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Isolation and Confirmation of Human T-Cell Leukemia Virus Type 2 From Peripheral Blood Mononuclear Cells

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Summary

Human T-cell leukemia virus type 2 (HTLV-2) was first isolated from leukemia patients, but has been found to be endemic among asymptomatic groups worldwide, including certain American Indian tribes (1). The virus infection is associated with a low incidence of disease among infected subjects, but has been found in patients with neurologic disorders and contributes to bacterial sepsis in AIDS patients (2,3). Polymerase chain reaction (PCR) and virus isolation techniques revealed that a high percentage of HTLV seroreactivity among intravenous drug users and blood donors in the United States is caused by HTLV-2 (4). Among serologic methods, enzyme-linked immunosorbent assays (ELISA) using whole virus preparations or in combination with recombinant and synthetic peptides are used as a primary screen for the infection. Antigen-capture systems have increased the sensitivity and accuracy in verification of HTLV-2 culture systems. The verification of HTLV-2 infection and detection of new strains of related viruses has been enhanced by employing virus-isolation methods using primary lymphocytes. Lymphocyte culture methods have also been used to test transformation properties of the virus and create stably expressing cell lines. This chapter briefly summarizes the biology of HTLV-2 infection and disease and details methods to isolate and verify the virus in lymphocyte cultures.

Keywords

Human T-cell leukemia virus type 2; HTLV-2; human T-cell leukemia virus type 1; HTLV-1; lymphoma; retrovirus; cell culture; antigen; replication; isolation; detection

1. Introduction

The first human retrovirus described, human T-cell leukemia virus type 1 (HTLV-1), is considered the cause of adult T-cell leukemia/lymphoma (ATLL) and is associated with chronic inflammatory diseases such as HTLV-1 myelopathy/tropical spastic paraparesis (HAM/TSP) (5). Human T-cell leukemia virus type 2 (HTLV-2) was later isolated from a patient with hairy cell leukemia (6) and has subsequently been determined to be endemic among persons sharing many of the same risk factors as those with HTLV-1 infection (7–12). The virus infection is also endemic among certain American Indian groups in North and South America and among at-risk groups worldwide (3,7). Detailed studies of the pathogenesis of HTLV-2 infection have not been reported, in part, due to the low incidence of disease among infected subjects. However, the infection is associated with neurologic disorders and contributes to bacterial sepsis in AIDS patients. With the development of polymerase chain reaction (PCR) techniques it was discovered that a high percentage of

⁷Several important caveats must be kept in mind in the application of PCR to detect HTLV-2 infection. The procedure detects the DNA of HTLV-2 and does not provide information concerning the replication capacity or infectious nature of the virus infection. Because of potential cross-contamination problems and detection of endogenous retrovirus sequences, the criteria of a positive PCR test should ideally include detection of HTLV-2 using primers from at least two separate portions of the genome.

HTLV seroreactivity among intravenous (IV) drug users and blood donors in the United States is caused by HTLV-2 (14–16). However, gaps remain in the literature regarding disease associations, transmission routes, and epidemiologic features of HTLV-2 infections.

Serologic surveys to detect antibodies against HTLV-1 and HTLV-2 remain a primary tool for virologists and epidemiologists interested in population-based studies of HTLV infection. The most widely used system of antibody detection is the enzyme-linked immunosorbent assay (ELISA), using whole-virus preparations for antigen preparations or in combination with recombinant and synthetic peptide-based immunoassay systems. The development of ELISA-based antigen-capture systems to detect small quantities of HTLV-1 and HTLV-2 core antigens has increased the sensitivity and accuracy of HTLV culture systems and largely replaced measurement of reverse transcriptase activity to verify cultures that express these highly cell-associated viruses. The use of PCR-based methods has dramatically enhanced the ability to detect HTLV-2 proviral DNA and has allowed significant improvements in virus detection compared to the labor-intensive procedures of virus isolation. The verification of HTLV-2 infection and detection of new strains of related viruses, however, has also been through virus isolation (11,17,18). Long-term culture of lymphocytes has been employed to test transformation properties and antigen expression of HTLV-2. This is owing, in part, to the fact that leukocytes derived directly from infected subjects are often negative when tested by conventional methods (e.g., immunofluorescence). This chapter briefly summarizes the biology and clinical features of HTLV-2 infection and disease. Virus isolation methods to detect HTLV-2 infection, to isolate new strains of the virus, and for basic comparative studies have expanded the knowledge base of this important human retrovirus.

The HTLV family shares structural features in common with all retroviruses, including the viral genes for group-specific antigens (*gag*), reverse transcriptase (*pol*), and envelope (*env*) proteins. However, HTLV-1 and HTLV-2 contain unique pX region genes, which code for important regulatory (i.e., Tax and Rex) and accessory proteins (e.g., p28 of pX ORF II of HTLV-2) at the 3' end of the provirus (Table 1). HTLV-2 shares 60% amino acid identity with HTLV-1 and shares genome structures and in vitro biological properties. As a result, HTLV-2 remains an important comparative model for the study of the deltaretroviruses. Examination of the genome sequence of the deltaretrovirus family of retroviruses that includes HTLV-1, HTLV-2, simian T-lymphotropic virus (STLV), and bovine leukemia virus (BLV) reveals conserved organization of genes within the 3' end of these viruses. Like HTLV-1, proteins encoded in the pX region of HTLV-2 are likely to be essential for viral replication during the natural infection (19).

Retroviruses initiate their replication by binding to specific host cellular receptors through complementary viral envelope components. Following penetration of the host cell, single-stranded viral RNA with associated nucleoproteins and reverse transcriptase is released to allow synthesis of a DNA copy of the viral RNA. The resulting DNA copy (provirus) typically integrates into the host genome and initiates synthesis of viral RNA synthesis including copies of the genome. Repeated nucleotide sequences (terminal repeat segments) at either end of the viral genome, in concert with viral and host genes, control viral replication. During productive phases of retrovirus replication, progeny viruses bud from host cell membranes (often obtaining host proteins in the process) and are released to infect other cells. Transcriptional blocks of virus replications, defective viral genomes, and highly regulated viral encoded proteins regulate the degree of virus expression within the host cell.

This ability of this group of retroviruses to activate cellular genes allows HTLV-1 and HTLV-2 to overcome cellular controls and cause inappropriate expression of particular genes (e.g., host transcriptional factors), which contribute to lymphocyte proliferation and

eventual transformation of the cell (20). These viruses are difficult to detect by conventional methods (e.g., *in situ* hybridization, immunofluorescence) within infected tissues. The predominant cell target of HTLV-1 appears to be the CD4⁺ lymphocyte, while CD8⁺ lymphocytes appear to be preferentially transformed by HTLV-2. The basis for the differential cell tropism is not understood, but may be related to post entry mechanisms (21).

HTLV-2, like HTLV-1 is highly cell associated and is transmitted from infected mothers to their offspring, primarily via breast milk. Virus culture and PCR studies of cord blood samples suggest that HTLV-1 and HTLV-2 are not transmitted *in utero*. Injection of infected blood is a common means of transmission of both viruses. In blood, the virus is associated with the cellular fractions that account for virtually all transmission of the infection. Parenteral exposure to the virus from needle sharing is a predominant mode of transmission of the virus among drug abusers (1,22–25). Procedures used for virus isolation and subsequent confirmation of viral antigens of HTLV-2 need to take into account the cell-associated nature of this group of viruses. Particular attention is also required in cell target preparation and in methods to test for viral antigen or nucleic acid in lymphocyte cultures.

2. Materials

1. Complete RPMI-1640 supplemented with 15% fetal bovine serum (FBS), 1% streptomycin/penicillin, and 1% glutamine (complete RPMI, cRPMI).
2. Human interleukin (hIL)-2 added at 10 U/mL as either purified or recombinant IL-2.
3. Normal uninfected human peripheral blood mononuclear cells (PBMC) obtained and maintained in complete RPMI-1640 media.
4. PBMC used for co-culture; prestimulated for 4 d with hIL-2 (10 U/mL) and phytohemagglutinin (PHA, 2 µg/mL) to activate for optimal infectivity prior to co-culture with test subject cells.
5. Viral core antigen production to test cell culture supernatants by enzyme-linked immunosorbent assay (ELISA) (Zeptomatrix, Buffalo, NY).
6. Western blot lysis buffer: 1% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, 20 µg/mL leupeptin, 20 µg/mL aprotinin, 1 mM sodium orthovanadate, 1 mM PMSF.
7. HTLV-2 polyclonal antiserum.
8. Genomic DNA extracted from cell cultures obtained by commercial affinity column.
9. HTLV-2 specific oligonucleotide primers.

3. Methods

3.1. Isolation of HTLV-2

The detection of HTLV-2 by virus isolation is dependent on activation of T lymphocytes and cell culture conditions that allow for the long-term growth of lymphocytes. The virus is transmitted by cell-to-cell transfer, and successful infection of cell cultures with cell-free virus preparations have generally been unsuccessful. Because of these biologic factors, virus isolation procedures require that test subjects' PBMC or tumor material be stimulated (e.g., by mitogens) and monitored for the presence of viral reverse transcriptase, antigens, or nucleic acids. In addition, coculturing with appropriate uninfected target cells (e.g., mitogen-stimulated PBMC) will enhance the ability of detection of HTLV-2 from infected persons.

The development of sensitive and specific antigen capture assays has greatly improved the ability to detect HTLV-2 from infected cell cultures. These assays detect soluble antigens (primarily major core antigens) in cell culture supernatants using specific antibodies to capture antigen. The bound antigen–antibody complex is detected colorimetrically. By comparison of the optical density readings of test samples with standard preparations, the quantity of viral antigen within the culture can be estimated. Alternate procedures to detect the presence of HTLV-2 in cell cultures include immunofluorescence assay (IFA) and avidin–biotin complex methods that allow visualization of viral antigens from the surface of infected cells (Table 2).

Infection of uninfected, but mitogen activated human PBMC is an effective method to amplify the number of HTLV-2 infected cells.

1. This is performed by co-culture with test subjects PBMC or lymphocytes with target uninfected PBMC prestimulated for 4 d with hIL-2 (10 U/mL) and phytohemagglutinin (PHA, 2 µg/mL) (“activated”) in cRPMI at a 1:1 ratio (*see* Notes 1 and ²).
2. Co-culture is performed in 24-well plates or 25-cm² flasks in complete RPMI. Care must be taken not to cross-contaminate 24-well plates; separation of individual subject cultures in separate plates is recommended.
3. Supernatants are then tested for viral core antigen by antigen capture assay at 3– 4 d intervals through 14 d of culture. Samples may be frozen in 1-mL aliquots and tested in batches.
4. Typically, positive cultures are detected by antigen capture assay within 2–3 d, but may not become positive until 7–14 d postculture. Cell clumping is common in early cell cultures due to mitogen stimulation and cell outgrowth of transformed cells (*see* Note 3).
5. Cultures negative for viral antigen or by PCR after 2 wk are generally considered negative and rarely produce positive results. Cells should be monitored for viable cell counts by standard dye procedures and split when cell growth exceeds 1.5×10^6 cells/mL. Cell splitting should result in cell concentrations of no less than 5×10^5 cells/mL to maintain cell-to-cell contact in the culture.

3.2. Antigen Capture Assay to Confirm HTLV-2 Core Antigen in Cultures

Viral core antigen production is used to test cell culture supernatants by ELISA (Zeptomatrix) according to the manufacturer's protocol (*see* Notes 4 and ⁵).

¹Cultures derived from human PBMC must be handled with universal biosafety precautions, i.e., assumed to carry blood-borne pathogens. In addition, long-term cell cultures used to produce HTLV-2 antigen should also be tested for the presence of other human viruses including the herpes viruses, Epstein-Barr virus, and the like, which may contaminate PBMC-derived cultures. Pretesting of blood samples from donors is recommended to eliminate common blood-borne pathogens, but this does not preclude the use of universal precautions.

²Cultures that become rapidly (within 24 h) acidic or basic should be considered suspicious for the presence of bacterial, mycoplasma, or fungal contamination and appropriately tested. Cultures should be observed daily to check for cell growth and to monitor potential contamination.

³Standard viable cell counts are useful to monitor the growth of HTLV-2 infected cell cultures. Cell clumping may occur and is expected until mitogenic stimulation and following transformation by HTLV-2. Cultures should be maintained between 1×10^6 and 1.5×10^6 cells/mL for optimal cell-to-cell contact and growth.

⁴The viral antigen capture assay used to measure cell culture supernatants by ELISA can be used to test for HTLV-1 antigen production, but will not differentiate these viruses. Monitoring cultures for the presence of reverse transcriptase is of limited value because of the low levels produced by this class of retrovirus. In addition, the measurement of this retrovirus enzyme is not specific for HTLV-2 and will also detect the presence of other retroviruses (e.g., HIV) and potentially detect endogenous host polymerases.

1. Collect cell supernatant after centrifugation at 1200 rpm for 10 min to remove cell debris. Samples (1 mL) may be tested directly or frozen at -20°C at tested when multiple samples are to be tested to reduce costs.
2. Virus antigen production may be detected as early as 2–3 d in culture. Cultures with low numbers of infected cells may take 7–14 d to rule out as negative.
3. Serial passage of the cells with supernatant samples are taken at each split (every 3–4 d) using fresh complete RPMI media.

3.3. Western Blot Assay for Confirmation of HTLV-2 Proteins From Cell Cultures

Western blot analysis is an alternative to evaluate the expression of cell-associated viral proteins and to confirm antigen capture or PCR results.

1. 5×10^6 HTLV-2-infected cells are lysed in standard Western blot buffer and 20 μg total protein separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
2. After transfer to nitrocellulose, viral proteins are detected by an anti-HTLV-1 or -2 human antiserum using commercially available secondary antibodies and detection systems.
3. HTLV-2 proteins are visualized with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) using chemiluminescence. Control positive (e.g., Mo-T-cell line) and negative cell lysates (e.g., Jurkat T-cells) should be included to rule out nonspecific bands.

3.4. Polymerase Chain Reaction for Detection for HTLV-2 From Cell Cultures

HTLV-2 antigen expression is limited from freshly isolated subject cells as a result of an active immune response and perhaps transcriptional silencing of the provirus *in vivo*. As a result the direct detection of viral proteins from cells or tissues is difficult. Therefore, methods used to detect viral nucleic acids have been employed to detect the presence of HTLV from infected cells (Table 3).

Techniques to detect relatively large copy numbers of viral nucleic acid include both Southern blot assays and *in situ* hybridization. Each assay is dependent on the sensitivity and specificity of the probe used to detect proviral DNA, as well as the methods and tissues used in the preparations. *In situ* hybridization uses radiolabeled probes that consist of portions of the viral genome (e.g., single-stranded DNA), which are applied to specifically treated slides of cells or tissues. This technique has the major advantage of morphological identification of infected cells; however, it is technically difficult and less sensitive than gene amplification methods (e.g., PCR). Southern blot procedures are similar to *in situ* hybridization in preparation of labeled probes; however, the detection is based on identification of viral nucleic acids from blotted membranes. Restriction enzymes with unique cutting sites are used to allow predictions of the integration of HTLV from lymphoma cells or cell cultures. The ability of Southern blot to detect monoclonal integration of HTLV-I in tumor cells has been employed diagnostically to predict the status of ATLL patients. Direct Southern blot of HTLV, like *in situ* hybridization, is technically difficult and is not sensitive to detect HTLV in low numbers of infected cells (e.g., seropositive but asymptomatic patients). Thus, PCR

⁵Unlike the situation for HIV-infected persons, these HTLV-2 antigen capture assays have not been useful to detect viral antigen directly from patients' bodily fluids (e.g., serum). This limitation of antigen detection directly from patients is caused by several factors, including complexing of antigens with patients' antibodies, but is principally due to the low levels of HTLV-2 antigens because of the cell-associated nature of the viral infection.

has become the method of choice to confirm the presence of HTLV-2 nucleic acid from cell cultures.

The application of PCR for the detection of HTLV-2 has allowed more sensitive and specific detection of the virus. The use of specific primers and probes have allowed investigators to discriminate the nucleic acids of HTLV-1 from closely related retroviruses like HTLV-2. The procedure has the major advantage of amplification of the viral signal. However, because of the sensitivity of the assay, strict adherence to control procedures is necessary to avoid cross-contamination. The development of PCR procedures for HTLV-1 and HTLV-2 has allowed investigations to determine the prevalence and potential disease associations of HTLV-2 (1,8,26,27). In addition, PCR can be used to directly diagnose individuals with HTLV-2 infection without the need for virus isolation.

1. DNA for PCR can be prepared from cells simply by lysing in the presence of detergents or by using more rigorous methods of phenol extraction.
2. Primer sequences for HTLV-2 are readily available (1,8,26,28,29) and can be purchased as oligonucleotides from commercial sources. Amplified products are detected after separation in agarose or acrylamide gels followed by transfer and probing by hybridization with labeled probes. Specific detection of HTLV-2 can be based on primer or probe design depending on preference of detection (*see* Notes 6–8).
3. Appropriate controls should include both negative and positive cell controls, molecular weight standards, sensitivity controls (serially diluted positive controls), and specificity controls (related but different viral cell lines) (*see* Note 6).
4. Controls and test samples should be prepared in a consistent manner to prevent variability in the methods of cell preparation and to avoid contamination of test samples.

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⁶Laboratory space used for all procedures involving DNA extraction should be separated physically from rooms used for amplification of the target DNA to prevent contamination of primer pairs and probes.

⁸A variety of alternative PCR methods (e.g., real-time PCR) have been developed to test for HTLV-1 and HTLV-2 from patient samples and cell cultures (27).

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Table 1
Human T-Lymphotropic Virus Type 2 (HTLV-2) Genomic Organization

Gene	Function	Protein product (kD)
Long terminal repeat (<i>LTR</i>)	Regulation	Noncoding
Group-specific antigen (<i>gag</i>)	Core proteins	p53 precursor p19 matrix p24 capsid p15 nucleocapsid
Protease (<i>pro</i>)	<i>gag</i> cleavage	p14 protease ^a
Polymerase (<i>pol</i>)	Replication	p62 transcriptase ^a p32 integrase ^a
Envelope (<i>env</i>)	Envelope	p61-68 precursor gp46 surface gp21 transmembrane
pX ORF IV	Regulation	p37 Tax (transactivation)
pX ORF III	RNA regulation	p24, p26 ^b Rex (regulator of splicing)
pX ORF II	<i>a</i>	p28 ^{c,d}
pX ORF I	<i>a</i>	p10 ^c
pX ORF V	<i>a</i>	p 11 ^c

^aDefined for HTLV-1, not defined for HTLV-2.

^bRex phosphorylation determines size.

^cPredicted protein product.

^dMay regulate viral RNA through posttranscriptional mechanism (P. Green, personal communications).

Table 2
Methods to Detect Human T-Lymphotropic Virus Type 2 (HTLV-2) Antigens in Cell Culture

Methods	Comments
Immunofluorescence assay	Simple to perform for detection of HTLV from infected cells. Cost-effective, labile signal.
Avidin-biotin complex assay	Easily performed for detection of HTLV from infected cells. Stable signal; allows slide record.
Antigen capture assay	Excellent sensitivity for HTLV-2 core antigen in cell culture supernatants. Will cross-react with HTLV-1 core antigen. Microtiter plate to test multiple samples.

Table 3
Diagnostic Methods For Detection of Human T-Lymphotropic Virus (HTLV) Nucleic Acids in Cell Cultures

Method	Procedure	Comment
Southern blot assay	DNA from HTLV-2 cells used with labeled complementary probes typically of long terminal repeats	Sensitivity determined by probes and conditions of assay. Method to estimate copy number in cell lines
<i>In situ</i> hybridization	HTLV-2 DNA or RNA in tissues, complementary probes	Excellent to determine cell type expressing virus. Less sensitive directly from patient for RNA <i>in situ</i> owing to low level of transcriptionally active cells
Polymerase chain reaction	DNA or RNA from HTLV-2 cells	Excellent sensitivity, useful to differentiate from HTLV-1, contamination a potential problem