THE YALE JOURNAL OF BIOLOGY AND MEDICINE 62 (1989), 103-114

Differential Diagnosis of Human Retrovirus Infections in the Laboratory

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Received February 13, 1989

Several human retroviruses have been discovered in the past decade, the majority of which have been implicated as etiological agents of severe disease entities. Thus, there is a clear need for accurate identification of human retroviruses in the laboratory. In this review, the classification and general properties of human retroviruses are outlined. Methods for detecting the presence of antibodies are reviewed. In addition, the principles of methods used for isolating and identifying retroviruses are discussed. Finally, techniques which detect the presence of retroviruses directly in clinical specimens without prior amplification in culture are summarized. Clearly, the ability to differentiate and recognize the different types of retrovirus is important for the proper treatment of diseases caused by these viruses.

INTRODUCTION

Retroviruses have been thrust into the spotlight in the past five years because of the epidemic produced by its most notorious member, the human immunodeficiency virus type 1 (HIV-1). Other retroviruses such as HIV type 2 (HIV-2) and the human T-leukemia virus type I (HTLV-I) have also carried out their deadly activity, but on a less dramatic scale. There is little doubt that the retroviruses have made their mark among the infectious diseases. Consequently, there is a clear need to differentiate and understand the methods used to identify each of the viruses in the laboratory.

These retroviruses as a class are characterized by the possession of a RNA genome and a reverse transcriptase enzyme. They also share a similar structure, consisting of a lipid membrane containing antigenic glycoproteins which encloses an inner protein core containing other antigenic proteins, two RNA strands, and the reverse transcriptase. In contrast to retroviruses of other species, the human retroviruses do not infect reproductive cells; thus, they are not transmitted endogenously to offspring. Instead, transmission is exogenous, as a transmissible, infectious agent.

The purpose of this paper is to review how the various human retroviruses differ from each other insofar as laboratory diagnosis is concerned. First, nomenclature, disease association, and general characteristics are discussed. Second, tests for the

Abbreviations: AIDS:acquired immune deficiency syndrome ARV:AIDS-associated retrovirus ELISA: enzyme-linked immunosorbent assay HAM:HTLV-I-associated myelopathy HIV-1, HIV-2: human immunodeficiency virus type 1, type 2 HTLV-I, HTLV-III: human T-leukemia virus type I, type III IFA: indirect immunofluorescence assay LAV: lymphadenopathy-associated virus LTR: long terminal repeat PCR: polymerase chain reaction RIPA: radioimmunoprecipitation TSP: tropical spastic paraparesis WB: Western blot

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TABLE 1
Nomenclature of Human Retroviruses

Group	Characteristic	Virus Name	Year Reported [References]	Disease Association
Human immunodeficiency virus (HIV)	Cytopathic	HIV-1[a]	1983 [1–4]	Acquired immune deficiency syndrome
		HIV-2	1986 [5]	Acquired immune deficiency syndrome
Human T-cell leukemia/lymphoma virus (HTLV)	Transforming	HTLV-I	1980 [6]	Adult T-cell leukemia/lymphoma (ATLL) Tropical spastic paraparesis (TSP) HTLV-I-associated myelopathy (HAM)
		HTLV-II	1982 [14]	Not established[b]
		HTLV-III	1983 [1–4]	See HIV-1
		HTLV-IV	1986 [18]	None[c]
		HTLV-V	1987 [20]	Not established[d]

[a]Previously called HTLV-III, LAV, or ARV
[b]Isolated from patients with hairy-cell leukemia
[c]This virus was initially thought to be similar to HIV-2. Subsequently, it was found to be indistinguishable from the simian immunodeficiency virus [19]
[d]Isolated from a patient with cutaneous T-lymphoma/leukemia

demonstration of antibodies are reviewed. Finally, principles of retrovirus culture and direct detection methods are summarized.

THE TWO MAJOR GROUPS OF HUMAN RETROVIRUSES

Nomenclature and Disease Association

Human retroviruses are of two major classes, distinguished by their primary pathologic effects on T cells (Table 1). Cytopathic retroviruses, which have been termed human immunodeficiency viruses (HIV), cause cell death and result in immunodeficiency. HIV-1, the first retrovirus found to be the etiologic agent of the acquired immunodeficiency syndrome (AIDS), was previously called human immunodeficiency virus (HIV), human T-leukemia virus type III (HTLV-III), lymphadenopathy-associated virus (LAV) and AIDS-associated retrovirus (ARV) [1–4]. The human immunodeficiency virus type 2 (HIV-2), previously named lymphadenopathy-associated virus type 2 (LAV-2), was isolated in 1986 from AIDS patients in West Africa [5].

The second major group of human retrovirus comprises transforming viruses. The human T-leukemia virus type I (HTLV-I), the first human retrovirus to be identified, has been implicated as the causative agent of adult T-cell leukemia-lymphoma [6–8] and tropical spastic paraparesis (TSP) or HTLV-I-associated myelopathy (HAM) [9–13]. HTLV-II was originally isolated from a patient with hairy-cell leukemia [14]. The implication of HTLV-II in a specific disease has been unclear because HTLV-II has been isolated from a total of only four patients [14–17]; two of these patients had hairy-cell leukemia [14,15]. Another retrovirus, initially named HTLV-IV, was isolated in West Africa in 1986 [18]. Subsequently, it was found that HTLV-IV isolates were indistinguishable from simian immunodeficiency virus isolates and were not independent viral isolates [19]. In 1987, a new distinct human retrovirus

TABLE 2
Comparison of Cytopathic and Transforming Retroviruses of Humans

1.	Similarities
	RNA genome
	Reverse transcriptase
	Integration of viral genome into host DNA
	Structure
	Genomic organization
	Infection of T cells
	Latency
2.	Differences
	Nucleotide sequences
	Antigenic reactivity
	Effect on T cells

(HTLV-V) was isolated from a patient with a cutaneous T-cell lymphoma/leukemia [20].

Comparison of Cytopathic and Transforming Retroviruses

Characteristics of cytopathic and transforming viruses are listed in Table 2 [6,7, 14,20,21]. In addition to similar structures, both groups of retroviruses share a similar genomic organization with three major structural genes (*gag, env,* and *pol*). Each of these genes codes for a precursor protein, which is processed into one or more structural elements. These genes are flanked by sequences called long terminal repeats or LTRs. In addition, several genes that regulate the expression of other viral genes have been identified. The HTLVs have one regulatory gene, called *tat* gene for transacting activator; the protein product of this gene mediates the transcriptional activation of the viral LTR and genes linked to it. The HIVs have at least three regulatory genes called *tat III, sor,* and *3′ orf*. The *tat III* gene is a transactivator gene that produces a 14 kD gene product. The functions of the *sor* and *3′ orf* genes are unclear at this time; however, it is known that they code for 23 kD and 27 kD gene products, respectively. Both groups of viruses primarily infect a subset of T cells and some neural cells, and they have a long incubation period.

Despite these similarities, the two groups of viruses are only distantly related, as determined by comparisons of nucleotide sequence and protein cross-reactivity. The genomes of HTLV-I, HTLV-II and HTLV-V are related; HTLV-I and HTLV-II share approximately 50 percent of their genome. Likewise, HIV-1 and HIV-2 are approximately 40 percent homologous. HTLVs, however, are not related to HIVs. As noted before, the effects on cells of these two groups of virus are strikingly different, since the cytopathic viruses usually kill the cells they infect and the transforming viruses induce cell proliferation.

TESTS FOR THE DEMONSTRATION OF ANTIBODIES

The demonstration of specific antibodies to retroviruses can, in the majority of cases, provide adequate evidence of ongoing infection, as retroviral antibodies generally persist for life. Enzyme-linked immunosorbent assays (ELISA) for the detection of antibodies to retroviruses were initially developed to screen blood and blood products. A serum that is found repeatedly positive by the ELISA screening test is further tested

by a supplementary method to confirm the presence of specific antibodies. The majority of screening and confirmatory tests make use of viral antigens prepared by growing the virus in culture; thus, antibodies to a mixture of viral proteins are probed in these assays. Recently, novel methods that make use of recombinant HIV-1 antigens and detect antibodies to one or more specific viral proteins have been described [22,23].

Screening Tests

The most frequently used assay, the ELISA, was developed for diagnosis of HTLV-I, HIV-1, and HIV-2 infections in 1983, 1984, and 1987, respectively [24–26]. Eight ELISA kits for HIV-1 and three ELISA kits for HTLV-I are now licensed in the U.S. for antibody testing. Although HIV-2 ELISA kits are available commercially, they are not yet licensed in the U.S.

HTLV-I and HTLV-II are very closely related and thus exhibit extensive serologic cross-reactivity. As a result, HTLV-I ELISA kits do not distinguish between HTLV-I and HTLV-II antibodies. In contrast, the degree of immunologic cross-reactivity between HTLV-I and HIV-1 in ELISAs appears to be very small, although some antigenic relationship has been suggested [27]. HIV-1 antibody-positive patients have been evaluated for reactivity to HTLV-I by ELISA [28] or by indirect immunofluorescence assay (IFA) [29]. Two of 35 patients were positive in the first study [28] and none of 363 were positive in the second study [29].

Because of the high degree of immunologic cross-reactivity between the *gag* and the *pol* gene products of HIV-1 and HIV-2 [5,30], a number of studies have determined whether HIV-1 ELISAs could be used to detect antibodies to HIV-2 [31,32]. In one study [31], 43 sera positive for HIV-2 antibody were tested by nine HIV-1 ELISAs. The overall prevalence of positive results ranged between 28 and 93 percent. Most of the ELISAs were less sensitive in detecting antibody to HIV-2 in sera from people with AIDS-like disease than in sera from asymptomatic HIV-2-infected individuals [31]. A number of reports, including two cases in the U.S., have suggested that anti HIV-1 ELISAs are inadequate for diagnosis of HIV-2 infections [33,34]; however, use of peptide antigens representing a conserved region of the transmembrane protein allows testing for type-specific antibodies against HIV in an ELISA [35,36]. Hence, specific antibodies in sera from patients infected with HIV-1, HIV-2, or both viruses can be identified.

Other screening tests have been used. They include passive hemagglutination for HIV-1 and HTLV-I [37–39] and indirect immunofluorescence [29,40–42].

Confirmatory Tests

Antigens coded by genes of the various retroviruses can be serologically cross-reactive in ELISA tests; thus it is important to utilize an assay that can discriminate between antibodies to specific proteins. The most frequently used of these tests is the Western blot test (WB). Other supplementary tests available include IFA and radioimmunoprecipitation (RIPA). In contrast to IFA, WB and RIPA detect antibodies to the specific viral gene products that have been separated by gel electrophoresis. Antibodies to retroviral gene products that are routinely used in laboratory diagnosis are listed in Table 3. Products of the *tat* gene are regulatory proteins which affect the level of expression of other viral genes. For HTLV-I, the 40 kDa protein encoded by the *tat* gene is essential for the transforming effect of the virus, and the antibody is detectable in the serum of HTLV-I-infected patients. Antibodies to the gene products of the *tat, sor,* and *3' orf* genes of HIV-1 have been detected in some patients but do not

TABLE 3
Antibodies to Gene Products of Human Retroviruses That Can Be Detected in Sera from Infected Individuals

Major Genes	General Description of Gene Products	Gene Products of		
		HIV-1	HIV-2	HTLV-I/HTLV-II
Core (*gag*)	Precursor of *gag* protein	p55	p53–55	p53
	gag protein	p24	p25	p24
	gag protein	p18	p16	p19
Envelope (*env*)	Precursor of *env* glycoprotein	gp160	gp130–140	gp61
	Outer *env* glycoprotein	gp120	gp105	gp46
	Transmembrane *env* glycoprotein	gp41	gp36	p21
Polymerase (*pol*)	Reverse transcriptase component	p65	p68	p62
	Reverse transcriptase component	p51		p32
	Endonuclease component	p31	p33	p14
Trans-Activator (*tat*)				p40X(HTLV-I) p37X(HTLV-II)

at present play a role in diagnosis. Antibodies to the HIV-1 *3' orf* regulatory factor have been recently found to be present and to persist in seronegative individuals who were infected with HIV-1 as demonstrated by polymerase chain reaction (PCR) DNA amplification [43].

Although there are many similarities in the protein products of the various retroviruses, cross-reactivity usually is detected only for the core proteins, particularly p24. Indeed, in WBs, antibody-positive sera give a strong signal with the transmembrane glycoprotein of the corresponding serotype alone. For example, an HIV-2-positive sera will often react weakly with p24 in an HIV-1 WB but will usually react strongly with both core and envelope proteins on an HIV-2 WB. Examples of reactivities of serum from HIV-1, HTLV-I, and dually infected individuals in HIV-1 and HTLV-I WBs are shown in Fig. 1. Neither the ELISA nor the WB for HTLV-I can distinguish between antibody to HTLV-I and antibody to the closely related HTLV-II. Criteria for calling a sample WB positive vary from laboratory to laboratory. It is generally accepted, however, that antibodies to at least one core and one envelope protein should be present. In most cases, use of one of the confirmation tests is sufficient. In some situations, however, use of both WB and RIPA is helpful because of complementary features. Thus, the WB often favors the detection of core proteins, whereas the RIPA favors the detection of envelope proteins. For example, antibodies to the HTLV-I gp61 and gp46 can be seen more easily using RIPA.

IFA has been used to confirm the presence of HIV-1 antibodies; various neoplastic T-cell lines infected with HIV-1 including CEM, Hut 78, and H9 can be used [40–42]. HTLV-I-transformed cell lines, MT-1 and MT-2, or Molt-3 cells have been utilized successfully in HTLV-I IFA [29,44]. An HTLV-II-transformed cell line has also been used in IFA [29]. Sera that reacted with HTLV-I were tested on HTLV-II slides, and specimens were considered positive if they reacted with both antigens. Adsorption tests determined whether antibody responses were more closely related to HTLV-I or to HTLV-II [29].

ISOLATION AND IDENTIFICATION OF HUMAN RETROVIRUSES

The principles of the procedures used for isolating and identifying HIV-1 have been outlined before [45]. Isolation and identification of human retroviruses is a two-stage,

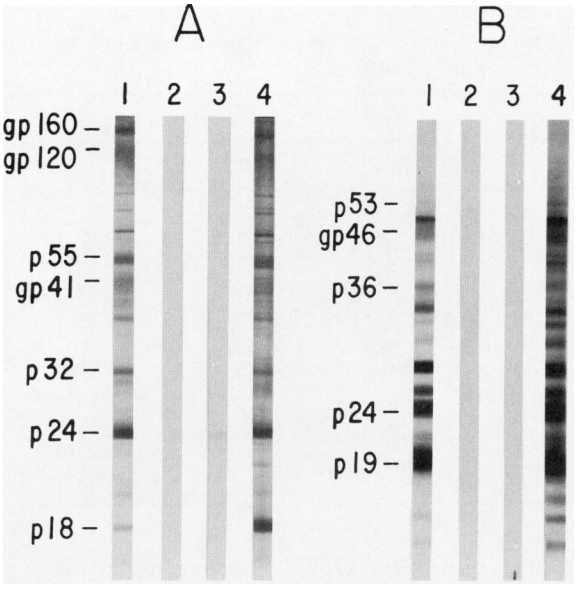

FIG. 1. Reactivity of sera from HIV-1 and HTLV-I ELISA-positive patients with HIV-1 (lanes A1 to A4) and HTLV-I (lanes B1 to B4) proteins in Western blots: Lanes A1 and B3: serum from an HIV-1-positive, HTLV-I-negative individual; Lanes B1 and A3: serum from an HIV-1-negative, HTLV-I-positive individual; Lanes A2 and B2: serum from an HIV-1-negative, HTLV-I-negative individual; Lanes A4 and B4: serum from an HIV-1-postive, HTLV-I-positive individual.

elaborate, and time-consuming procedure (Fig. 2). In the first stage, susceptible cell cultures are co-cultivated with the patient specimen for several weeks. In the second stage, viral enzymes or antigens that are released in culture supernatants or present in the cells are identified.

Usefulness of Retrovirus Isolation

Although diagnosis of retroviral infections is more easily obtained by detecting antibodies in the patients' sera, management of patients can, in many circumstances, be significantly improved if the presence of the virus can be demonstrated in the patient. We have discussed before the need and value of HIV-1 isolation *(1)* for confirming the diagnosis of HIV-1 in individuals who have no detectable antibodies or in whom the significance of seropositivity is unclear; *(2)* for disease staging; and *(3)* for monitoring antiviral therapy [45]. Isolation of other retroviruses may help to confirm the diagnosis of retrovirus infections in cases where antibody results are negative or difficult to interpret. Most important, virus isolation attempts allow the demonstration of retroviruses as causative agents of new disease entities and the discovery of new viruses.

Cytopathogenicity and Host Cell Range

Primary lymphocyte cultures, prepared from the blood of normal donors and stimulated with a mitogen such as phytohemagglutinin and a T-cell growth factor such as interleukin 2, are the most sensitive for the isolation of the cytopathic retroviruses [45]. Because HIV-1 and HIV-2 produce cell death, fresh donor lymphocytes have to be added weekly to the cultures.

In contrast to the cytopathic retroviruses, the transforming retroviruses eventually do not require addition of fresh lymphocytes or T-cell growth factor for their growth. Initially, cells from the patients are grown *in vitro* in the presence of T-cell growth factors or cells from the patients are co-cultivated with stimulated donor lymphocytes. HTLV-I-infected cells will continue to thrive without addition of fresh lymphocytes.

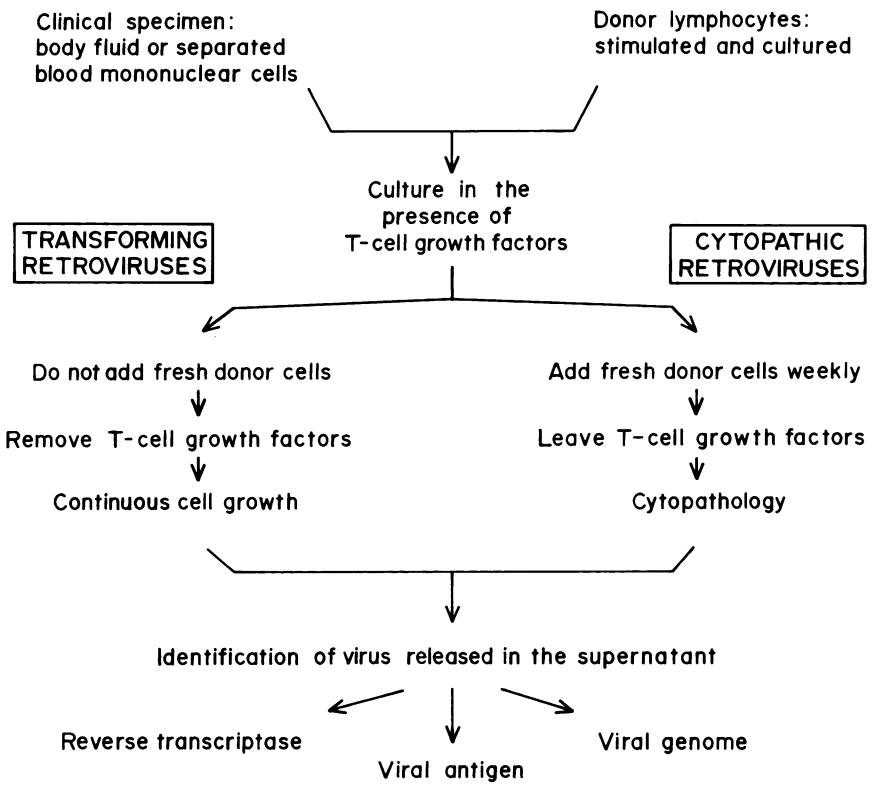

FIG. 2. Flow chart of various steps involved in isolation and identification of human retroviruses.

After several weeks in culture, T-cell growth factors can be removed from the culture media, as HTLV-I- and HTLV-II-infected cells do not require exogenous T-cell growth factors for their continued growth. For both groups of viruses, cell viability and cytopathology should be monitored. In addition, the cell cultures are evaluated at weekly intervals throughout the culture period for virus expression and release.

Human retroviruses are known to infect human cells which bear the CD4 surface antigen. This antigen is a marker for the helper inducer T-cell subset. HIVs have been shown to infect T lymphocytes, monocytes and macrophages, and B lymphocytes and cells of neuronal or glial origin, some but not all of which bear CD4 [21]. HTLVs can readily infect helper inducer T lymphocytes, but much less is known about their ability to grow in other cell types. Cells infected with HIV-1 or HIV-2 will show, to various degrees, a characteristic cytopathic effect consisting of multinucleated giant syncytial cells with ring formation. These cells die over a period of a few days. Many HIV-1 isolates possess markedly different abilities to replicate and induce cytolysis in different host cell types [35,46–51]. Syncytium-inducing isolates often have higher infectivity than isolates that do not induce syncytia. HIV-1-infected individuals may carry multiple HIV-1 genotypes with distinct cytopathogenic and cell tropism [52]. Recently, a new HIV-2 isolate that did not cause cytopathic effect in susceptible T cells was recovered from an Ivory Coast patient [50]. HTLV-I and HTLV-II do not produce cell death; instead, these viruses will generally transform T4 cells in five to ten weeks. HTLV-I can also show syncytia formation [53].

Methods of Retrovirus Identification

Methods of identification rely most often on the demonstration of viral proteins or viral genome in supernatants of infected cells. Less frequently, identification of viral antigens or viral genome in infected cells is attempted. The measurement of reverse transcriptase is broadly utilized as the primary method for the detection of all retroviruses. The assay is based on identifying the enzyme's capacity to assemble DNA from a RNA template. For HIV-1 and HIV-2, it takes, in general, 7 to 28 days of co-cultivation of patients' specimens with susceptible cells to accumulate sufficient enzyme for detectability in the supernatant. Release of detectable reverse transcriptase activity into the supernatants of HTLV-I-infected cells can take much longer, up to 12 weeks [54]. The reverse transcriptase assay does not allow for the identification of each type of retrovirus.

Detection of specific viral antigens allows for identification of each type of retrovirus. Antigen capture ELISA kits for detection of HIV-1 antigens are available; however, kits for detection of the other retrovirus antigens are not available commercially.

Other methods of retrovirus identification involve the identification of retrovirus nucleic acids. Dot blot hybridization techniques have been employed to identify HIV-1 [55] and HIV-2 [56]. In this assay, cytoplasmic RNA prepared from cultured cells is denatured, spotted on nylon filter, and detected with labeled viral DNA. In addition, PCR has been used to monitor cultures for the presence of proviral sequences of HIV-1, HIV-2 [57,58], and HTLV-I [59,60]. The PCR takes advantage of an enzyme that uses a defined segment in a strand of DNA as a template for assembly of a complementary strand. During each reaction, the number of DNA strands doubles. The reaction takes only a few minutes and is repeated up to 40 times; a discrete strand of DNA can therefore be amplified to as many as one million copies. A monoclonal antibody solution hybridization assay for the measurement of viral RNA in patients' specimens has also been described recently [61]. The assay involves a reaction in solution between a biotinylated DNA probe and the putative RNA target in the sample. Labeled hybrids are detected by using a solid-phase anti-biotin antibody and an enzyme-labeled monoclonal antibody specific for DNA-RNA hybrids.

DIRECT DETECTION OF RETROVIRUSES

Methods for detecting retroviruses directly in cells or body fluids from infected individuals are less cumbersome and faster than virus isolation procedures. Except for the PCR method, however, they do not include amplification of the virus and thus tend to be less sensitive than virus isolation. The antigen capture ELISA method has been used to detect HIV-1 antigen in serum and cerebrospinal fluid [62]. In this assay, antibodies that recognize epitopes of the viral protein are coated to beads or plates and are used to catch the antigen. A positive reaction is identified with a probe antiviral antibody and a labeled secondary antibody. This method is useful for early detection of HIV-1 infection because antigenemia can be demonstrated as early as two weeks after infection, one month prior to the appearance of antibodies; however, antigen detection systems cannot detect virus that has entered a latent state with low or absent expression of structural proteins.

The use of nucleic acid hybridization techniques has the advantage of not requiring production of proteins by the virus. HIV-1 has been detected directly within lymphocytes, macrophages, and other cells by in situ hybridization [63,64]. The recently

developed PCR method allows for the detection of infectious agents that are present in cells at very low copy numbers and that are not detectable by current methodologies. The PCR DNA amplification technique has been utilized to identify the presence of proviral sequences of HIV-1 directly in DNA isolated from peripheral blood mononuclear cells of seropositive individuals [58]. HTLV-I has been detected in HTLV-I seronegative patients with T-cell leukemia [59] and in the blood of patients with chronic progressive myelopathy [13]. In addition, proviral sequences from HIV-1 and HIV-2 have been detected by PCR in a person dually infected [57].

CONCLUSION

Several human retroviruses that are capable of producing significant morbidity and mortality have been discovered in recent years. Laboratory identification of the particular retroviruses involved in the various diseases is important to improve patient management. It is necessary for laboratories to consider the differential diagnosis of the various types of human retroviruses because the number of pathogenic human retroviruses has increased in recent years and because mixed infections with HIV-1 and HTLV-I [54,65,66] and with HIV-1 and HIV-2 [57] have been reported. With continuing advances in antiviral therapy and increased knowledge of the pathogenesis of these viruses, the correct identification of the viral etiology will have more immediate clinical application.

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