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Detection of Human Immunodeficiency Virus Type 1 RNA in Plasma Samples from High-Risk Pediatric Patients by Using the Self-Sustained Sequence Replication Reaction

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There is an urgent need for rapid and sensitive methods to assess human immunodeficiency virus (HIV) infection in infants and children. We evaluated an approach by using the self-sustained sequence replication reaction (3SR) to amplify HIV type 1 (HIV-1) RNA directly. The amplified RNA product was then detected by bead-based sandwich oligonucleotide capture hybridization and rare earth metal chelate time-resolved fluorescence. The sensitivity of this technology was determined to be less than 12 HIV-1 RNA copies with an amplification level of 10^{10}-fold with purified HIV-1 RNA. Plasma samples from 19 high-risk pediatric patients younger than 5 years of age were examined, and results were compared with viral culture of patient plasma. Results from plasma culture and 3SR amplification agreed for 14 of these patients and disagreed for 5. Of the five samples which did not agree, four were positive by 3SR and negative by culture and one was positive by culture and negative by 3SR but became positive by 3SR at a subsequent testing. We conclude that 3SR amplification coupled with time-resolved fluorescence is a promising technology for investigating the relationship between the presence of HIV-1 RNA in plasma and progression of disease in HIV-infected pediatric patients. This technology should be important in the assessment of HIV-1 infection, in evaluating drug therapies, and in understanding the pathogenesis and transmission of the virus.

A major problem in evaluating a human immunodeficiency virus type 1 (HIV-1) infection in pediatric patients is that there are no standard methods to assess the extent of disease and efficacy of therapy. Maternal transfer of antibody across the placenta during gestation results in virtually all infants born to HIV-infected mothers being seropositive at birth; however, only 30 to 50% of these babies are actually infected with the virus (2), and determining changes in antibody titer has very limited value. The p24 antigen assay as well as other markers, such as CD4 counts, beta 2 microglobulin, neopterin, and the symptomatology used to assess HIV-1-infected adults, are generally unreliable in infants (2).

In adults, HIV-1 can be isolated from both peripheral blood mononuclear cells (PBMCs) and plasma. The success rate of isolation is usually much higher for PBMCs than plasma (1). Ehrnst et al. (10) reported the isolation of HIV-1 during all stages of infection in adults, with highest isolation (100%) during the AIDS stage and lowest (~50%) during the asymptomatic stage. Ho et al. (13) also found a strong correlation between the stage of disease and the titer of HIV in the plasma but not the titer of virus in PBMCs.

Because of the increasing number of infected infants born to HIV-1-infected mothers, the early onset of clinical symptoms, the high mortality rates in infants, and because there are no good markers for the extent of HIV disease in pediatric patients, the development of nucleic acid amplification systems such as the polymerase chain reaction (PCR) (11, 17) and RNA transcription-based systems, including the transcription-based amplification system (TAS) (15) and the self-sustained sequence replication (3SR) reaction (12), lend promise to the sensitive detection and assessment of HIV-1 infection in these patients. The TAS and PCR amplification systems were recently compared for sensitivity of detection of HIV-1 sequences in 86 PBMC specimens from 30 HIV-1-positive patients. TAS and PCR had similar detection sensitivities: 93 and 95%, respectively (8). In this paper we report the development and evaluation of a system that uses 3SR amplification, bead-based sandwich capture hybridization (23), rare earth metal chelate-labeled probes (18), and time-resolved fluorescence (6, 7) for direct detection of HIV-1 RNA in plasma. These results were compared with viral culture of patient plasma.

MATERIALS AND METHODS

Specimens. Thirty 3- to 5-ml samples of heparin-anticoagulated peripheral blood were drawn from 19 HIV-1 high-risk pediatric patients (five were newborns, eight were <1 year, three were <2 years, and three were 3 to 5 years old) at the University of California Davis Medical Center. All patients were born to seropositive mothers. Plasma was separated from cells by centrifugation at 800 × *g* for 15 min. Plasma was further clarified by centrifugation at 950 × *g* for 30 min and then placed in plasma culture or tested for the presence of viral RNA by using 3SR amplification.

Plasma culture. One milliliter of patient plasma was mixed with 10^7 phytohemagglutinin (Difco, Detroit, Mich.)-stimulated PBMCs from HIV-1-seronegative donors. Lympho-

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TABLE 1. Sequences of HIV-1 synthetic oligonucleotides

Oligonucleotide	Orientation	Sequence (5'-3')	Position[a]
Primer			
1	Sense	GGT TTT GCG ATT CTA	6465–6479
2	Antisense	(AAT TTA ATA CGA CTC ACT ATA GGG A)[b] TAG CAT TGT CTG TGA	6631–6645
Probe	Sense	(Aminolink–TTTTT)[c] AAT TAG GCC AGT AGT ATC AAC TCA ACT GCT	6551–6580
Capture	Sense	(Aminolink–TTTTT)[c] AGT CTG GCA GAA GAA GAG GTA GTA ATT AGA	6591–6620

[a] Ratner et al. (19).
[b] T7 promoter sequence.
[c] Aminolink coupling agent for chelate and bead attachment and 5-T-base spacers.

cytes were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 20 U of interleukin 2 (Cellular Products, Buffalo, N.Y.) per ml, 2 μg of polybrene (Sigma) per ml, 5×10^{-5} M 2-mercaptoethanol (Sigma), and 10 U anti-alpha interferon (ICN Immunobiologics, Lisle, Ill.) per ml. The supernatants were tested biweekly for HIV-1 p24 antigen by using a commercial solid-phase sandwich enzyme-linked immunosorbent assay (E. I. DuPont, Wilmington, Del.). A culture was considered positive when the absorbance values of the test were more than two times the values for samples from seronegative PBMCs and when positive values were noted on at least three consecutive samplings, indicating viral replication. Supernatants from cultures of HIV-1-negative plasma were consistently negative when tested by the antigen assay. Cultures reported negative were held for 4 weeks.

Preparation of RNA for amplification. RNA was isolated from the plasma samples as described by Chomczynski and Sacchi (4), with minor modification. Briefly, 100 μl of clarified plasma was mixed with 1.0 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0)–0.5% sarcosyl–0.1 M 2-mercaptoethanol)–100 μl of 2 M sodium acetate, pH 5.0–1.0 ml of water-saturated phenol–200 μl of chloroform-isoamyl alcohol (49:1). The solution was vortexed briefly and incubated on ice for 15 min. The RNA fraction was recovered by centrifugation at 10,000 × *g* for 20 min at 4°C and was followed by ethanol precipitation of the aqueous layer. RNA was pelleted by centrifugation at 16,000 × *g* for 20 min at room temperature. To ensure high purity, the RNA pellet was resuspended in solution D, ethanol precipitated, dried, and resuspended in 0.1% diethylpyrocarbonate-treated water.

Synthetic oligonucleotides. The sequences of the oligonucleotides used are given in Table 1. Sequences were derived from the complete nucleotide sequence of the HIV-1 genome (19). The DNA oligomers were chemically synthesized on a Applied Biosystems DNA synthesizer (model 380 B) and were purified by using a Poly-Pak oligonucleotide purification cartridge (Glen Research, Sterling, Va.). The envelope-specific primers used in this study have been successfully used by others to amplify a 298-base RNA fragment of HIV-1 by 3SR amplification (12). The target-specific probe is complementary to a nonprimer portion of the amplified product.

3SR amplification. Figure 1 summarizes the steps involved in the 3SR reaction. This amplification reaction was performed in a total volume of 50 μl containing 5 to 10% of the total extracted patient plasma RNA or 4.0 μl of diluted and purified HIV RNA (dilution range, 10^4 to 10^{-1} copies), 0.125 μg of each primer, 1 mM (each) the four deoxynucleoside triphosphates, 6 mM (each) the four ribonucleoside triphosphates, 40 mM Tris-HCl (pH 8.1), 30 mM $MgCl_2$, 20 mM KCl, 4 mM spermidine trihydrochloride (Sigma), 10 mM dithiothreitol, 10% dimethyl sulfoxide, and 15% sorbitol. All stock solutions and diluted reagents were made in diethylpyrocarbonate-treated water. Amplification reactions were heated to 65°C for 2 min followed by temperature equilibration to 42°C. AMV reverse transcriptase (15 U) (Life Sciences, St. Petersburg, Fla.), T7 RNA polymerase (50 U) (Stratagene, La Jolla, Calif.), and *Escherichia coli* RNase H (3 U) (Bartels Division, Baxter Diagnostics Inc., Seattle, Wash.) were added. Enzymes were mixed by gentle pipetting, and the reaction tubes were incubated at 42°C for 90 min. After amplification, the tubes were placed on ice for approximately 10 min and briefly spun in a microcentrifuge.

Preparation of labeled probes. The rare earth metal chelate label was functionalized for attachment to the oligonucleotide probe by dissolving 7.0 mg of the reagent in 100 μl of 0.2 M LiOH. To this solution was added 100 μl of 0.5 M sodium carbonate and 200 μl of water. Five microliters of thiophosgene was added in chloroform to the chelate solution. The solution was stirred and then dried under reduced pressure. Twenty optical density units (57 nmol) of the 30-base oligonucleotide probe with a 5-thymidine-base spacer and functionalized on the 5' end with 6-aminohexyl phosphate (aminolink) were dissolved in 0.1 M sodium borate, pH 9.5. The chelate solution and oligomer solution were combined and reacted for 12 h with constant stirring. The probe was purified by using reverse-phase high-pressure liquid chromatography which was followed by gel filtration. Purified probe was verified by 14% polyacrylamide gel electrophoresis followed by terbium staining of the chelate and ethidium bromide staining of the DNA. These staining reactions ensured that the probe preparation was uncontaminated with unlabeled DNA probe. The specific activity of the probe (2000 time-resolved counts per fmol) was determined by directly capturing 1 and 10 fmol of probe on complementary beads. The average time-resolved counts per femtomole was calculated by subtracting the background and dividing by the number of femtomoles used in duplicate direct capture assays.

A radioactive oligonucleotide probe was made by 5' end labeling with [γ-^{32}P]ATP (Amersham, Arlington Heights, Ill.) by using T4 polynucleotide kinase (Stratagene) as described by Sambrook et al. (20) Unincorporated label was removed from the labeled probe by using G-50 nick columns (Pharmacia, Uppsala, Sweden).

Capture bead preparation. Preparation of polystyrene capture beads (Seradyn, Indianapolis, Ind.) followed the protocol described by Lund et al. (16), with slight modification. Briefly, 200 μl of carboxymethyl latex beads (0.8 μm) were prewashed three times in distilled water. After the final

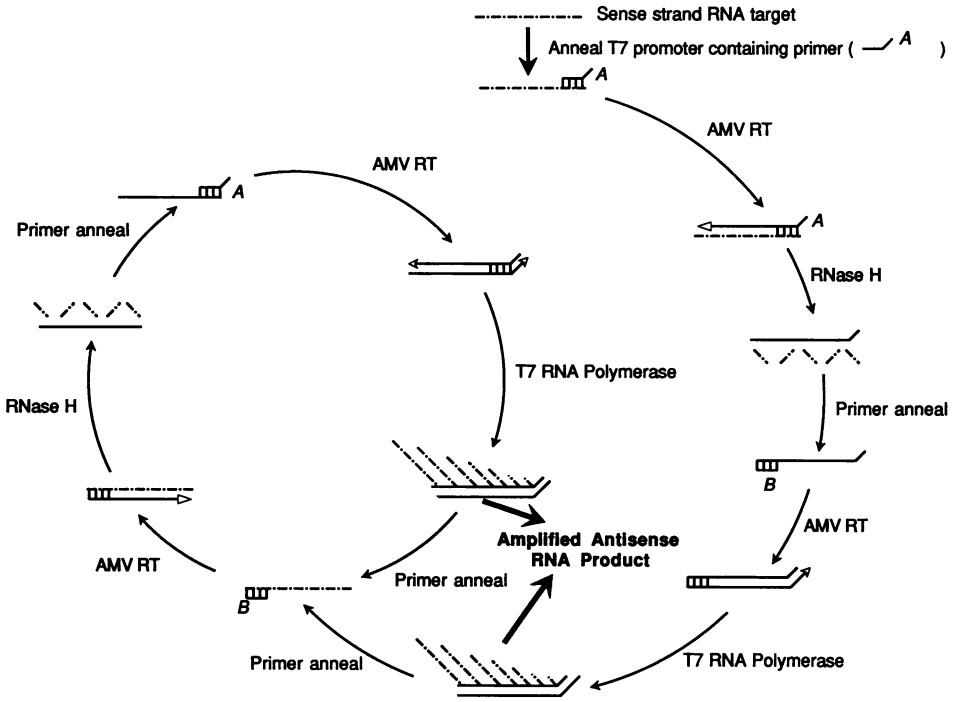

FIG. 1. 3SR amplification reaction. 3SR utilizes simultaneous reverse transcription and RNA transcription to produce multiple RNA copies of the target sequence by means of cDNA intermediates. Oligonucleotide primers A and B are used to prime cDNA synthesis, with primer A containing the promoter sequence required by the T7 RNA polymerase molecule. cDNA synthesis requires the digestion of RNA in the RNA-DNA duplex by the enzyme RNase H. The cDNAs are then used to produce RNA copies of the target sequence. The target RNAs can then further serve as templates for additional amplification of the target sequence.

wash, the beads were resuspended in 0.2 ml of 0.1 M imidazole buffer, pH 6.0. The bead suspension was mixed with 20 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 0.6 optical density units of capture oligonucleotide resuspended in 0.1 M imidazole buffer, pH 6.0. The beads were allowed to react overnight with gentle agitation and then washed four times in 1× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate. Beads were resuspended in wash buffer and stored at 4°C.

Bead-based sandwich assay. Aliquots of the 3SR amplification mixture (1.0 to 10.0 μl) were analyzed by bead-based sandwich capture hybridization. The hybridization solution (50 μl) contained 10 μl of a suspension of capture polystyrene beads (5 μg/μl) to which was bound the HIV-1 complementary 30-base capture sequence, 0.75 M NaCl, 0.075 M sodium citrate, 25 mM morpholinepropanesulfonic acid (MOPS) pH 7.0, and either 100 fmol of rare earth metal chelate-conjugated oligonucleotide probe or 100 fmol of ^{32}P-labeled probe. The hybridization solution was pipetted into a microtiter plate (Pandex Division, Baxter Diagnostics Inc., Mundelein, Ill.) (or microfuge tube for the ^{32}P-labeled probe control assay), covered, and incubated at 50°C for 60 min. The beads were washed twice at room temperature with 0.1× SSC. A solution of 3.5 μM terbium in 0.2 M magnesium chloride and 1.5 μM EDTA was added to the beads and incubated for 10 min at room temperature. Beads were read in either a fluorescence particle concentration (14) time-resolved instrument (Baxter MicroScan Research Instrument) at the excitation wavelength of 340 nm and the emission wavelength of 545 nm or a liquid scintillation counter (Beckman, Fullerton, Calif.). Figure 2 illustrates the principles of the bead-based sandwich time-resolved fluorescence assay for the detection of HIV-1.

RESULTS

Sensitivity. The sensitivity of the 3SR procedure was determined by amplifying decreasing amounts of purified HIV-1 RNA (Fig. 3). By using 3SR amplification, the bead-based sandwich capture assay and a rare earth metal chelate-labeled probe or a ^{32}P-labeled probe, less than 12 copies of a 298-base RNA fragment could be detected. It is evident that the two probe-labeling methods, rare earth metal chelate and [γ-^{32}P]ATP, gave similar levels of sensitivity. The limit of sensitivity for the time-resolved fluorescence instrument used in this study is currently 100 amol, with a signal at least 1.5 times that of background.

An additional interesting observation was that the level of amplification did not decrease with increasing concentrations of starting HIV-1 RNA target. This is a different result than was previously observed for a 3SR primer pair used to amplify *E. coli* (3), as well as a different result than has been observed for PCR (9).

Detection of HIV-1 in plasma by culture and 3SR. The results of 3SR and viral plasma culture from plasma samples collected from 19 high-risk pediatric patients less than 5 years of age are as follows: Three patients were positive by both viral culture and 3SR, 11 patients were negative by both methods, 4 patients were positive by 3SR and negative by culture, 1 patient was positive by culture and negative by 3SR but subsequently became positive by 3SR at next testing (within 1 month). All patients who tested positive by 3SR

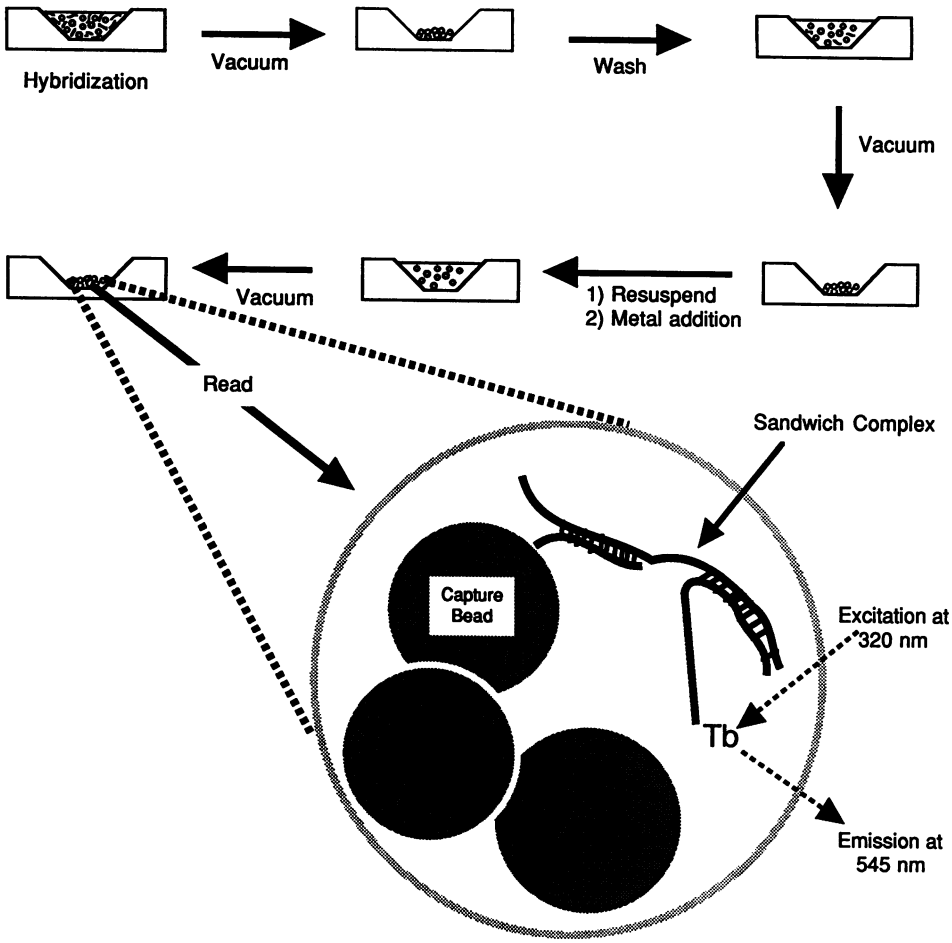

FIG. 2. The bead-based sandwich time-resolved fluorescence assay for the detection of HIV-1. In the time-resolved fluorescence hybridization and detection assay, the 3SR-amplified target sequence is mixed with target-specific capture beads and target-specific probe. The sandwich complex, composed of target, capture beads, and Tb^{3+}-labeled probe, is washed and resuspended and terbium is added. After incubation with the terbium solution, the beads are read in a time-resolved fluorometer at the excitation wavelength of 320 nm and the emission wavelength of 545 nm.

were additionally positive by culture and HIV DNA positive by PCR assay of PBMCs (data not shown).

DISCUSSION

Diseases caused by HIV are rapidly becoming a major cause of early childhood morbidity and mortality (2). More than 50% of infants who acquire the virus vertically show symptoms by the age of 1 year, and approximately 80% show signs by the age of 2 years (21). The period between infection and the development of symptoms is often much shorter in young children than adults; however, the exact period is unpredictable. As more therapeutic choices become available, the need for evaluation of disease will become increasingly important.

In this study, we describe technology which combines the self-sustained sequence replication reaction and time-resolved fluorescence for the detection of HIV-1 RNA in plasma from high-risk pediatric patients. The 3SR technique, unlike PCR, can directly amplify RNA from a sample in an isothermal fashion and produce very high levels of amplification of the specific-single-stranded target in 90 min or less. We report here a 3SR sensitivity of less than 12 HIV-1 RNA copies in 90 min with an amplification level of $>10^{10}$-fold. We have combined this powerful amplification technique with a detection system that employs polystyrene bead capture of amplified target with a rare earth metal chelate-labeled oligonucleotide probe and time-resolved fluorescence detection. We have compared this technology with plasma viral culture for the rapid and sensitive detection of the HIV-1 virus. Results show that this technology is at least as sensitive and significantly faster than plasma culture for the detection of HIV-1 RNA in plasma.

The rare earth metal chelate used as a label in this assay has several unique properties which make the time-resolved technology suitable as a direct probe-based detection system. The chelate-labeled probes are stable for more than 2 years in a lyopholized state at −20°C; exhibit a large Stoke shift, exciting in the 320-nm range and emiting in the 500- to 600-nm range; and have a life span in the millisecond range, longer than sample autofluorescence, and the individual sample reading time is very fast (22).

The ultimate use of this technology is not limited to detection of HIV infection in pediatric patients (which can also be done very effectively by PCR on PBMCs), but evaluation of the extent and progression of disease. HIV RNA rather than DNA was chosen to be detected because the RNA form of HIV is especially associated with viral

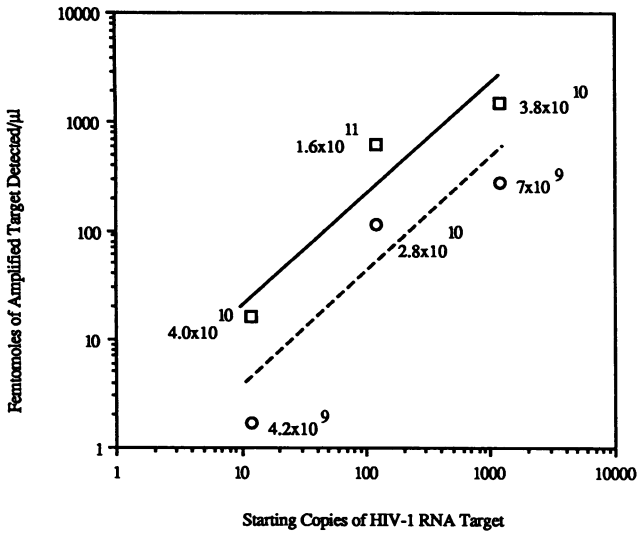

FIG. 3. Sensitivity of the 3SR procedure. 3SR amplification was performed on different amounts of purified HIV-1 RNA. Amplified product was detected by using a bead-based sandwich assay and time-resolved fluorescent (○)- or ^{32}P (□)-labeled probes. Femtomoles of target detected was determined by dividing the time-resolved counts corrected for background by the specific activity of the probe. Amplification levels were determined by dividing the time-resolved counts per microliter by the specific activity of the probe. This value was then divided by the femtomoles of target being amplified in the 3SR reaction.

activity and replication. The choice of plasma is especially appropriate for regularly monitoring pediatric populations, because HIV RNA in plasma correlates with disease progression in adults (5) and because of the limited sample size that is available from neonates and infants. Additionally, zidovudine treatment decreases the amount of virus in plasma, but not PBMCs, from adults (13).

Improved assessment of HIV-1 infection will become increasingly important as antiretroviral therapy is further developed and a better understanding of the events related to the infection of the infant emerge. 3SR amplification coupled with time-resolved fluorescence is a promising technology for investigating the relationship between the presence of HIV-1 RNA in plasma and the progression of disease in HIV-infected pediatric patients because it is at least as sensitive as plasma culture, can be performed in hours instead of weeks, and is potentially automatable. This technology should be important in the assessment of HIV infection, evaluating drug therapies, and understanding the pathogenesis and transmission of HIV-1.

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