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# Use of a Generic Polymerase Chain Reaction Assay Detecting Human T-Lymphotropic Virus (HTLV) Types I, II and Divergent Simian Strains in the Evaluation of Individuals With Indeterminate HTLV Serology

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In countries with a low prevalence of human T-lymphotropic virus (HTLV) infection, indeterminate HTLV serologies are a major problem in blood bank screening because of the uncertainties about infection in these cases. The recent discovery of two new types of simian T-lymphotropic viruses (STLV), which give an HTLV-indeterminate serology, raises the question whether indeterminate serologies in humans may be linked to new types of HTLV. Starting from a Tax sequence alignment of all available primate T-cell lymphotropic virus strains (PTLV), including the two new types STLV-PH969 and STLV-PP1664, we developed generic and type-specific nested polymerase chain reactions (PCRs). The generic PCR proved to be highly sensitive and cross-reactive for all four types of PTLV, while the discriminatory PCRs had a high sensitivity and a specificity of 100%. There was no cross-reactivity with human immunodeficiency virus (HIV), ensuring correct interpretation of results from coinfected patients. Among the 77 serologically indeterminate samples tested, 6 were found to be HTLV-I PCR positive and 1 was HTLV-II PCR positive. Sequencing of one of the HTLV-I PCR positives excluded PCR contamination, and revealed a divergent type of HTLV-I. The majority of the seroindeterminate samples (91%) were however HTLV-PCR negative, and no new types of HTLV were found. This new assay can identify otherwise undetected HTLV-I or HTLV-II infections and is a useful tool of screening for new types of HTLV among seroindeterminate samples. ***J. Med. Virol. 52:1–7, 1997.***


**KEY WORDS:** nested PCR; diagnostics; PTLV

## INTRODUCTION

The human T-cell lymphotropic virus type I (HTLV-I) was first isolated in 1980 from a patient with cutaneous T-cell lymphoma [Poiesz et al., 1980]. In 1982, HTLV-II was identified from a patient with hairy cell leukemia [Kalyanaraman et al., 1982]. HTLV-I is etiologically linked to adult T-cell leukemia [ATL; Miyoshi et al., 1981; Seiki et al., 1983; Yoshida et al., 1982], to tropical spastic paraparesis [TSP; Gessain et al., 1985; Hirose et al., 1986], HTLV-associated myelopathy [HAM; Osame et al., 1987; Roman and Osame, 1988], and other inflammatory conditions, but the majority of HTLV-I infected people remain asymptomatic throughout life [Kondo et al., 1987; Kaplan et al., 1990; Goubau et al., 1990]. Although initially isolated from a leukemic patient, no clear etiological link has been established between HTLV-II and any type of leukemia or lymphoma [Hjelle et al., 1991]. However, a number of TSP/HAM-like cases

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have been reported in HTLV-II infected patients [Jacobson et al., 1993; Hjelle et al., 1992; Murphy et al., 1993; Harrington et al., 1993]. Serological cross-reactivity with HTLV was found in some old world monkeys and apes, leading to the isolation of STLV-I [Komura et al., 1984; Watanabe et al., 1985], a simian T-lymphotropic virus that is associated with lymphoma and lymphoproliferative diseases [Homma et al., 1984]. An STLV-II was reported in a new world spider monkey [Chen et al., 1994], but this could not be confirmed by other groups. HTLV (and also STLV) is transmitted between sexually active partners, from mother to child mainly through breastfeeding, and by blood-to-blood contact such as transfusion or needle sharing.

HTLV-I is endemic in Central and West Africa, the Caribbean, and parts of South America, Japan, and Melanesia/Australia. Molecular phylogenetic analysis has shown that HTLV-I most likely arose as a zoönotic infection through multiple species crossings from nonhuman primates to humans, and that species crossing of STLV-I also occurs among nonhuman primates [Koralnik et al., 1994; Liu et al., 1996; Goubau et al., 1996a; Ibrahim et al., 1995; Saksena et al., 1994; Crandall, 1996]. Thus, HTLV-I and STLV-I do not belong to independent phylogenetic lineages and should be called primate T-cell lymphotropic virus type I (PTLV-I). Recently two new types of STLV have been found in Africa. Two wild-caught Hamadryas baboons from Erythrea had indeterminate HTLV serology. From one of these, a third type of PTLV, PTLV-L, distantly related to both PTLV-I and PTLV-II, was isolated and characterized [Goubau et al., 1994; Van Brussel et al., 1996]. Another new STLV, PTLV-PP, was isolated from captive and wild-caught [Liu et al., 1994b; Giri et al., 1994] bonobos (pygmy chimpanzees), only found in the wild in Zaire. Infected bonobos had a HTLV-I-like or indeterminate serology. Although distinct, this virus is more closely related to HTLV-II than to HTLV-I [Vandamme et al., 1996].

The standard approach to HTLV diagnosis is to screen sera by particle agglutination or enzyme-linked immunoassays and to confirm infection by Western blotting [Taylor et al., 1996]. HTLV-I-positive sera react in Western blot minimally with the native Gag proteins p19 and p24, with one Env antigen (recombinant rgp21, included in many commercial Western blot kits, or native gp46) and should be typable either through their reactivity with HTLV-I type-specific recombinant or synthetic peptides or through differential titration in immunofluorescence. HTLV-II sera usually react weakly in HTLV-I Western blots, mainly with the p24 Gag protein and the rgp21, and are typable in type-specific assays. The serological reactivity of the two new STLV-types would be classified as HTLV indeterminate. During screening for HTLV antibodies, indeterminate Western blot results are often found in screening positive sera [Biggar et al., 1993; Garin et al., 1994; Goubau et al., 1990, 1993]. Because of the uncertainties about infection in these people, mostly healthy blood donors, it is unclear whether they should be notified and how they could be counseled. Using polymerase chain reaction (PCR) on lymphocytes of people with persistent indeterminate HTLV serological results, it has been shown that the vast majority are not infected with HTLV-I or -II [Cavalcanti et al., 1993; Delaporte et al., 1991; Vallejo and Garcia-Saiz, 1995; Maloney et al., 1992]. It may be worthwhile to exclude also the possible presence of divergent viruses in these cases.

We have shown that the currently used HTLV primers fail to amplify efficiently the two new types of STLV. We have therefore developed a new sensitive generic PCR that is able to detect the two existing HTLV/STLV types and the two new STLV types. This new generic PCR may enable us to detect other divergent HTLV-like viruses, if present, which would have been missed with conventional serology and PCR. In addition, four type-specific nested PCRs were developed, which discriminate among the four types of HTLV/STLV after a generic amplification in a first round.

## MATERIALS AND METHODS

### Origin of the Tested Samples

The following patient samples were used to evaluate the screening assay: 10 HTLV-I-seropositive and 7 HTLV-seronegative samples from the Equateur province of Zaire [Liu et al., 1994a; Vandamme et al., 1994]; 2 HTLV-II-seropositive, 11 HTLV-seronegative, and 10 HTLV-seroindeterminate samples from Efe (Mbuti) pygmies in Zaire [Goubau et al., 1996b]; 5 HTLV-I-seropositive, 1 HTLV-II-seropositive, and 12 HTLV-seroindeterminate samples from Gabon [Delaporte et al., 1991]; 5 HTLV-I-seropositive and 17 HTLV-seronegative samples from the state of Ceara in northeastern Brazil, 12 of them suffering from a HTLV negative TSP [de Castro Costa et al., 1995]; 54 HTLV-seroindeterminate samples from Bavarian blood donors (Germany); 1 HTLV-II-seropositive and 2 HTLV-seroindeterminate samples from the United Kingdom; 1 HTLV-II-seropositive sample from Italy. Depending on their origin, the samples had been tested with different screening assays and Western blots. The samples from Gabon were tested using a first-generation Western blot which does not include rgp21 or type-specific peptides.

### Sample Preparation

For provirus detection in patient samples, $10^6$ peripheral blood mononuclear cells were pelleted and lysed in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, Perkin-Elmer) containing 2 mM $MgCl_2$, 0.5% Tween-20, 0.5% NP40, and 100 μg/ml proteinase K (Boehringer Mannheim, stabilized proteinase K solution) for 1 hr at 56°C. The DNA was extracted using phenol/chloroform (Life Technologies), precipitated with ethanol, and dissolved in Milli-Q water (Millipore system). All samples were treated in this way except for the German samples which were sent to us as extracted DNA. For extracting the DNA of the control cell lines and the English samples, the Qiagen blood kit was used (Westburg). DNA

TABLE I. Primers in the *tax* Gene Used for the Generic and the Discriminatory HTLV PCRs

| PCR | Primers | Sequence 5′-3′ | Orientation |
|---|---|---|---|
| Generic outer | AV45 | GGACGCGTT(A/G)TC(A/G)GCTC | Sense |
| | AV46 | (G/T)GG(A/G)GAIAG(C/T)TGGTA(G/T)AGGTA | Antisense |
| Generic inner | AV42 | CTCCCCTCCTTCCCCAC | Sense |
| | AV43 | CCA(G/C)(A/G)(G/T)GGTGTAIAIGTTTTGG | Antisense |
| HTLV-I inner | AV49 | CCCTCCTTCCTCCAGGCCAT | Sense |
| | AV80 | GGTCTGGAAAAGACAGGGTTG | Antisense |
| HTLV-II inner | AV50 | TCAATCAATGCGAAAGCACACC | Sense |
| | AV81 | TAGGTATAGGCATACTACGGTT | Antisense |
| STLV-PH969 inner | AV51 | ACAATTGCCTCGAGCTCACCC | Sense |
| | AV82 | GAGGCACACGACGGAGCT | Antisense |
| STLV-PP1664 inner | AV52 | ACGGGTGCCTATACCCAACTC | Sense |
| | AV83 | GGTACAAGCAAACTACGGTTC | Antisense |

from the pygmy samples was extracted as described in Goubau et al. [1996b].

### PCR

DNA from $10^5$ cells was used for amplification in a 50 μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM nucleotide triphosphates, 0.2 μM outer or 0.5 μM inner primers and 0.025 U/μl AmpliTaq (Perkin-Elmer), with 1.5 mM $MgCl_2$ for the generic primers and 2 mM for the discriminatory inner primers (see Table I). Cycling conditions on a GeneAmp PCR System 9600 (Perkin-Elmer) were 30 sec 94°C, 15 sec 50°C, 45 sec 72°C for the outer generic PCR; on a Triothermobloc (Biometra) 30 sec 95°C, 15 sec 52°C, 1 min 72°C for the inner generic PCR; and 30 sec 95°C, 15 sec 50°C, 1 min 72°C for the inner discriminatory PCR. The outer fragment was amplified for 45 cycles, 2 μl was transferred to the inner PCR, and amplified for 25 cycles. A globin PCR was performed using the primer pair PC03/KM38 to assure the quality of the DNA [Vandamme et al., 1995]. Amplification products were separated on a 6% polyacrylamide gel and visualized by ethidium bromide staining. The images were processed on a videoimager (ImageMaster VDS, Pharmacia Biotech, Roosendaal, The Netherlands). Positive controls for the PCR were MT-2 cells harboring HTLV-I MT2 (kindly provided by Luc Montagnier, Institut Pasteur, Paris, France), SI-5 cells harboring HTLV-I Mel5 (kindly provided by Richard Yanagihara, NIH, Bethesda, MD), 729 cells harboring HTLV-II Mo (kindly provided by Helen Lee, Abbott Laboratories, North Chicago, IL), clone 19 cells harboring HTLV-II (kindly provided by Dana Gallo, Viral & Rickettsiai Disease Laboratory, Berkeley, CA), Gu cells harboring HTLV-II Gu [Salemi et al., 1996], PH969 cells harboring STLV-PH969 [Goubau et al., 1994], PP1664 cells harboring STLV-PP1664 [Liu et al., 1994b]. Negative controls for the PCR were Hut-78 cells (kindly provided by Institut Pasteur, Brussels, Belgium) and ACH-2 cells (kindly provided through the AIDS reagent project of the Medical Research Council, UK).

### Primer Development and Synthesis

The PCR primers were designed using the *tax* gene alignment of all available HTLV and STLV strains. The primer sequences for the generic nested PCR and the four discriminatory inner PCRs are given in Table I. Primers were developed and analyzed using the Oligo software (Medprobe, Oslo, Norway). Synthesis was done by Pharmacia Biotech and Perkin-Elmer/Applied Biosystems.

## RESULTS

### Detection Limit and Specificity of the New PCR Assay

Tenfold dilution series of HTLV-I MT2 [Miyoshi et al., 1981], HTLV-I Mel5 [Gessain et al., 1993], HTLV-II Mo [Seiki et al., 1983], HTLV-II clone 19 [Gallo et al., 1991], HTLV-II Gu [Salemi et al., 1996], STLV-PH969 [Goubau et al., 1994], and STLV-PP1664 cells [Liu et al., 1994b] in HTLV/STLV-negative Hut78 cells were used to determine the sensitivity and specificity of the PCR assay, and were used as positive controls during the screening of patient samples. Negative controls were Hut78 cells and the human immunodeficiency virus (HIV)-1-positive ACH2 cells. The generic primers AV45-46 were used in the outer PCR of the generic and the four-discriminatory PCRs. In this way, the same outer PCR product can be used for the five different inner PCRs, ensuring parsimonious use of precious materials. These five nested protocols all reached a detecion limit of one (HTLV-I MT-2, HTLV-II Mo and Gu, STLV-PH969, and STLV-PP1664) to five (HTLV-I Mel5, HTLV-II clone 19) infected cells with a DNA input derived from $10^5$ cells (Fig. 1). All HTLV/STLV types were detected efficiently by the nested generic PCR. The four discriminatory PCRs were very specific for each of the four HTLV/STLV types; no cross-reactivity was seen at any concentration of proviral DNA (Fig. 1).

### Performance of the New PCR Assay on Patient Samples

A total of 138 patient samples were tested (see Materials and Methods) with the generic nested PCR. Negative samples were tested with the PC03/KM38 globin primers [Vandamme et al., 1995; Vandamme, 1994] to ensure the quality of the extracted DNA. The two samples with a negative globin PCR were excluded from the study. As summarized in Table II, 24 of the 25 seropositives were detected by the generic PCR, as well

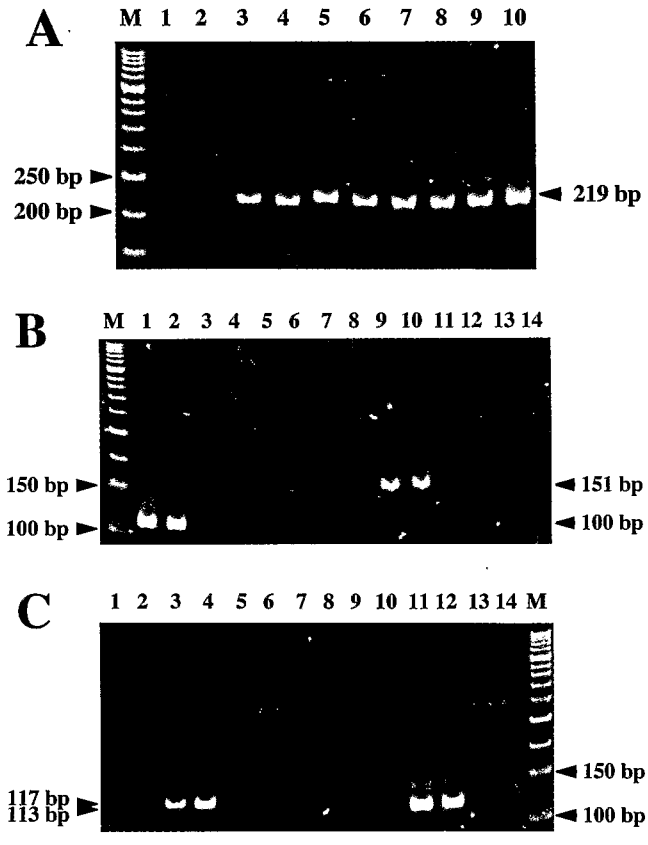


Fig. 1. Specificity and detection limit of the HTLV generic and discriminatory PCRs. A dilution series of infected cells in Hut-78 cells was used to determine the detection limit of the PCR on genomic DNA using the specified primers (see Materials and Methods and Table I). All amplifications are performed on DNA from $10^5$ cells. The number of HTLV infected cells in the dilutions is mentioned, otherwise undiluted samples are used. The HTLV/STLV-specific band is indicated with an arrow. M: Marker XIII from Boehringer Mannheim. **A:** Generic nested PCR. Lanes **1:** HUT78, **2:** ACH-2; **3:** one PP1664 cell; **4:** five PP1664 cells; **5:** one PH969 cell; **6:** five PH969 cells; **7:** five clone 19 cells; **8:** 10 clone 19 cells; **9:** one MT-2 cell; **10:** five MT-2 cells: **B:** Lanes 1 to 7, HTLV-I-specific inner primers; lanes 8 to 14, HTLV-II-specific inner primers. Lanes **1:** five MT-2 cells; **2:** one MT-2 cell; **3:** clone 19; **4, 11:** PH969; **5, 12:** PP1664; **6, 13:** ACH-2; **7, 14:** HUT78; **8:** MT-2; **9:** 10 clone 19 cells; **10:** five clone 19 cells. **C:** Lanes 1 to 7, STLV-PP1664-specific inner primers; lanes 8 to 14, STLV-PH969-specific inner primers. Lanes **1, 8:** HUT78; **2, 9:** ACH-2; **3:** one PP1664 cell; **4:** five PP1664 cells; **5:** PH969; **6, 13:** clone 19, **7, 14:** MT-2; **10:** 10 clone 19 cells; **11:** one PH969 cell; **12:** five PH969 cells. MT-2 cells harbor HTLV-I, clone 19 cells harbor HTLV-II, PH969 cells harbor STLV-PH969, PP1664 cells harbor STLV-PP1664, HUT-78 cells are HTLV negative, ACH-2 cells are HTLV negative but HIV-1 positive.

as 7 of the 77 seroindeterminates. All seven were HTLV-I or HTLV-II positive by PCR. All 20 HTLV-I seropositives were positive in the new HTLV-I-specific PCR. Four of the five HTLV-II seropositives were detected in the HTLV-II-specific PCR. The one sample that was missed was an HTLV-II pygmy sample [Goubau et al., 1996b] that also tested negative with other HTLV generic and HTLV-II-specific primers. The six HTLV-seroindeterminate but HTLV-I-PCR-positive samples were one pygmy sample from Zaire and five samples from Gabon. The one HTLV-seroindeterminate but HTLV-II-PCR-positive sample was from an English intravenous drug user with a Caribbean sexual partner. All 34 seronegative samples were HTLV-PCR negative. No human samples were found positive for either of the two new STLV types, STLV-PH969 or STLV-PP1664. The performance of the generic and the specific PCRs towards the two new viruses was confirmed by analyzing the original baboon and bonobo blood samples from which the two new viruses had been isolated. Both blood samples were positive with the generic PCR. The four discriminatory PCRs performed as expected: the baboon blood sample (infected with STLV-PH969) was only positive with the PH969-specific primers and the bonobo blood sample (infected with STLV-PP1664) was only positive with the PP1664-specific primers.

### Performance of Previously Reported PCR Primers

All patient samples were also tested with the generic nested PCR TR101-104 [Maloney et al., 1992] and some were tested in addition with the generic single SK110-111 PCR [Kwok et al., 1988] linked to hybridization with the SK112 HTLV-I and SK188 HTLV-II-specific probes [see Liu et al., 1994a]. These tests gave results concordant with the new generic and discriminatory primers, including the seropositive PCR-negative and the seroindeterminate PCR-positive samples. The detection limit of the TR101-104 primers for the PH969 and PP1664 cell line were 1,000 and 1 positive cell in $10^5$ negative cells, respectively. The lymphocytes from the original baboon harboring STLV-PH969 were negative with these primers and those from the original bonobo harboring STLV-PP1664 were positive. The generic HTLV-I/HTLV-II/BLV PCR [Dube et al., 1994], using the M110-111 primers with the M115 generic probe, was able to detect HTLV-I (MT-2 cells) and HTLV-II (clone 19 cells), but was negative for STLV-PH969 and STLV-PP1664.

## DISCUSSION

Indeterminate HTLV serologies are observed frequently, especially in blood samples from Africa. Epidemiological surveys suggest that the majority of the HTLV-indeterminate results are due to nonspecific reactivities of these sera with HTLV antigens [Mauclère et al., 1995]. To establish proper epidemiological data, there is a need to verify indeterminate serologies using PCR techniques. PCR results are especially needed for blood donors with indeterminate serologies: are they infected with HTLV-I or -II, or maybe with a divergent or a new HTLV type? In most cases these blood donors are not informed, but their blood is discarded routinely.

The recent discovery of two new types of PTLV in baboons and bonobos [Goubau et al., 1994; Liu et al., 1994b; Van Brussel et al., 1996; Vandamme et al., 1996; Giri et al., 1994], presenting an indeterminate serology, could perhaps help to solve some uncertainties of indeterminate serologies found in humans. Generic HTLV PCR primers, developed using the genomic sequence of HTLV-I and HTLV-II, perform badly on these new STLV strains. We have therefore developed a sensitive set of PCR primers that should allow the detection of new

TABLE II. Performance of the PCR Screening Test for the Detection of HTLV-Related Sequences

| Serostatus | Number of samples | Number of generic nested HTLV-PCR positives | Number of discriminatory inner PCR positives | | | |
|---|---|---|---|---|---|---|
| | | | HTLV-I | HTLV-II | STLV-PH969 | STLV-PP1664 |
| HTLV-I positive | 20 | 20 | 20 | 0 | 0 | 0 |
| HTLV-II positive | 5 | 4 | 0 | 4 | 0 | 0 |
| HTLV indeterminate | 77 | 7 | 6 | 1 | 0 | 0 |
| HTLV negative | 34 | 0 | 0 | 0 | 0 | 0 |

HTLV-like viruses, if present in humans. Using an alignment of all available HTLV-I STLV-I, and HTLV-II strains, and the new STLV-PH969 and STLV-PP1664 *tax/rex* gene regions, a generic nested PCR primer set was developed that allowed the detection of one to five infected cells in $10^5$ negative cells. This HTLV-generic PCR was highly sensitive (96% of seropositives) when tested on 138 lymphocyte samples collected in Europe, Africa, and South America. Neither the seronegatives nor the HIV-1 cell line ACH-2 were HTLV PCR positive, showing a good specificity of the new generic primers. Only one HTLV-II seropositive was missed. This sample was isolated from an Efe pygmy, and was also negative with classical generic and HTLV-II-specific primers. This is probably due to a low proviral load, combined with a lower DNA input in the PCR for the pygmy samples. The new generic PCR was able to detect seven HTLV-positive samples among the seroindeterminates. Four sets of specific inner primers were developed to discriminate the four types of PTLV. All four specific nested PCRs, using the same generic outer PCR, could detect one to five infected cells in $10^5$ negative cells. There was no cross-reactivity among the four types, even at a high input of one of the other three types of PTLV DNA. The sensitivity on the patient samples was 100% among HTLV-I seropositives and 80% among the HTLV-II seropositives, additionally both blood samples from the original monkeys harboring the two new STLV viruses were correctly identified. The same HTLV-II pygmy sample that was negative in the generic PCR was also negative in the new HTLV-II-specific PCR. Six of the seven HTLV-seroindeterminate samples that were positive with the generic HTLV PCR were typed as HTLV-I, the other was an HTLV-II strain. Five of the seroindeterminate samples that were identified as HTLV-I by PCR had been tested with a first-generation Western blot which did not include recombinant proteins, thereby lacking specificity. The sixth sample came from a pygmy and the results of the present PCR typing have been confirmed by sequencing of a part of the *tax/rex* gene [Goubau et al., 1996b]. The PCR typing correlated completely with the serotyping in our study population. None of our samples reacted with the PTLV-L or PTLV-PP, specific primers.

In conclusion, using *tax/rex* sequences of HTLV-I, STLV-I, and HTLV-II, and the new types of STLV, new highly sensitive generic and discriminatory PCR primers were developed that should allow the identification of HTLV-related sequences, if present, in HTLV-indeterminate blood samples. The majority of these indeterminate samples tested negative for HTLV DNA, revealing the presence of a few HTLV-I strains in Africa and one HTLV-II in England in patients with indeterminate serologies. These findings show that some HTLV-I and HTLV-II remain undetected with the currently used Western blots. Modifications to enhance their sensitivity are needed to detect all HTLV infections. Although some of these infections might be caused by divergent strains, all HTLV strains presently found in HTLV-seroindeterminate samples could be typed as HTLV-I or HTLV-II and no new HTLV-related sequences were found.

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