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Development of molecular methods for detection and epidemiological investigation of HIV-1, HIV-2, and HTLV-I/II infections

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SUMMARY

The work presented here was initiated to determine the possibilities of molecular methods for the detection and epidemiological investigation of HIV and HTLV infections.
We present the results of a literature research and describe the development and partial evaluation of a new PCR method for the amplification of RNA and DNA sequences of the HIV-1 *pol*, *env* and *gag*, HIV-2 *ltr* and HTLV-I/II *tax/rex* genes.
For the amplification of viral RNA, samples were treated with guanidium thiocyanate and sodium lauryl sarcosinate to disrupt the virus and to inactivate RNAses. Paramagnetic beads were used to extract the RNA, followed by solid-phase reverse transcription for cDNA synthesis. DNA or cDNA was amplified using a two-step PCR protocol in which the product from the first PCR was further amplified in a second PCR with nested primers combined with a touch-down temperature protocol to enhance sensitivity and specificity.
A pilot study showed that in all peripheral blood mononuclear cell (PBMC) samples from seven HIV-1-infected individuals of CDC class II, proviral HIV-1 DNA was detected using three primer sets. HIV-1 RNA could be detected in the plasma from ten of fifteen HIV-1-infected individuals of CDC class II. Together with data from the literature, our results indicate that PCR methods are useful in the detection of HIV-1 infections and complementary to conventional methods such as enzyme immunoassays and Westernblot. They are especially useful when conventional methods do not allow a diagnosis to be made, for example in newborns of HIV-infected mothers, in monitoring of the viral load, and in patients with idiopathic CD4+ T-lymphocytopenia.
Using the same method, we amplified HIV-2 and HTLV-I RNA and DNA sequences. However, clinical evaluation of these PCR methods must be conducted.
This newly developed method may possibly be used in molecular epidemiological studies as we were able to directly sequence the product of the HIV-1 *env* np-PCR, the V3 variable region of the gp120 gene. However, molecular epidemiology must be conducted at the clonal level with more than one isolated part of the viral genome. For molecular epidemiological studies of HIV-1, for example, the variable regions V3, V4, and V5 of the gp120 gene together are useful targets.
Promising methods for obtaining materials for PCR as well as serology with regard to epidemiology on the streets are the use of finger-prick blood spots on filter paper and saliva collection. Further studies are needed to determine the value of these methods in molecular epidemiological investigation.

SAMENVATTING

Het onderzoek dat hier wordt gepresenteerd werd gestart om de mogelijkheden van moleculaire methoden voor detectie en epidemiologisch onderzoek van HIV en HTLV infecties te onderzoeken.

We presenteren de resultaten van een literatuurstudie en beschrijven de ontwikkeling en gedeeltelijke evaluatie van een PCR methode voor de amplificatie van RNA en DNA sequenties van HIV-1 *pol*, *env* en *gag*, HIV-2 *ltr* en HTLV-I/II *tax/rex* genen.

Voor de amplificatie van viraal RNA werden de monsters behandeld met guanidine thiocyanaat en natrium-lauryl-sarcosinaat om het virus kapot te maken en om RNAses te inactiveren. Paramagnetische bolletjes werden gebruikt om het RNA te extraheren gevolgd door solid-phase reverse-transcriptie voor cDNA synthese. Een twee-staps PCR protocol werd gebruikt voor de amplificatie van DNA of cDNA, waarbij het produkt van de eerste PCR verder wordt geamplificeerd in een tweede PCR met nested primers gecombineerd met een touch-down temperatuur protocol, om de gevoeligheid en specificiteit te verhogen.

Een pilotstudie laat zien dat in alle perifere bloed mononucleaire cellen (PBMC) monsters van zeven HIV-1 geïnfecteerde individuen uit CDC klasse II, proviraal HIV-1 detecteerbaar was gebruik makend van drie primersets. HIV-1 RNA was detecteerbaar in plasma van 10 van vijftien HIV-1 geïnfecteerde individuen uit CDC klasse II. Samen met gegevens uit de literatuur geven onze resultaten een indicatie dat PCR methoden bruikbaar zijn voor detectie van HIV infecties als toegevoegde methode aan de conventionele methoden zoals de enzym-immunoassays en de westernblot. Ze zijn speciaal geschikt als met de conventionele methoden geen duidelijke diagnose gesteld kan worden, zoals bijvoorbeeld bij borelingen van HIV geïnfecteerde moeders, bij het volgen van de hoeveelheid aanwezig virus en bij patiënten met een idiopatische CD4+ T-lymfocytopenie.

Met dezelfde methode zijn HIV-2 en HTLV-I RNA en DNA sequenties geamplificeerd. Echter, deze methoden moeten nog klinische geëvalueerd worden.

Deze nieuw ontwikkelde methode is mogelijk bruikbaar voor moleculair epidemiologische studies omdat het product van de HIV-1 env np-PCR, het V3 variabele gebied van glycoproteïne gp120 gen, direkt te sequencen was. Echter, moleculaire epidemiologie moet uitgevoerd worden op het nivo van moleculaire kloons van het virus in meer dan één gebied van het virale genoom. Voor moleculair epidemiologische studies van HIV-1 zijn, bijvoorbeeld, de variabele gebieden V3, V4 en V5 van het gp120 gen geschikte kandidaten. Veelbelovende methoden om materialen voor PCR en serologie bij epidemiologische veldstudies te verkrijgen zijn het gebruik van vingerprik-bloed op filter papier en van speeksel afname. Verdere studies zijn nodig om de waarde van deze methoden in moleculair epidemiologisch onderzoek vast te stellen.

1. INTRODUCTION

At present, infection with HIV-1, HIV-2, or HTLV-I/II is usually determined by the detection of antibodies against antigenic constituents of either of the viruses, for example, in the patient's blood with enzyme immunoassays (EIAs) and Westernblot assays (WB), and occasionally by detection of virus-specific antigen with EIAs. However, in some cases a clear-cut result cannot be obtained by these methods: for example, (i) in newborns or children of HIV-seropositive mothers when maternal antibodies are present and antigen levels are too low to be detected (45); (ii) during the early phase of infection (30, 49); and (iii) in patients with idiopathic CD4+ T-lymphocytopenia (71). Furthermore, there are rare cases of indeterminate results of antibody EIAs and WB which may lead to misinterpretation of seropositivity (34). Sometimes a clear determination of the virus type based on the antibody profile in WB is not possible, as is the case in dual and even triple infection with different virus types (3, 40). Culture of the virus can sometimes solve these problems; however, culture of retroviruses requires a special safety laboratory, and well-trained personnel and is very laborious and expensive. Furthermore, a typing system for the cultured virus must be available.

Once the diagnosis is made, a continuous monitoring of the viral load in the blood of a seropositive patient may give prognostic information about the progression of the disease and is useful in monitoring anti-retroviral therapy. HTLV-I/II can only be detected in PBMC, because it is not present as free virus in serum or plasma (10, 48). HIV-1 and HIV-2, on the other hand, can be detected in both PBMC and serum or plasma. Conventional methods to measure viral load in plasma are the quantitative virus-specific antigen EIA (58, 65) and quantitative plasma virus culture (28, 46, 58, 65). The conventional method to measure viral load in PBMC is quantitative cell culture (46, 65). Both methods, antigen EIA and culture, have disadvantages. Viruses can be trapped in immune complexes leading to unreliable results in both methods (57, 59, 63). Antigen detection can, however, be improved by treatment at low pH (59, 63) or by boiling (63) of serum. Quantitative virus culture not only has the disadvantages of retrovirus culture mentioned above, but is also very difficult to standardize and gives results that are difficult to interpret (53). Neither method is very sensitive (58, 72). All the conventional methods depend on variably reacting systems. The antibody response of the infected person can be used in the diagnosis of an infection only when the response meets certain criteria (77), and can even hinder the measurement of antigen in plasma or the culture of virus from plasma. Donor PBMC are used in virus culture from the patient's plasma or PBMC which introduces unknown variables in the efficiency of the culture.

An alternative which has been successfully used by many others is molecular detection and monitoring of the viral load using the polymerase chain reaction (PCR) to amplify the viral RNA or proviral DNA sequences (10, 17, 27, 41, 46, 70). The antibody response does not hinder PCR. Furthermore, RNA or DNA extraction methods and PCR methods can easily be standardized. However, PCR methods are expensive and require well-trained personnel and several separated laboratory rooms must to avoid contamination. In spite of its disadvantages, the PCR method may be the answer to the diagnostic problems mentioned above (3, 30, 34, 43, 44, 45, 49) and is, to date, the most sensitive method for monitoring the viral load in anti-retroviral therapy (52, 64, 68, 72) and progression of disease (7, 32, 52, 54, 58, 64, 72). Molecular typing of HIV-1 is also useful in studying the prognosis of disease progression (23, 51, 75).

Another area of investigation is the epidemiology of the retrovirus infections. Transmission of the virus, spreading of the infection, and the virus (sub)types involved have been studied by several authors using PCR and sequencing of welldefined parts of the genome followed by phylogenetic analysis (for HIV-2 and HTLV-I/II, see references 9 and 20; HIV-1 is discussed in more detail below).
HIV-1 has been divided into 7 genotypes (A-G) based on nucleotide sequences of the *gag* gene, to obtain insight in its worldwide geographic distribution. However, the distribution of variants is complex and no clear picture of the transmission pathway has so far emerged (47). The transmission of HIV-1 and its divergence in the host over time have been studied using molecular methods to determine, for example, which virus types are involved (in transmission of mother to child: 62, 76), what the rate of divergence is (in transmission via factor VIII batches to hemophiliacs: 8; follow-up study: 16, 36, 38 and 66), and if it is possible to establish the transmission pathway (transmission from a dentist to patients: 4, 5, 6 and 56; transmission from a surgeon to patients: 29).
Two major problems in this kind of investigation are the selection of the part of the genome to be sequenced and the method of phylogenetic analysis to be used. The following parts of the HIV-1 genome have been used in the studies mentioned above: *gag* p17, *gag* p24, and *env* gp120 different regions. Results of phylogenetic analysis may differ with the part of the genome which has been used for sequencing (8, 66, 67). However, the part of the HIV-1 genome that is used most often is the immunodominant V3 loop of the gp120 envelope glycoprotein. The problems which may occur depending on the method of phylogenetic analysis are clearly demonstrated by the commentary on the initial article in which the transmission of HIV-1 from a dentist to his patients was described (56). Depending on the method used, there was evidence that the dentist had infected his patients (56) and that he could not have infected his patients (18, 69). Thus, phylogenetic analysis must be carefully used and the results interpreted with caution.

Whether viral RNA extracted from serum or plasma or DNA extracted from PBMC, has to be used, depends on the type of application. HTLV-I/II can only be detected by PCR in PBMC, whereas HIV-1 and HIV-2 can be detected by PCR in both PBMC and serum or plasma. Furthermore, when initiating a research project, it must be taken into account that the amount of HIV-1 RNA and DNA changes during the course of the infection (7), the sequences of RNA and DNA are different at one point in time and are changing discontinuously (67), and there is extensive intrapatient sequence variation (24, 50). It has been shown that at the presumed time of virus transmission, "late" viral RNA sequences of free virus in the blood of the virus donor are most closely related to the "early" proviral DNA sequence in the PBMC of the virus recipient (26). Also, in anti-retroviral therapy in HIV-1 infection, mutations which cause resistance to the drug first appear in the viral RNA sequences of free virus in the blood and later in the proviral DNA in PBMC (68). Therefore, depending on the type of application, choices must be made whether RNA or DNA will be used, if consensus or cloned sequences are needed, and when the samples should be taken.

Because we wanted to cover both molecular detection and molecular epidemiological investigation and take into account the reported differences between RNA and DNA sequences, we needed a standardized PCR method to amplify both viral RNA and DNA. Thus, the aim of our study was the development of a PCR method suitable for both the amplification of HIV-1, HIV-2, HTLV-I, or HTLV-II RNA and DNA which can be used for detection and for amplification of parts of the genomes which can be used for direct sequencing.

2. MATERIALS AND METHODS

2.1 PCR and virus-culture controls

The cell lines U1, U937HIV-2_{MS}, and MT-2 (AIDS Research and Reference Reagent Program Catalog, nos. 165, 127, and 237, respectively) were used as positive controls for HIV-1, HIV-2, and HTLV-I DNA-PCR, respectively. The supernatant of the cell lines H9/HTLV-IIIb (AIDS Research and Reference Reagent Program Catalog, no 400), U937HIV-2_{MS}, and MT-2 were used as positive controls for HIV-1, HIV-2, and HTLV-I RNA-PCR, respectively.
The parental H9 cellline (AIDS Research and Reference Reagent Program Catalog, no 87), which is negative for retrovirus sequences, was used as a negative control for the DNA-PCR. Healthy donor plasma was obtained from the Blood Bank Utrecht to serve as a negative control in the PCR for retroviral RNA sequences.
For quantitation of infectious HIV-1, we used the supernatant of H9/HTLV-IIIb cells as a positive control and culture medium as a negative control.

The cell line U1, which shows minimal constitutive expression of HIV-1, has two integrated copies of HIV-1 proviral DNA per cell (22, 60). The cell line U937HIV-2_{MS} yields high levels of cell-free HIV-2_{MS}, but the amount of HIV-2 proviral DNA copies per cell is not known (35). The cell line MT-2 has at least 8 integrated copies of HTLV-I proviral DNA per cell; one of which is a complete genome and the others are defective (39). The part of the HTLV-I genome which we have chosen as a target for amplification is present in at least 6 copies. The cell line is a continuous producer of HTLV-I virions. The H9/HTLV-IIIb cell line is a constitutive producer of HIV-1 virions.

The cell lines were cultured at a density of 4-7 x 10^5/ ml in 50 ml RPMI 1640 medium (Gibco BRL, Life Technologies B.V., Breda, The Netherlands) supplemented with 11% fetal calf serum, 20 U/ml human recombinant interleukin-2 (Boehringer-Mannheim B.V., Almere, The Netherlands), 0.05 mM β-mercaptoethanol, 100 U/ml streptomycin, 100 U/ml penicillin, and 2 mM glutamin in Nunclon 80-cm^2 culture flasks (A/S Nunc, Denmark), at 37° C and 5% CO_2. The medium was changed and the amount of cells adjusted twice weekly.

2.2 PCR of HIV-1, HIV-2 and HTLV-I sequences

2.2.1 Selection of primers for PCR

A two-step PCR protocol with nested primers was used. The selected nested primer pairs are listed in Table 1.

Table 1 Description of the primers used

Primer	Description	Virus	Sequence (5'-3')	Gene and Position[1]
JA4[2]	outer, sense	HIV-1	G A A G G C T T T C A G C C C A G A A G	gag (1271-1290)
JA5	inner, sense	HIV-1	A C C A T C A A T G A G G A A G C T G C	gag (1398-1417)
JA6	inner, antisense	HIV-1	T A T T T G T T C C T G A A G G G T A C	gag (1529-1510)
JA7	outer, antisense	HIV-1	T C T C C T A C T G G G A T A G G T G G	gag (1567-1548)
JA9	outer, sense	HIV-1	C A C A G T A C A A T G T A C A C A T G	env (6952-6971)
JA10	inner, sense	HIV-1	A A A T G G C A G T C T A G C A G A A G	env (7006-7025)
JA11	inner, antisense	HIV-1	A C A A T T T C T G G G T C C C C T C C	env (7338-7319)
JA12	outer, antisense	HIV-1	A C A G T A G A A A A A T T C C C C T C	env (7378-7359)
JA17	outer, sense	HIV-1	T A C A G G A G C A G A T G A T A C A G	pol (2326-2345)
JA18	inner, sense	HIV-1	G G A A A C C A A A A A T G A T A G G G	pol (2376-2395)
JA19	inner, antisense	HIV-1	A T T A T G T T G A C A G G T G T A G G	pol (2505-2486)
JA20	outer, antisense	HIV-1	C C T G G C T T T A A T T T T A C T G G	pol (2592-2573)
AM1	outer, sense	HIV-2	T G A G A C T G C A G G G A C T T T C	ltr (9380-9398)
AM2[3]	inner, sense	HIV-2	A G G A G C T G G T G G G G A A C G	ltr (9432-9449)
AM3	inner, antisense	HIV-2	G G A A C A C C C A G G C T C T A C C	ltr (9575-9557)
AM4[4]	outer, antisense	HIV-2	A G C A A G C G T G G A G C C G T C T	ltr (9631-9613)
PW1[5]	outer, sense	HTLV-I	C G G A T A C C C A G T C T A C G T G T	tax/rex (7359-7378)
		HTLV-II[6]	T . . . . C	tax/rex (7248-7268)
PW3	inner, sense	HTLV-I[6]	. . . . A . . C	tax/rex (7375-7394)
		HTLV-II	G T G T T T G G C G A T T G T T G T A C A	tax/rex (7264-7283)
PW4	inner, antisense	HTLV-I	T C G A T G G G G T C C C A G G T	tax/rex (7499-7483)
		HTLV-II[6]	. . . .	tax/rex (7388-7372)
PW2	outer, antisense	HTLV-I	G A G C C G A T A A C G C G T C C A T	tax/rex (7517-7499)
		HTLV-II[6]	. . . . T . . C	tax/rex (7406-7388)

1) Gene and position according to the following sequences:
   HIV-1 reference : HIV-1 isolate HXB2, GenBank accession no. K03455.
   HIV-2 reference : HIV-2 isolate ROD, GenBank accession no. M15390.
   HTLV-I reference : HTLV-I proviral sequence of leukemia cell DNA, GenBank accession no. J02029.
   HTLV-II reference : HTLV-II proviral sequence of infectious clone, GenBank accession no. M10060.
2) JA primers as described by Albert and Fenyö (1).
3) identical to SK89 (61).
4) the underlined nucleotide is in the ROD isolate a G, however, all other HIV-2 sequences used for the alignment (see text) have an A at that site.
5) PW1 en PW3 are identical to the outer sense and inner sense primers described by Tuke et al. (73). PW2 is one nucleotide shorter at the 3' end than the outer antisense primer described by Tuke et al. (73). PW4 is 3 nucleotides shorter at the 5' end and 3 nucleotides longer at the 3' end than the inner antisense primer described by Tuke et al. (73).
6) mismatched nucleotides are indicated; a dot means that the nucleotides are identical in both sequences.

Three nested primer sets were selected for detection of HIV-1 sequences: JA4-JA7 (*gag* gene), JA9-JA12 (*env* gene), and JA17-JA20 (*pol* gene) designed by Albert and Fenyö (1). These primer sets were chosen because they were specially designed for

nested PCR and they are located in three different genes. HIV-1 could be detected in all HIV-seropositive patients studied with these primer sets (1, 2). The nested primer set JA9-JA12, which amplifies the coding sequence of the V3 loop of gp120 envelop protein, was also selected for use in the molecular epidemiological investigation of HIV-1 infection.

The LTR region was selected for detection of HIV-2 sequences. The AM2 primer was identical to SK89 (61), whereas the other 3 primers of the nested set were designed by us (see Table 1) based on the alignment of the sequences of the HIV-2 strains HIV2BEN, HIV2DI94, HIV2ISY, HIV2ROD, HIV2CAM2, and HIV2NIHZ obtained from GenBank.

The nested primer sets selected for the detection of HTLV-I and HTLV-II are essentially the same as those described by Tuke et al. (73), with minor modifications of the inner and outer antisense primers (see Table 1). These primer sets have been used successfully for the detection and differentiation of HTLV-I and HTLV-II infections (73).

2.2.2. DNA preparation

DNA was isolated from cells using the method essentially as described by Kawasaki (37). Briefly, 1 x 10^6 cells were washed twice in phosphate buffered saline (PBS) and vortexed in 100 µl isotonic high-pH buffer (50 mM Tris-HCl at pH 8.5, 140 mM NaCl, 6 mM $MgCl_2$, 0.5% Nonidet P-40, and 5U RNAguard [Pharmacia Biotech B.V., Woerden, The Netherlands]), after which the nuclei were pelleted for 10 seconds in a microfuge. The supernatant containing cytoplasmic RNA was either discarded or stored for future use. The nuclei were resuspended in 100 µl K-buffer (50 mM Tris-HCl at pH 8.5, 50 mM NaCl, 6 mM $MgCl_2$, 0.5% Nonidet P-40, 0.5% Tween 20, and proteinase K [Sigma; Brunschwig chemie, Amsterdam, The Netherlands] to 60 µg/ml). After incubation for one hour at 60° C, the proteinase K was heat-inactivated for 10 minutes in boiling water. The DNA preparation was either used immediately or stored at -70° C.

2.2.3. RNA preparation

Three different protocols for the isolation of viral RNA were evaluated:

1) The conventional protocol:
   Guanidium thiocyanate (GTC) disruption of the virus followed by phenol-chloroform extraction and 2-propanol precipitation of the RNA
2) The SDS/Dynabeads protocol:
   SDS disruption of the virus followed by extraction of the RNA with oligo$(dT)_{25}$-coated paramagnetic beads (Dynabeads® Oligo$(dT)_{25}$, Dynal AS., Oslo, Norway)

3) The GTC/Dynabeads protocol:
   GTC/detergent disruption of the virus followed by extraction of the RNA with oligo$(dT)_{25}$-coated paramagnetic beads.

Each protocol is briefly described below.

The conventional protocol

This protocol was essentially the one described by Chomezynski and Sacchi (15). Briefly, 60 µl plasma or control was mixed with 4 µl Poly A (Boehringer-Mannheim; 4 mg/ml), 300 µl denaturation fluid (4 M GTC, 25 mM Na-citrate pH 7, 0.5% sodium lauryl sarcosinate, 0.1 M β-mercaptoethanol), 240 µl phenol equilibrated in 10 mM Tris-HCl at pH 7.5, and 240 µl chloroform/isoamylalcohol (49:1) and shaken for 10 minutes, after which the precipitate was pelleted for 20 minutes at maximum speed in a microfuge. The water phase was mixed with 200 µl phenol and a 200 µl chloroform/isoamylalcohol mixture, shaken for 10 minutes, and centrifuged for 5 minutes at maximum speed in a microfuge. The water phase was then mixed with a 400 µl chloroform/isoamylalcohol mixture, shaken for 10 minutes, and centrifuged for 5 minutes at maximum speed in a microfuge. Finally, the water phase was mixed with 35 µl 3 M NaAc (pH 5.2) and 400 µl 2-propanol and incubated overnight at -20° C. The precipitate was pelleted by centrifugation at maximum speed for 20 minutes in a cool (4° C) microfuge. The pellet was washed once with 80% ethanol of -20° C, dried, redissolved in 10 µl 1x PCR buffer (50 mM Tris-HCl at pH 8.5, 50 mM NaCl, 6 mM $MgCl_2$, 2 mM DTT), and immediately transferred to the first PCR reagents mix of the nested-primer reverse-transcription PCR (npRT-PCR).

The SDS/Dynabeads protocol

A modification of the protocol described by Chiodi et al. (14) was used. Serum, plasma, and control specimens (250 µl) were each mixed with 50 µl Vanadyl ribonucleosyl complexes (VRCs, 200 mM; Gibco BRL) and 10 µl tRNA (Boehringer-Mannheim; E. coli 5 mg/ml in lysis buffer). Lysis-buffer (LB) consisted of 10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 0.5 M LiCl, and 2% sodium dodecyl sulfate (SDS). Then, 175 µl 2x LB and 640 µl 1x LB were added and thoroughly mixed, whereafter 50 µl (250 µg) prewashed oligo$(dT)_{25}$-coated paramagnetic beads were added and mixed. After an incubation of at least 4 minutes on ice, in which polyadenylated RNA was allowed to bind to the beads, the beads were washed four times in 300 µl of washing buffer (10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 0.15 M LiCl) using a magnetic particle concentrator (Dynal MPC®-M, Dynal AS., Oslo, Norway). After the last washing, the supernatant was completely removed and the beads were resuspended in 10 µl 1x PCR buffer and immediately transferred to the first PCR reagents mix of the npRT-PCR.

The GTC/Dynabeads protocol

The Dynal AS protocol for the extraction of mRNA from cells and tissues with detergent in the presence of GTC (21) was used and further developed by us for the isolation of retroviral RNA from serum, plasma, or culture supernatant, as will be described in this report. The sample (250 µl) was mixed thoroughly with 400 µl extraction buffer (4 M GTC, 0.1 M Tris-HCl at pH 8.0, 0.5% sodium lauryl sarcosinate, 1% DTT). Then, 445 µl dilution buffer (29 mM Tris-HCl at pH 8.0, 1.46 M LiCl, 2.9 mM EDTA) was added and thoroughly mixed, whereafter 50 µl (250 µg) prewashed oligo$(dT)_{25}$-coated paramagnetic beads were added and mixed. After an incubation of at least 4 minutes on ice, the beads were washed twice in 300 µl washing buffer with SDS (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.15 M LiCl, 0.1% SDS) using a magnetic particle concentrator. The beads were washed another 3 times with washing buffer without SDS (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 0.15 M LiCl), after which the supernatant was completely removed and the beads were resuspended in 10 µl 1x PCR buffer and immediately transferred to the first PCR reagents mix of the npRT-PCR.

2.2.4. Nested-primer reverse-transcription polymerase chain reaction (npRT-PCR) and nested-primer polymerase chain reaction (np-PCR)

We used npRT-PCR for the detection of retroviral RNA, for which we combined the solid-phase reverse-transcription (cDNA) reaction and the first PCR reaction with outer

Table 2 Amounts of primers to be used in 50-µl reagents mix and amplicon length of the second PCR product

Primer set	Type	Amount of each primer (pmol)	Amplicon length (bp)
JA4-JA7	outer	1	296
JA5-JA6	inner	20	131
JA9-JA12	outer	1	426
JA10-JA11	inner	10	332
JA17-JA20	outer	5	266
JA18-JA19	inner	20	129
AM1-AM4	outer	1	251
AM2-AM3	inner	10	143
PW1-PW2	outer	1	158
PW3-PW4	inner	20	124

primers in one tube. The sample (10 µl) was added under the oil layer to the cDNA/first PCR reagents mix (40 µl). The final concentrations in 50 µl were: 50 mM Tris-HCl (pH 8.5), 50 mM NaCl, 6 mM $MgCl_2$, 2 mM DTT, 1 mM of each dNTP (Boehringer-Mannheim), 1 U AmpliTaq DNA polymerase (Perkin-Elmer Nederland B.V., Gouda, The Netherlands), 5 U RNAguard (Pharmacia), 2 U AMV-RT (Promega Corporation, Leiden, The Netherlands), and outer primers (for the amount of primers, see Table 2).

Table 3 Temperature cycle program for npRT-PCR and np-PCR

Program step		Type	Cycles	Description	Time	Temperature
npRT-PCR	np-PCR					
1		temp. cycle	1	reverse transcription		
				1) primer annealing	10 min.	20° C
				2) reverse transcription	1 hour	42° C
				3) reaction termination	10 min.	95° C
2	1	temp. cycle	15	first PCR amplification		
				1) denaturation	30 sec.	95° C
				2) primer annealing	30 sec.	50° C
				3) extension	1 min.	72° C
				(time extended each cycle with 2 sec.)		
3	2	temp. cycle	1	final extension	10 min.	72° C
4	3	temp. cycle	2	second PCR, touchdown amplification		
				2 cycles each temperature step		
				1) denaturation	30 sec.	95° C
				2) primer annealing	30 sec.	65-56°C
				(1° C lower every 2 cycles)		
				3) extension	1 min.	72° C
5	4	temp. cycle	30	second PCR, final amplification		
				1) denaturation	30 sec.	95° C
				2) primer annealing	30 sec.	55° C
				3) extension	1 min.	72° C
				(time extended each cycle with 2 sec.)		
6	5	temp. cycle	1	final extension	10 min.	72° C
7	6	soak	1	storage		4° C

We used np-PCR for the detection of retroviral provirus DNA. The sample (10 µl) was added under the oil layer to the first PCR (40 µl) reagents mix. Final concentrations were the same as those for the cDNA/first PCR reaction of the npRT-PCR, with exception of RNAguard and AMV-RT. Five µl of the product of the (cDNA/)first PCR was amplified in the second PCR with inner primers. The final concentrations in the

second PCR (50 µl) were the same as in the first reaction of the np-PCR (for the amount of primers, see Table 2).

The synthesis of cDNA and the amplification of cDNA or DNA templates were performed on an automated DNA thermal cycler (Perkin-Elmer) with the temperature protocol outlined in Table 3. Only the reverse-transcription step was omitted when amplifying DNA. The nested reaction was performed as a touchdown PCR to improve the specificity (19).

The final PCR product (10 µl) was analyzed by electrophoresis on a 4% agarose gel (Nusieve 3:1, FMC Bioproducts, Rockland, USA) and stained with ethidium bromide. The specific length of the amplicon for each primer set is shown in Table 2.

2.2.5. Determination of sensitivity of PCR

Serial ^{10}log dilutions of DNA preparations from a known amount of control cells were used to determine the sensitivity of the np-PCR. The determination of the sensitivity of the npRT-PCR was performed for HIV-1 only, because only the culture supernatant of the H9/HTLV-IIIb cell line could be easily titrated. Serial ^{10}log dilutions of plasma spiked with culture supernatant were used to determine the sensitivity. The HIV-1 virion concentration of the culture supernatant was determined by $TCID_{50}$ end-point titration on MT-2 cells. Throughout the study, we used a stock culture supernatant containing 2.9×10^5 $TCID_{50}$ HIV-1/ml.

2.3. Restriction-enzyme analysis of the HTLV-I amplicon

The amplicon of the predicted size (see Table 4) of the second PCR was typed by restriction-enzyme analysis using the endonucleases Sau3A and Taq1, as described by Tuke et al. (73).

Table 4 Fragment length of HTLV-I and HTLV-II PW2-PW3 amplicon, undigested and digested with Sau3A or Taq1

Virus	Fragment length (bp)		
	Undigested	Digested with Sau3A	Digested with Taq1
HTLV-I	124	103, 21	121, 3
HTLV-II	124	124	68, 53, 3

2.4. Clinical samples

A total of fifteen CDC class II patients with a complete WB pattern was selected for the study. Eight patients (patients 1 to 8) were treated at the University Hospital Utrecht and freshly prepared plasma samples were obtained from each of them. Quantitative virus culture of six samples was started within 2 hours after venipuncture. Four patients received anti-retroviral therapy, AZT alone or in combination with ddC or ddI. The remaining seven patients were seropositive hemophiliacs (patients 9 to 15) from the Van Creveldclinic in Utrecht. Heparinized whole blood samples and clothed whole blood samples were obtained from one venipuncture. None of these seven patients received anti-retroviral therapy. The samples were processed within 2 hours after venipuncture. Plasma or serum was separated from cells of the heparinized sample or from the bloodcloth of the clothed sample, respectively, by centrifugation for 10 minutes at 900xg and stored at -20° C for the HIV-1 PCR and p24 antigen assays. PBMC were isolated from the cell fraction of the heparinized sample by Ficoll-Paque (Pharmacia) gradient centrifugation in LeucoSep tubes (Greiner B.V., Alphen a/d Rijn, The Netherlands) and subsequently frozen at -135° C in culture medium with 10% DMSO. They were rapidly thawed in handwarm water for immediate DNA isolation.
The npRT-PCR of plasma samples and np-PCR of PBMC samples were carried out with all three HIV-1 primer sets. Serum samples were only tested with the HIV-1 *pol* primer set in the npRT-PCR assay. All samples had been freeze-thawed only once when tested.

2.5. Detection of HIV-1 p24 antigen

Serum, plasma, and culture supernatant were assayed for HIV-1 p24 antigen using a commercial enzyme immunoassay (Abbott HIVAG-1 Monoclonal; Abbott Laboratories, North Chicago, IL, USA). The results are expressed as ratios of the sample O.D. value and the cutoff O.D. value or in pg p24/ml of sample.

2.6. End-point dilution plasma culture of HIV-1

We used a modification of the method described by Ho et al. (28). Briefly, plasma was cleared of all debris by centrifugation for 15 minutes at 3000xg. Decreasing volumes (1000 µl in twofold and 250 µl, 62 µl, 16 µl and 4 µl in fourfold) were incubated for 24 hours at 37° C and 5% CO_2 with 2×10^6 3-day phytohemagglutinin-activated PBMC from healthy blood-bank donors in 1.5 ml culture medium (RPMI 1640 with 11% fetal calf serum, 20 U/ml interleukin-2 [human, recombinant, Boehringer-Mannheim], 0.05 mM β-mercaptoethanol, 100 U/ml streptomycin, 100 U/ml penicillin, 2 mM glutamin). After 24 hours, the cells were washed three times with culture medium, resuspended in

150 µl culture medium with 2 µg/ml polybrene, and transferred to Transwell inserts (Costar Europe Ltd, Badhoevedorp, The Netherlands; pore size 0.4 µm), which were fitted in wells of a Mark II 24-well tissue-culture cluster (Costar; type 3424) filled with 900 µl culture medium supplemented with 2 µg/ml polybrene. The cells were cultured for 4 weeks at 37° C and 5% CO_2. The inserts with the cells were transferred to a new 24-well tissue-culture cluster filled with fresh medium twice weekly. The medium left in the old tissue-culture cluster was assayed for HIV-1 p24 antigen. A culture was considered positive when the ratio of the HIV-1 p24 antigen assay was $\geq$1.00. The titer was calculated as the highest dilution which became positive after 4 weeks of culture.

3. RESULTS

3.1. PCR method to detect retroviral RNA and DNA

3.1.1. Optimization of PCR parameters

For reasons of convenience, we wanted to use the same protocol for all viruses as much as possible. Therefore, we optimized the reactions in such a way that the same temperature-cycle protocol and the same reagents mix could be used. Also for reasons of convenience, we used a two-step PCR protocol with nested primers, because the nested reaction improves the specificity to the extent that probe hybridization is not needed for confirmation (2, 81). We selected a temperature protocol and a reagents mix to start with and then we optimized the other parameters. We wanted to have a reaction in which the product of the first PCR is not visible when analyzing the product of the second PCR. This can be achieved in two ways: first, by reducing the number of cycles of the first PCR and second, by reducing the concentration of the primers of the first PCR, both resulting in lower amounts of product in the first PCR. Because it is more convenient to use different primer concentrations than different temperature-cycle protocols when performing reactions with different primer sets at the same time, we first optimized the number of cycles of the first PCR and then the concentration of each primer set. Finally, the primer concentration of the second PCR was optimized in such a way that the total PCR was most sensitive, detecting the lowest amount of DNA templates and, most specifically, showing only product with a specific length.

Temperature-cycle protocol and reagents mix

Based on the calculated Tm values of the primers with the computer program OLIGO (MedProbe A.S., Oslo, Norway), we selected one temperature-cycle protocol to start with (see Table 3). The annealing temperature for the first PCR was set at 50° C and for the second PCR at 55° C. To make the amplification more specific, a touch-down protocol was used for the second PCR (19). The reagents mix that is successfully used at our laboratory for the amplification of influenza virus RNA sequences was selected for this PCR method (11).

Number of cycles

The numbers of cycles in the first and in the second PCR were optimized with the JA17-JA20 primer set (5 pmol primers JA17 and JA20 each in the first PCR and 10 pmol primers JA18 and JA19 each in the second PCR) and HIV-1 RNA samples extracted from dilutions of culture supernatant containing 7200, 72, and 0.72 $TCID_{50}$

HIV-1, using the SDS/Dynabeads protocol. The first PCR was performed with 10, 20 and 30 cycles. The second PCR was performed according to the touch-down protocol followed by 30 cycles at 55° C. At 10 cycles, there was no visible product in the first PCR at any concentration, while even 0.72 $TCID_{50}$ HIV-1 could be detected after the second PCR (data not shown). At 20 cycles, there was visible product in the first PCR with 7200 $TCID_{50}$ HIV-1 (data not shown). Because we wanted to have no visible product in the first PCR and no reduction in sensitivity, we selected 15 cycles as the number of cycles for the first PCR. Because 0.72 $TCID_{50}$ HIV-1 (approximately 0.5 infectious particle) could be detected, the cycle count of the second PCR was not further optimized. Furthermore, these results showed that the cDNA reaction combined with the first PCR was as effective since 0.72 $TCID_{50}$ HIV-1 could be detected. Therefore, the cDNA reaction also needed no further optimization. Because the cDNA reaction was so effective, we concluded that the same temperature-cycle protocol, without the cDNA step, is also applicable to amplification of proviral DNA sequences.

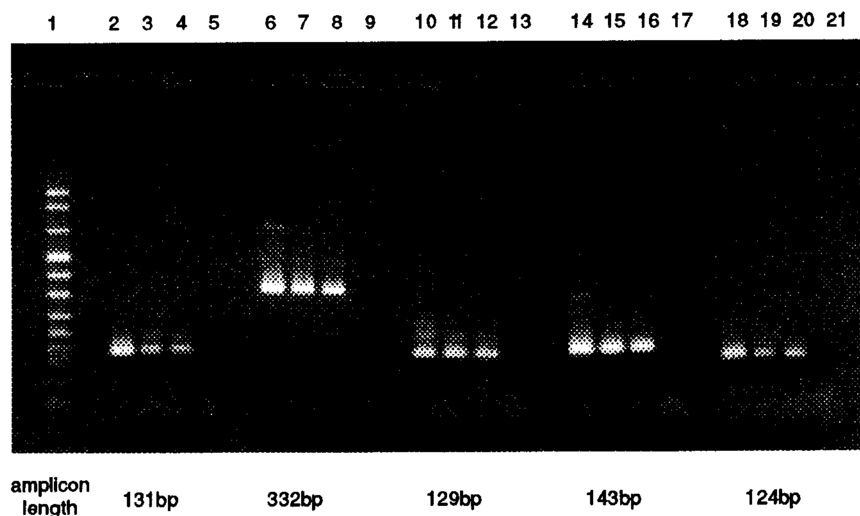

Fig. 1. Amplification of HIV-1, HIV-2, and HTLV-I proviral DNA with np-PCR from ^{10}log dilutions of DNA derived from cell lines. **Lane 1**: molecular weight marker. **Lanes 2-5, 6-9, 10-13**: HIV-1, ^{10}log dilutions containing DNA of 10^4, 10^0, 10^{-1}, or 10^{-2} U1 cells. **Lanes 2-5**: amplification with JA4-7 primers. **Lanes 6-9**: amplification with JA9-12 primers. **Lanes 10-13**: amplification with JA14-17 primers. **Lanes 14-17**: HIV-2, ^{10}log dilutions containing DNA of 10^4, 10^2, 10^1 or 10^0 U937HIV-2_{MS} cells, amplification with AM1-4 primers. **Lanes 18-21**: HTLV-I, ^{10}log dilutions containing DNA of 10^4, 10^0, 10^{-1}, or 10^{-2} MT-2 cells, amplification with PW1-4 primers.

Primer concentration

The concentration of each primer set was optimized using serial ^{10}log dilutions of positive control-cell DNA in PCR buffer corresponding to the DNA of 10^4 to 10^{-2} cells.

The optimal primer concentrations we found are shown in Table 2. Only at the highest template concentration and 30 cycles the first PCR show a faint band of product with specific length, except for the primer set JA4-JA7 (data not shown). These results show that amplification occurred at the concentrations used, and it may be supposed that product is also formed at 15 cycles. No specific product was formed when using parental H9 cell line control DNA. When analyzing the product of the second PCR performed with the normal temperature-cycle protocol, product of the first PCR (faint bands above the clearly visible product of the second PCR) was barely visible (Fig. 1 lanes 2-4, 6-8, 10-12, 14-16, 18-20), even at high template input (Fig. 1 lanes 2, 6, 10, 14, 18). Also, no specific product was formed when using parental H9 cell line control DNA (not shown).
At the primer concentrations used for the first PCR, cDNA was effectively synthesized and amplified with all the primer sets when using RNA as a template (data not shown). This supports our conclusion that the protocol is also suitable for npRT-PCR.

3.1.2. Sensitivity and specificity

At the optimal primer concentrations, the lowest detectable amount of DNA with np-PCR corresponded with 10^{-1} U1 cells for HIV-1 and all the JA primer sets (Fig. 1, JA17-20 lane 4, JA4-7 lane 8, JA9-12 lane 12). For HIV-2 and the AM primer set the lowest detectable amount corresponded with 10 U937HIV-2_{MS} cells (Fig. 1, lane 16). For HTLV-I and the PW primer set, it corresponded with 10^{-1} MT-2 cells (Fig. 1, lane 20). Only product of predicted length was formed.

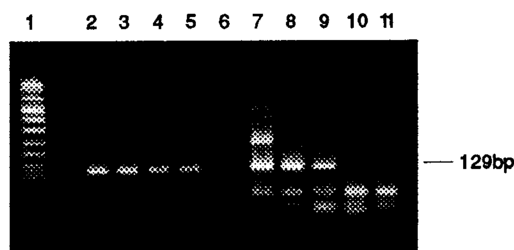

Fig. 2. Two-step amplification of HIV-1 proviral DNA with nested primers (np-PCR) compared with one-step amplification with a single primer pair (sp-PCR). **Lane 1**: molecular weight marker. **Lanes 2-6, 7-11**: ^{10}log dilutions of DNA, derived from H9/HTLV-IIIb cells, containing DNA of 10^3, 10^2, 10^1, 10^0, or 10^{-1} cells. **Lanes 2-6**: np-PCR amplification with nested primer set JA17-20. **Lanes 7-11**: sp-PCR amplification with primer pair JA18-19 only.

We compared the sensitivity of the np-PCR using the nested primer set JA17-JA20 with that of a PCR using the single inner primer pair (sp-PCR) JA18-JA19, with serial

^{10}log dilutions of positive control cell DNA (H9/HTLV-IIIb) corresponding with DNA of 10^3 to 10^{-1} cells. The temperature-cycle program of the sp-PCR consisted of the touch-down program of the protocol with the nested primers followed by 45 cycles at 55° C. The total number of cycles was the same for both PCRs. We detected DNA in the sample one dilution step further with the np-PCR than with the sp-PCR (Fig. 2, lane 5 versus lane 10). At high cellular DNA concentrations there were additional bands with the sp-PCR (Fig. 2, lane 7) while there was only a product band of predicted length with the np-PCR (Fig. 2, lane 2).

The specificity was further tested by applying the PCRs for each virus, HIV-1, HIV-2, and HTLV-I, with primers designed for the two other viruses. None of the primers amplified sequences of the virus for which it was not designed (data not shown).

The sensitivity of the npRT-PCR was evaluated with the primer set JA17-JA20 only with serial ^{10}log dilutions of plasma spiked with HIV-1 virions (0.72 to 0.0072 $TCID_{50}$). RNA was extracted using the GTC/Dynabeads protocol. We detected 0.072 $TCID_{50}$ HIV-1 with this protocol (Fig. 3, lane 3).

3.1.3 Comparison of three RNA extraction methods

Three different methods were evaluated for their easy of performance and sensitivity to extract (retroviral) RNA from serum or plasma: (i) the conventional method, (ii) the SDS/Dynabeads method, and (iii) the GTC/Dynabeads method.

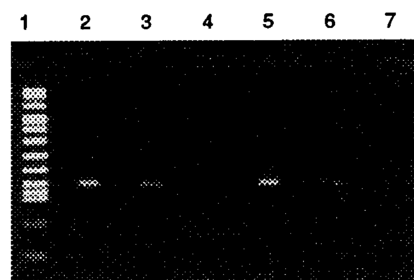

Fig. 3. Comparison of the GTC/Dynabeads protocol with the conventional protocol for isolation of HIV-1 RNA from plasma. Detection of RNA by npRT-PCR amplification with nested primer set JA17-20. **Lane 1**: molecular weight marker. **Lanes 2-4, 5-7**: ^{10}log dilutions of plasma containing 0.72, 0.072 or 0.0072 $TCID_{50}$ HTLV-IIIb. **Lanes 2-4**: results with GTC/Dynabeads protocol. **Lanes 5-7**: results with conventional protocol.

A major problem in the extraction of RNA from plasma is the existence of RNAses in plasma. Without taking precautions to destroy or inhibit the RNAses in plasma, RNA

corresponding with even 2.9 x 10^4 $TCID_{50}$ HIV-1 was totally degraded within the time necessary to extract the RNA with the SDS/Dynabeads protocol (data not shown). To circumvent this problem, we compared two methods in which retroviral RNA is extracted with oligo(dT)$_{25}$-coated paramagnetic beads, making use of the fact that retroviral RNA is polyadenylated. The two methods differ in how RNAase activity is managed and how the virus is disrupted. In the SDS/Dynabeads method, RNAses are inhibited by adding VRCs and tRNA to the sample, whereafter the virus is disrupted by SDS treatment. In the GTC/Dynabeads method, the RNAses are degraded and at the same time the viruses are disrupted by adding GTC and detergent to the sample. We also compared the GTC/Dynabeads method with the conventional method of RNA extraction in which GTC was also used to degrade RNAses and to disrupt the virus, but where RNA is extracted by phenol and chloroform/isoamylalcohol followed by 2-propanol precipitation. The methods were evaluated with npRT-PCR using the primer set JA17-JA20 only with serial ^{10}log dilutions of plasma spiked with HIV-1 virions.

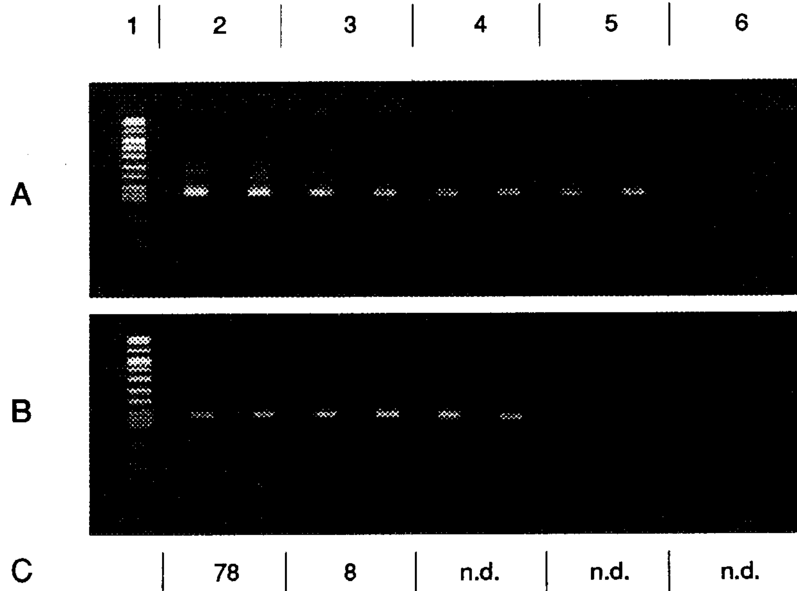

Fig. 4. Comparison of two methods for HIV-1 RNA extraction from plasma with Dynabeads oligo(dT)$_{25}$, and their relation to HIV-1 p24 antigen detection. **A.** Results with GTC/Dynabeads isolation protocol. **B.** Results with SDS/Dynabeads protocol. **A/B.** Detection of RNA by npRT-PCR amplification with primers JA17-20. **Lane 1**: molecular weight marker. **Lanes 2-5**: duplicates of ^{10}log dilutions of plasma containing 72, 7.2, 0.72, or 0.072 $TCID_{50}$ HTLV-IIIb. **Lane 6**: HIV-1 antibody-negative donor plasma. **C.** Abbott p24 Ag EIA of the same samples as A/B, numbers indicate pg p24/ml of sample (n.d. = not detectable).

The GTC/Dynabeads method was as sensitive as the conventional method, extracting RNA from the ^{10}log dilutions down to 0.072 $TCID_{50}$ HIV-1 (Fig. 3, lane 3 versus lane 6).

The SDS/Dynabeads method was less sensitive, extracting RNA from the ^{10}log dilutions only down to 0.72 $TCID_{50}$ HIV-1 (Fig. 4B, lane 4).

Finally we compared the two Dynabeads methods with the HIV-1 p24 antigen EIA, another method to indirectly detect virions in plasma. We tested the same samples used for npRT-PCR in the HIV-1 p24 antigen EIA. The EIA was 100-fold less sensitive than the GTC/Dynabeads protocol followed by npRT-PCR (Fig. 4C).

3.2 Restriction-enzyme analysis of HTLV-I amplicon

The product of the np-PCR of MT-2 cell DNA amplified with the PW1-4 primer set was digested with either Sau3A or Taq1. The restriction pattern was as predicted (Fig. 5, Table 4).

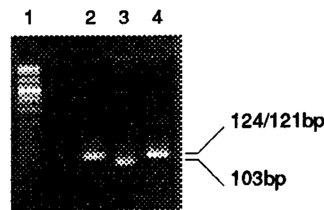

Fig. 5. Amplification by np-PCR of HTLV-I proviral DNA derived from the MT-2 cell line, and digestion analysis of the amplification product. Amplification was conducted with PW1-4 primers. **Lane 1**: molecular weight marker. **Lane 2**: undigested amplification product. **Lane 3**: Sau3A-digested amplification product. **Lane 4**: Taq 1-digested amplification product.

3.3 HIV-1 RNA and DNA detection in clinical samples

Principally, to get an idea of the usefulness of the method for detecting RNA or DNA in clinical samples, we tested 7 paired serum/plasma/PBMC samples and 8 single plasma samples from a total of 15 CDC class II patients. PBMC and plasma samples were examined with all three HIV-1 primer sets, serum samples with HIV-1 *pol* primers only. The results are shown in Table 5 and Table 6. Some relvant aspects of the method we used for quantitative plasma virus culture are described in the next section.

All PBMC samples were positive with at least one primer set, 6 (85.7%) with at least two primer sets, and 4 (57.1%) were positive with all three primer sets. Five (33.3%) of the plasma samples were negative with all three primer sets, one of which was obtained from a patient on anti-retroviral therapy (AZT/ddI). The plasmas of three other patients on anti-retroviral therapy (AZT, AZT/ddI, AZT/ddC), however, were positive

Table 5 Pilot study of HIV-1 PCR on clinical samples of CDC class II patients and its relation to relevant factors in HIV infection

Patient no.	Sample	Anti-retroviral therapy	CD4 count /mm^3 [$]	p24 Ag ratio	Plasma viral load (titer)	PCR		
						GAG	POL	ENV
1	plasma	AZT	94	0.27	1/16	+	+	+
2	plasma	-	446	0.42	1/62	+	-	+
3	plasma	-	576	**2.80 (+)**	1/125	+	+ *	+
4	plasma	AZT/ddC	33	0.45	undiluted	+	+ *	+
5	plasma	-	375	0.45	1/125	+	+ *	+
6	plasma	-	3	**2.18 (+)**	1/100	-	- *	-
7	plasma	AZT/ddI	221	0.28	n.d.	-	- *	-
8	plasma	AZT/ddI	6	0.25	n.d.	+	+ *	+
9	plasma	-	346	0.33	n.d.	-	+ *	+
	serum			0.22		n.d.	+	n.d.
	PBMC					+	-	+
10	plasma	-	243	0.29	n.d.	+	+	+
	serum			0.23		n.d.	+	n.d.
	PBMC					+	+	+
11	plasma	-	899	0.29	n.d.	-	-	-
	serum			0.22		n.d.	+	n.d.
	PBMC					+	+	+
12	plasma	-	21	0.32	n.d.	-	-	-
	serum			0.29		n.d.	-	n.d.
	PBMC					+	+	+
13	plasma	-	570	0.26	n.d.	+	+	+
	serum			0.22		n.d.	+	n.d.
	PBMC					+	+	+
14	plasma	-	331	0.39	n.d.	-	+	-
	serum			0.28		n.d.	+	n.d.
	PBMC					-	+	+
15	plasma	-	669	0.25	n.d.	-	-	-
	serum			0.24		n.d.	-	n.d.
	PBMC					-	+	-

n.d. = not done

[$] CD4 counts were performed at University Hospital Utrecht.

* both SDS/Dynabeads and GTC/Dynabeads protocol were used for RNA extraction, while only the GTC/Dynabeads protocol was used for the rest.

with all three primer sets. Ten (66.6%) of the plasma samples were positive with at least one primer set, 9 (60%) with at least two primer sets, and 7 (46.6%) with all three primer sets. It was not possible to detect HIV-1 sequences in all HIV-1-infected individuals using only one primerset, neither with PBMC nor with plasma as sample source (Table 6).

Table 6 Cumulative results of HIV-1 PCR based on sample source and amplified region of the HIV-1 genome

Sample source	Total no. tested	Number positive per region of the HIV-1 genome and percentage of total		
		GAG	POL	ENV
Plasma	15	8 (53%)	9 (60%)	9 (60%)
Serum	7	n.d.	5 (71.4%)	n.d.
PBMC	7	5 (71.4%)	6 (85.7%)	6 (85.7%)

n.d. = not done

There was almost no difference when using serum or plasma as a source for HIV-1 RNA. One of 7 serum/plasma sample pairs gave inconsistent results (patient 11, Table 5); the serum was positive, whereas the plasma was negative.

3.4 End-point dilution plasma culture of HIV-1

We chose the method described by Ho et al. (28) because they reported 100% culture-positive results in 54 patients in the CDC classes II/III and IV. We modified the method, however, to make refreshment of the culture medium easier and less sensitive to contamination with bacteria or fungi. Ho et al. cultured for four weeks in one 24-well tissue-culture cluster. We used removable inserts (membrane pore size 0.4 µm) in which the PBMC preincubated with the sample were added. The inserts were then easily transported to a new 24-well tissue-culture cluster for culture medium refreshment.

We conducted two experiments to technically validate the method. Inserts with different pore sizes were tested for their ability to retain PBMC. We found that almost no PBMC passed through the membrane with a pore size of 0.4 µm. We also wanted to assay the culture medium on the outside of the insert in the well of the 24-well tissue-culture cluster for HIV-1 p24 antigen. Therefore, we tested whether the virus diffuses through the membrane (poresize 0.4 µm) by incubating a standard amount of HIV-1 (culture supernatant 1/1600), with or without PBMC, in the inserts for four days ($n = 5$). The average ratios of the p24 antigen assay of the medium on the outside and the inside of the insert were respectively 1.53 ± 0.15 SD and 4.11 ± 0.34 SD with PBMC, and 1.54 ± 0.15 SD and 4.05 ± 0.15 SD without PBMC. These findings show that the virus diffuses through the membrane, but is not equally distributed. Nevertheless, enough virus is transported through the membrane to detect a positive culture. The findings also show

that the PBMC do not obstruct the diffusion of the virus through the membrane. Furthermore, using this method we found that when a culture is positive, the O.D. value of the medium on the outside of the insert after 4 weeks of culture exceeds the O.D. range of the spectrophotometer.

4. DISCUSSION

We developed a simple, sensitive, and specific method for the amplification of retroviral RNA and DNA. It is a simple method in that the same temperature protocol and the same reagents mix can be used for the amplification of both RNA and DNA sequences of the HIV-1 *pol, gag* and *env*, HIV-2 *ltr* and HTLV-I/II *tax/rex* genes, without a reduction in sensitivity. It is also simple, because for the simultaneous preparation of RNA and DNA from cells, only a two step lysis is needed. These crude lysates can then be successfully amplified in npRT-PCR or np-PCR, respectively. Furthermore, the method for extracting mRNA from cells and tissues that we adapted from Dynal (GTC/Dynabeads protocol) and which we developed further for use in the extraction of (retroviral) RNA from plasma, serum or culture supernatant is less laborious than the conventional method. A final proof of its simplicity is that the method needs no probe-hybridization confirmation step (1, 81).

The method is sensitive because it detects DNA corresponding with 10^{-1} U1 cells (0.2 HIV-1 provirus sequences), 10 U937HIV-2_{MS} cells (30% positive; at least 3 HIV-2 provirus sequences), and 10^{-1} MT-2 cells (0.6 HTLV-I provirus sequences). The method, including RNA extraction, is also sensitive for reverse transcription and the amplification of RNA sequences. With this method, we were able to detect 0.072 $TCID_{50}$ HIV-1 and amplify HIV-2 and HTLV-I RNA sequences. The sensitivity of this method for the detection of these two latter viruses could not be determined, however, because we had no suitable cell line at our disposal on which the virus culture supernatant could be titrated. However, it may be assumed that the cDNA synthesis from HIV-2 or HTLV-I RNA is as effective as that from HIV-1 RNA, because they have almost the same RNA organization.

The results of the sensitivity study of the npRT-PCR with HIV-1 clearly showed that the combination of the reverse-transcription reaction with the first PCR in one tube is possible without reducing the efficiency of the cDNA synthesis. Furthermore, these results showed that cDNA is effectively synthesized from RNA coupled to a solid phase, the paramagnetic beads. This is simpler than the method used by Chiodi et al. (14), who first eluted RNA from the beads, separated RNA-containing eluate from the beads, and then performed PCR on the eluate.

The high sensitivity of the method was achieved by using a two-step PCR in which the product from the first PCR was further amplified in a second PCR with nested primers (np-PCR) instead of a PCR using a single primer pair (sp-PCR). The results obtained when these two PCR methods were compared, clearly showed a 10-fold increase in sensitivity when using np-PCR. The same results also showed that np-PCR is more

specific than sp-PCR, because at high DNA concentrations sp-PCR showed additional bands while np-PCR only showed products of the predicted length. The specificity of np(RT)-PCR was also shown when using either of the primer sets, only products of the predicted length were formed. Thus, with np(RT)-PCR we specifically amplified the fragment for which the primers were designed out of the mass of cell DNA or polyadenylated RNA content of serum or plasma. Finally, the specificity of np-PCR was shown when using primers designed for a specific virus, (HIV-1, HIV-2, or HTLV-I), no sequences of either of the other viruses were amplified.

For RNA amplification, we needed a simple method for isolating RNA from the sample. Our results showed that the GTC/Dynabeads method and the conventional method were equally sensitive: we isolated down to 0.072 $TCID_{50}$ HIV-1 from plasma. However, we preferred the GTC/Dynabeads method because it is simple, less time-consuming, and uses less harmful reagents. The SDS/Dynabeads method is one ^{10}log step less sensitive (down to 0.72 $TCID_{50}$ HIV-1 was isolated) than the two other methods, probably because the RNAses in the sample are inhibited rather than degraded, as is the case with the other two methods. Nevertheless, in our pilot study showed no difference was found between the two Dynabeads methods in the ability to isolate RNA from plasma.
When compared with p24 antigen detection, the GTC/Dynabeads method followed by npRT-PCR was at least 100-fold more sensitive in detecting HIV-1 in plasma. This increase in sensitivity is important for the diagnosis of HIV-1 infection in newborns and when monitoring HIV-1-infected persons undergoing anti-retroviral therapy.

We were also able to detect HTLV-I sequences. Subsequent restriction-enzyme analysis yielded the predicted patterns. Because we did not have HTLV-II-provirus-containing and HTLV-II-producing cell lines at our disposal, we were not able to demonstrate the amplification of HTLV-II sequences or the discrimination of the HTLV-I and HTLV-II amplicons. However, because the primers we used were almost the same as those used by Tuke et al. (73) and because the amplification and restriction analysis of the HTLV-I sequence did not present any problems, we assume that the amplification and restriction analysis of HTLV-II will not be a problem with our method.

Our pilot study on clinical samples of HIV-1-infected individuals using all three HIV-1 primer sets clearly showed that one primer pair does not suffice for 100% detection of RNA or DNA in all samples. In one plasma sample with a positive p24 antigen assay and a positive plasma virus culture (patients 6), we could not detect RNA with any of the three HIV-1 primer sets. Our results with PBMC are comparable with those reported by Albert and Fenyö (1, 2) who used the same primer sets: all PBMC-samples were positive with at least one primer set. However, all three primersets must be used to

avoid false-negative results. This phenomenon has also been reported by other investigators with other primer sets (31, 69, 81). Some report 100% detection with one primer pair, e.g. the SK38/SK39 primer pair (7, 33), whereas others cannot confirm this (72). Because of this and the known sequence variation of the HIV-1 genome, we prefer testing with several primer sets located in different genes to avoid false-negative results. Reported rates of detection of HIV-1 RNA in plasma range up to 100% (7, 58). We found only 66.6% of HIV-1-infected individuals positive for HIV-1 RNA with at least one primer set. We do not believe this is a sensitivity problem since, technically, the developed method can detect 0.072 $TCID_{50}$ HIV-1 in spiked plasma. A possible explanation is that HIV-1 must be actively replicating when it appears as free virus in the blood. It is also known that replication is low in CDC class II patients. Hence, the amount of plasma tested (250 µl) was perhaps too low to detect HIV-1 RNA in all of our cases, as was reported for CDC class II patients (58). In addition, treatment with anti-retroviral drugs may reduce the amount of circulating virus in the blood below the detection level (patient 7). Nevertheless, further research is needed to address this problem.

Use of our method for the detection of HIV-2 or HTLV-I/II RNA or DNA has only been evaluated on plasma samples spiked with the respective virus culture supernatants or on cell-line DNA. The results of these sensitivity studies are promising but need further clinical evaluation. This is especially true for HIV-2 since we designed our own primer set. On the other hand, we used a primer set for HTLV-I/II with minor modifications, which has been used successfully in the detection and differentiation of HTLV-I and HTLV-II infections (73). For the same reasons we used several primer sets to detect HIV-1, research should be conducted to develop additional primer sets for HIV-2 and HTLV-I/II for the confirmation of negative results with the currently used primer sets.

The JA9-JA12 primer set, which amplifies the V3 domain of the gp120 envelop glycoprotein of HIV-1, was also chosen to study the molecular epidemiology of HIV-1. In an initial study we successfully used the amplification product of the JA9-JA12 primerset for direct sequencing (not shown). This is in agreement with data reported by Albert and Fenyö (1). Thus, the method may be very suitable for investigating possible transmission pathways among cohorts, e.g. of intravenous drug-users (IVDUs), by direct sequencing of amplified parts of the viral genome.

The pilot study also clearly showed that both serum and plasma can be used as a source for the detection of virions with npRT-PCR and that the GTC/Dynabeads method effectively extracts RNA from both types of samples. Extracting plasma, serum, and PBMC as sources of viral RNA or proviral DNA can be done when the patients are treated at a hospital and venipuncture can be performed. However, when no trained

personnel is available (developing countries), when samples have to be taken on the streets (e.g., IVDUs), or when venipuncture blood is difficult to obtain (e.g., newborns), another source must be used. Capillary whole blood from a heel/finger prick spotted on filter paper (13) and saliva (25, 78) have been reported to be suitable alternatives for PCR. These materials are easy to obtain and need no immediate processing or freezing. The extraction and PCR amplification of DNA from filter-paper blood has been extensively investigated by several authors using spotted peripheral whole blood(12, 55, 79). They reported that the DNA of one infected cell in the spot can be detected and that the method is as sensitive as PCR on PBMC directly isolated from venipuncture whole blood. Moreover, DNA can be isolated from filter-paper blood after elution of antibodies for serology (80). Recently, Kunisada et al. (42) reported the detection and quantitation of HIV-1 proviral DNA and viral RNA from blood and serum, respectively, spotted on filter paper for long-distance transport. This is probably also possible with capillary finger/heel prick whole blood spotted on filter paper.
The existence of free HIV-1 in saliva (78) as well as the presence of proviral DNA sequences in cells excreted in saliva (25, 78) have been demonstrated. However, the excretion of infected cells in saliva is very variable (25) and taking saliva samples in a standardized way is difficult. The great advantage of using saliva, however, is that taking a sample is not invasive. Furthermore, saliva can also be used for HIV-1 serology (74). Since both filter-paper blood and saliva can be used for serologic and molecular methods, they offer a good alternative when obtaining venipuncture blood is difficult or impossible.

A case report (see Addendum) shows that the PCR method is a useful additional tool in the detection or exclusion of HIV infection when other assays do not allow a definitive diagnosis to be made. Several authors have reported similar cases with clinical symptoms related to AIDS, a weak or no reaction in antibody detection, and no detectable HIV by PCR or antigen detection (e.g. 71). This condition is often accompanied with very low CD4+ counts and is now known as idiopathic CD4+ T-lymphocytopenia.

Currently, our interest is focused on the following topics: (i) investigation whether finger-prick capillary blood spotted on filter paper or saliva can be used for studying the molecular epidemiology of HIV-1 spreading; (ii) construction of a plasmid, in which the primer sites of the primers we used are incorporated, which can be used as a positive control in RNA and DNA PCR and to make the PCR quantitative; and (iii) development of a quantitative PCR for monitoring the viral load in plasma and PBMC during anti-retroviral therapy as a more accurate alternative to quantitative virus culture and measurement of antigen levels with EIAs.

5. CONCLUSIONS

We developed a two-step PCR method using nested primers for the detection of HIV-1, HIV-2, or HTLV-I/II RNA or DNA. The assay is simple, sensitive, and specific, and the product can be directly used for sequencing purposes. The method may be very useful for the detection of HIV-1, HIV-2 and HTLV-I/II sequences in the diagnosis of infection with either of the viruses, as we demonstrated for HIV-1. We showed, in accordance with Albert and Fenyö (1, 2), that in all PBMC samples of HIV-1-infected individuals, proviral HIV-1 DNA can be detected using three primer sets located in different genes. HIV-1 could only be detected in the plasma of 66.6% of infected individuals, a problem that needs to be further addressed. The HIV-2 and HTLV-I/II PCR also need further clinical evaluation.

This new method may also be used in molecular epidemiological studies, since we could directly sequence the product of the HIV-1 *env* np-PCR. From a literature study on molecular epidemiology, we conclude that molecular epidemiology must be conducted at the clonal level with more than one isolated part of the viral genome. The variable regions V3, V4, and V5 together are useful targets for molecular epidemiology of HIV-1

The method of RNA extraction followed by solid-phase RT-PCR (with or without nested primers) is generally applicable to the amplification of polyadenylated RNA. In our laboratory, we used the same method successfully for the detection of influenza virus RNA, hepatitis C virus RNA, and polio virus RNA.

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ADDENDUM

Case report of a possible HIV-infected individual with clinical symptoms suggesting AIDS

A male bisexual individual born on 11 January 1922 was hospitalized with the following clinical symptoms: since 6 months Kaposi sarcoma, CMV duodenitis, HSV-2 esophagitis, and candidiasis suggesting a state of immunodeficiency. On admission, blood was taken for HIV diagnostic assays. The patient's serum tested negative for anti-HIV-1 and anti-HIV-2 antibodies in the Abbott 3rd generation EIA and negative for HIV-1 p24 antigen in the Abbott HIVAG-1 monoclonal assay.

Serum of the patient was submitted to our laboratory to test for HTLV-I antibodies. The ELISA for HTLV-I/II antibodies was negative. One month later, a whole-blood sample was submitted to our laboratory for further examination of possible HIV infection. The results of the tests conducted on the plasma are summarized in Table 1.

Table 1. Results of tests carried out with the second plasma sample

Assay	Results (ratio)	Interpretation
Vironostika HIV mixt Elisa	**3.41**	**weakly positive**
Abbott 3rd gen. HIV1/2 EIA	0.15	negative
repeated	0.26	negative
Abbott HIVAG-1 Mon. assay	0.36	negative
Western Blot	no reaction with HIV-1 proteins	negative
	no reaction with HIV-2 gp36	negative

The CD4/CD8 ratio (2.3) and the CD4+ lymphocyt count (690/mm^2) were normal.
With the PBMC isolated from the whole-blood sample, a culture for HIV was started. Twice weekly the culture supernatant was analyzed for the presence of HIV p24 antigen with the Abbott HIVAG-1 monoclonal assay (Table 2). Samples of the culture supernatants were stored at -30° C for future analysis. The culture was terminated after four weeks and all supernatants were analysed by npRT-PCR for the presence of HIV-1 *gag*, *env*, and *pol* sequences (Table 2).

The cells harvested at the end of the culture (day 27) and the original PBMC were analyzed for the presence of HIV-1 provirus using np-PCR with *gag*, *env* and *pol*

primers (Table 2). Both samples only had an amplicon with *env* primers, although these were not of the correct length but slightly smaller (± 250bp instead of 332bp). The amplicon included the variable V3 loop of gp120 in which sequence deletions are

Table 2. Results of the analysis of the culture supernatants and cells

Days of culture	p24 Antigen ratio (supernatant)	npRT-PCR (supernatant)			RT-PCR (cells)		
		gag	*pol*	*env*	*gag*	*pol*	*env*
patient's PBMC					-	-	+
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	n.d.	-	-	-	n.d.	n.d.	n.d.
7	**1.25 (+)**	-	-	-	n.d.	n.d.	n.d.
10	**1.21 (+)**	-	-	-	n.d.	n.d.	n.d.
13	0.79	-	-	-	n.d.	n.d.	n.d.
17	0.85	-	-	-	n.d.	n.d.	n.d.
20	0.74	-	-	-	n.d.	n.d.	n.d.
24	0.48	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
27	n.d.	-	-	-	-	-	+

n.d.= not done

known to occur (gaps). Because of the positive Vironostika and the very weak positive p24 antigen EIA at days 7 and 10 in culture, the amplicon was sequenced to exclude the possibility that we were dealing with a shortened V3 loop. The sequence was compared with known sequences of the *env* gene of HIV-0, HIV-1, and HIV-2 obtained from the GeneBank collection with the alignment option of the computer program Geneworks. No reliable dendogram could be made because too many mismatches and too long gaps were needed to obtain a reliable alignment with either of the sequences.

Conclusion:

Based on all the results, we conclude that the patient had no detectable infection with HIV-0, -1, or -2 at the moment the whole-blood sample was collected.