significant regional heterogeneity in the seroprevalence of infection by HTLV-1/2 in Brazilian pregnant women, with variations ranging from 0.0%

in the western Amazon [28] to approximately 1.0% in the state of Bahia [29, 30]. In the present study, a seroprevalence of HTLV-1 of 0.42% was found by WB analysis, indicating that the metropolitan area of São Luis is the region with the fourth largest seroprevalence among pregnant Brazilian women. This seroprevalence is almost twofold higher than that found among blood donors (0.15%) [31] and is highly similar to that found in a previous study in pregnant women in this region [26]. In Europe, a multinational study observed a seroprevalence of HTLV-1/2 infection six times higher in pregnant women than in blood donors, despite different prevalence rates among countries [32]. Variations of seroprevalence among pregnant women are found even in Africa, which is considered the area most endemic for HTLV-1 worldwide, as well as the place of origin of human retroviruses, with variations from ranging 0.2% in South Africa to 5.5% in Nigeria [5].

In recent years, molecular methods for the diagnosis of different types of infectious agents have revolutionized diagnostic medicine because of their high sensitivity, specificity, and rapidity [33]. This study is the first to use PCR as a molecular diagnostic screening method for HTLV-1 in pregnant Brazilian women, while previous studies have used this technique only as a confirmatory and discriminatory test [26, 30, 34–39]. In the present study, the PCR technique detected a prevalence of 0.7% (5/713) of HTLV-1 in the pregnant women who were analyzed. This prevalence is similar to that found in Bahia, which presents the highest incidence in pregnant women in the Brazilian literature [30, 34].

Only the presence of HTLV-1 was detected in the present study. Previous research performed in pregnant women [26] and blood donors in Maranhão [40] also detected the presence of HTLV-1 and HTLV-2, indicating that HTLV-2 is disseminated in the Maranhão population. It is of fundamental importance to know which type of HTLV is involved in the infection because HTLV-1 is the most pathogenic and is related to severe diseases, such as ATLL and HAM/TSP [2].

We observed that the average age of the 5 pregnant women who tested positive for HTLV-1 by CMIA and PCR was 25.6 years, which is in keeping with the findings reported by other authors [26, 34, 37, 41, 42]. However, a population-based study in Bahia's capital found a high number of seropositive women older than 50 years of age [25].

Factors related to poverty, such as lower income and lower education levels, may be associated with HTLV-1 infection, suggesting that these factors can influence the transmission and maintenance of the virus in the population [7, 27]. This influence was not observed in the present study, as no association was found between HTLV-1 infection and family income or schooling. These data are consistent with the findings of previous studies conducted in the states of Bahia [29, 30] and Rio de Janeiro [41]. However, Ribeiro et al. [43] and Nunes et al. [25] reported conflicting data.

We found that 60.0% of the HTLV-1-infected women reported beginning sexual intercourse at or below 16 years of age. The early initiation of sexual intercourse among young women may increase the risk of contracting sexually transmitted infections, which also becomes a risk factor for HTLV-1/2 infection [44].

Sexual and vertical transmissions are the main routes of HTLV-1 [45]. A population study carried out in Salvador, Bahia, concluded that sex might have been the main route of HTLV-1 infection in the evaluated population [25]. Thus, the increased risk of women acquiring HTLV infection may be related to the higher effectiveness of transmission from men to women and may be associated with a history of sexually transmitted diseases and mucosal lesions, which would increase the chances of transmission by cell-cell contact [46].

Other high-risk behaviors can increase the likelihood of the sexual transmission of HTLV, such as having multiple sexual partners and not using condoms during sex [47]. This study revealed that all of the infected women had a fixed sexual partner. Although 80.0% of these women affirmed that they occasionally or never used condoms during sex, these data agree with those reported for Rio de Janeiro, where most of the infected pregnant women did not use condoms [41].

HTLV-1 infection was higher among primigravidae (3/5–60.0%), in contrast to data reported by Oliveira and Avelino [35], Figueiró-Filho et al. [36], and Sequeira et al. [47], who observed a higher number of infected pregnant women with multiple pregnancies in Goiás, Mato Grosso, and Pará, respectively.

In the present study, 50% (1/2) of the infected women who had previous pregnancies breastfed their children for more than 6 months. Although no significant differences were observed between the HTLV-1-positive and HTLV-1-negative groups, prolonged breastfeeding for more than 6 months is a risk factor for HTLV-1/2 infection [6, 45] because vertical transmission can occur in approximately 20% of children breastfed by infected women [6]. Additionally, vertical transmission is also a risk factor for the development of HTLV-1-related diseases [27]. In Japan, the vertical transmission of this virus was significantly reduced by prenatal screening for HTLV-1/2 and by counseling seropositive mothers to avoid breastfeeding [6].

Statistical analysis showed no *significant difference between* the PCR-determined HTLV-1-positive and HTLV-1-negative groups after analysis of the sociodemographic variables and epidemiological data analyzed. These results are in keeping with studies carried out in Bahia [29, 30] and Rio de Janeiro [41]. The high prevalence of HTLV-1 observed in this study, as well as the similarity of sociodemographic and epidemiological profiles between the groups, demonstrates the need for HTLV-1/2 screening in

routine prenatal care for pregnant women, as suggested by other researchers [26, 29, 30, 37, 41].

In conclusion, the results obtained through both serological and molecular screening showed a high prevalence of HTLV-1 infection in pregnant women residing in the metropolitan area of São Luis, MA. These data highlight the extent of the problem and demonstrate that this metropolitan area is among the Brazilian capitals with the highest HTLV-1 prevalence in pregnant women. The high prevalence of HTLV-1 infection in the studied pregnant women demonstrates a need for the implementation of public health measures for the control and prevention of this infection in our population. As there is no cure, vaccine, or specific treatment for HTLV infection and its associated diseases to date, prevention is the best option to combat the spread of this virus. However, to this end, it is necessary to adopt effective public policies, including educating the population and health professionals. In addition, it is essential to incorporate HTLV-1/2 screening during the prenatal care of pregnant women, to provide free milk formula to the children of infected mothers and to make notification of this infection compulsory.

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Authors' contributions Maria de Fátima Castro Mendes, Bruna de Oliveira de Melo, and Hermerson Sousa Maia extracted the proviral DNA from the peripheral blood mononuclear cells (PBMCs) and performed molecular biology assays. Silvio Gomes Monteiro and Thiago Azevedo Feitosa Ferro performed the statistical analyses of the results. Conceição de Maria Fernandes da Silva Pinto selected the records of the patients of HTLV in the LACEN-MA database. José de Ribamar Oliveira Lima performed the serological tests chemiluminescent microparticle immunoassay (CMIA) and Western blotting (WB) analysis. Maria Rosa Quaresma Bomfim and Edel Figueiredo Barbosa Stancioli designed and conducted the study, analyzed the data, and composed the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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