

### Detection and Quantification of Human Immunodeficiency Virus RNA in Patient Serum by Use of the Polymerase Chain Reaction

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Human immunodeficiency virus (HIV) RNA was detected and quantified in the serum of HIV-seropositive individuals using the polymerase chain reaction (PCR) and a nonisotopic enzyme-linked affinity assay. Of 55 HIV-infected patients who were not receiving therapy, serum HIV RNA was detected in 9 of 19 who were asymptomatic, 11 of 16 with AIDS-related complex (ARC), and 18 of 20 with AIDS, with copy numbers ranging from 10<sup>2</sup> to >5 x 10<sup>4</sup>/200 µl of serum based on a relationship between absorbance and known copy number of gag gene RNA. Linear regression analysis demonstrated a correlation between infectious titer in 42 patient sera cocultured with donor peripheral blood mononuclear cells (PBMC) and PCR product absorbance (r = .70, P < .01). Serum HIV RNA detected by PCR also correlated with serum p24 antigen positivity, CD4 counts <400/mm<sup>3</sup>, and the presence of HIV-related symptoms or disease. Quantification of infectious HIV RNA in cell-free serum by PCR may be useful as a marker for disease progression or in monitoring antiviral therapy.

Quantification of viremia in human immunodeficiency virus (HIV) infection may be an important step in understanding both pathogenesis and treatment in patients with AIDS. Recently, quantitative plasma cultures have been shown to correlate with clinical disease, immunologic impairment, and quantitative assays of p24 core protein in the circulation [1, 2]. Culture techniques may be limited by the requirement for rapid processing, the variability in phytohemagglutinin (PHA)-stimulated donor cells, the long-term maintenance of infectious virus in culture, and the variation in the ability of clinical isolates of HIV to replicate in culture.

With the development of molecular techniques such as gene amplification, it has become possible to detect small numbers of HIV DNA or RNA copies. The polymerase chain reaction (PCR) has been widely applied to the detection of HIV proviral DNA and RNA from peripheral blood mononuclear cells (PBMC) in seropositive patients [3, 4]. In addition, HIV RNA has been detected in plasma [5] by extraction of RNA, reverse transcription, and cDNA PCR.

Here we describe a method to detect and quantify HIV RNA in patient serum using gene amplification of an HIV-specific gag gene sequence and quantitation of the product with a nonisotopic enzyme-linked affinity assay.

#### Patients and Methods

Whole blood samples were obtained by venipuncture from 15 seronegative healthy controls and 55 HIV-infected patients. HIV-seropositive patients were clinically assessed according to the Centers for Disease (CDC) criteria [6]. Nine were asymptomatic, 16 had AIDS-related complex (ARC), and 20 met the CDC criteria for AIDS. These corresponded to CDC class II, IVa, and IVc and IVd, respectively. No HIV-infected patient was receiving antiretroviral therapy at the time of specimen collection. Serum was separated within 1 h and stored at -70°C until use.

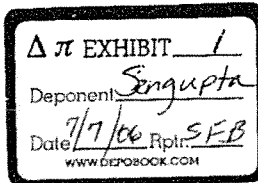
**RNA extraction, reverse transcription, and amplification of cDNA.** Total RNA from 200 µl of serum was extracted using guanidinium thiocyanate and reverse transcribed with M-MLV reverse transcriptase by methods previously described [7, 8]. Oligomers used for PCR included SK38, SK39, SK19, and SK145, all of whose sequences have been published previously [9]. Biotinylation of primer SK38 and horseradish peroxidase (HRP) labeling of probe SK19 were done as described [10]. PCR was carried out in a 100-µl reaction volume as previously described [11] for 30 cycles of amplification in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with the following program: 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, followed by a 10-min extension at 72°C. Negative and positive controls, which included both high- and low-copy-number HIV RNA and DNA, were added at each step. All samples were run in duplicate.

**Construction of gag cRNA standard.** To construct a standard, SK145 and SK39 were extended and modified to produce linker primers with EcoRI and KpnI restriction sites added to each primer respectively. HIV<sub>1</sub> DNA was amplified with this primer pair to yield a 300-bp gag gene product containing the desired restriction sites. Amplified DNA and plasmid pSP72 (Promega, Madison, WI) were digested separately with EcoRI and KpnI (New England Biolabs, Beverly, MA), then ligated under standard conditions in a 1:4 M ratio of pSP72 to insert. Transformation of DH5α-competent cells (BRL, Gaithersburg, MD) with the resulting ligated plasmid was carried out according to the supplier's protocol. A clone was ob-

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tained that included the 300-bp insert. The insert was then sequenced using the Sequenase 2 kit (United States Biochemical, Cleveland) under standard conditions to verify the correct sequence. Plasmid DNA was transcribed to RNA with a T7 polymerase in vitro transcription kit (Promega). The resulting plasmid and cRNA were quantitated on a spectrophotometer to obtain correct copy number.

**Enzyme-linked affinity assay.** To detect and quantitate PCR product, 5  $\mu$ l of PCR product and 45  $\mu$ l of 5 $\times$  saline-sodium phosphate-EDTA were heated to 95°C for 5 min and then cooled on ice to denature the sample. Then 1 pmol of SK19-HRP in 5 $\times$  Denhardt's solution was added to the denatured PCR product and hybridized for 1 h at 42°C. Next, 100  $\mu$ l of 2.54- $\mu$ m polystyrene avidinated beads (Eastman Kodak, Rochester, NY) ( $\sim$ 150 pmol of avidin) was added to each well of a 1.2- $\mu$ m topodync membrane-bottom plate (Pall Biosupport, East Hills, NY). The beads were washed with PBS by suspension and filtering on a vacuum filtration holder (Millipore, Bedford, MA). Hybridized PCR product was added to each well containing beads for 20 min. The bead-target-oligomer probe complex was then washed with PBS. A color substrate (o-phenylenediamine [Sigma, St. Louis]) was added to each well for 10 min. The reaction was stopped with 2 N H<sub>2</sub>SO<sub>4</sub> and vacuum-filtered into a clear polystyrene microtiter plate (Costar, Cambridge, MA). The absorbance was read at 490 nm on a plate reader (Dynatech, Alexandria, VA).

**Serum HIV culture.** Fresh FBMC from seronegative blood donors were stimulated in RPMI 1640 medium containing 5  $\mu$ g/ml PHA and 20% fetal calf serum for 3 days. Patient serum was serially diluted in 24-well culture plates (Costar) and cocultured with  $1 \times 10^4$  washed PHA-stimulated donor cells per well in duplicate as described by Ho et al. [1]. Stocks of HIV<sub>1</sub> were produced in acutely infected H-9 cells and their titer determined as the reciprocal of the end-point dilution of virus that resulted in p24 antigen production in H-9 cells after 28 days.

## Results

Detection and quantification of HIV RNA was first assessed in reconstruction experiments in which dilutions of HIV<sub>1</sub> or HIV<sub>1</sub> virus stock were added to HIV-seronegative donor serum or gag gene cRNA from the plasmid vector was reverse transcribed and amplified in parallel with extracted sera. Reverse transcription and amplification of known amounts of gag gene cRNA and infectious HIV<sub>1</sub> RNA and DNA alone yielded a relationship between absorbance values obtained in the enzyme-linked assay of PCR product and copy number of cRNA and DNA and TCID<sub>50</sub> of virus (figure 1). The absorbance values obtained were linear between  $10^3$  and  $5 \times 10^4$  input copies of gag cRNA,  $10^3$  and  $10^4$  TCID<sub>50</sub> of HIV<sub>1</sub> virus stock, and  $10^3$  and  $10^4$  copies of HIV DNA. The use of 5  $\mu$ l of PCR product in the affinity assay allowed quantification of input DNA and RNA over the widest range of input nucleic acid (10–50,000 copies) and the most linear response (100–10,000 copies; figure 1).

The sensitivity of each step of the assay was determined by the addition of dilutions of infectious virus to serum, cRNA to the reverse transcription reaction, or plasmid DNA to the amplification step. After 30 cycles of amplification, 10

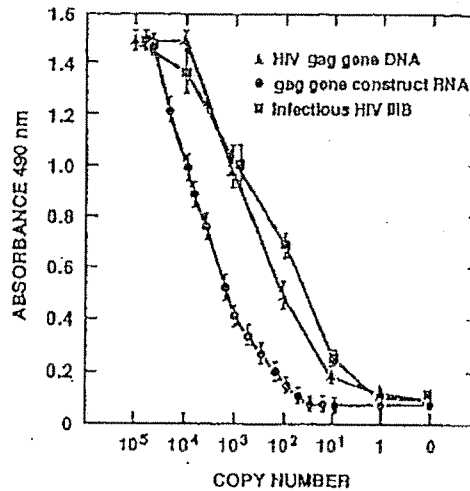


Figure 1. Quantification of infectious human immunodeficiency virus (HIV<sub>1</sub>) RNA, HIV gag gene DNA, and cRNA gag gene construct copy number by polymerase chain reaction.

TCID<sub>50</sub> of HIV<sub>1</sub>, 100 copies of cRNA, and 10 copies of HIV plasmid DNA gave an absorbance that was greater than a negative absorbance cutoff (0.135), defined as the mean absorbance obtained from 15 seronegative sera ( $0.084 \pm 0.017$ ) plus three standard deviations. The use of a greater number of cycles of amplification or >5% of the PCR product increased the sensitivity of the assay for lower copy number but decreased the dynamic range (data not shown).

To test the reproducibility of this assay, 11 sera, 9 from HIV-positive patients and 2 from seronegative controls, were subjected to separate extraction, reverse transcription, and amplification on the same day. Linear regression analysis demonstrated good correlation of mean absorbance values from separate extractions and reverse transcription ( $r = .98$ ,  $P < .01$  and  $r = .94$ ,  $P < .01$ , respectively, two-tailed *t* test) done on the same day. When the same serum samples were extracted on different days, correlation of mean absorbance was somewhat less ( $r = .83$ ,  $P < .01$ , two-tailed *t* test). When intraassay variability of the enzyme-linked affinity assay was tested, multiple replicates of the same PCR sample yielded an absorbance that varied <10% between wells.

A series of experiments was carried out to determine the likely origin of signal obtained from extracted sera in this assay. Several lines of evidence point to genomic HIV RNA within virus particles as the source. This was supported by ultracentrifugation, in which signal from sera was found in the pellet fraction, adsorption of signal to immobilized CD4, and deletion of reverse transcriptase to extracted sera, with subsequent amplification resulting in signals below the established cutoff (data not shown).

Sera from 38 of 55 HIV antibody-positive patients demonstrated a positive signal, that is, an absorbance value  $> 0.135$ . When the results of PCR were analyzed by the patient's clinical status, 9 of 19 asymptomatic, 11 of 16 ARC, and 18 of 20 AIDS patients had detectable signal. Symptomatic patients, those with ARC and AIDS, were more likely to have detectable signal than asymptomatic patients (29/36 vs. 9/19,  $P < .03$ ,  $\chi^2$ ). The mean absorbance values for each clinical group increased from  $0.142 \pm 0.05$  in asymptomatic patients to  $0.267 \pm 0.27$  and  $0.355 \pm 0.31$  in ARC and AIDS patients, respectively (figure 2). The mean absorbance value for all symptomatic patients (those with ARC and AIDS) was significantly greater than that for asymptomatic patients ( $0.316 \pm 0.29$  vs.  $0.142 \pm 0.05$ ,  $P < .01$ , two-tailed  $t$  test).

Measurement of viral RNA extracted from serum in this assay was compared with antigen production in cocultures of 10-fold dilutions of serum with PHA-stimulated donor cells. Figure 3 shows the relationship between stage of disease, infectious titer as measured by p24 antigen production in cocultures of serum dilutions, and PCR absorbance in 42 sera. Three sera were culture-positive and negative by PCR (one each asymptomatic, ARC, and AIDS), while 15 were PCR-positive and culture-negative (6 asymptomatic, 5 ARC, and 4 AIDS). Twenty-four sera showed concordance between assays: 7 negative (5 asymptomatic, 1 ARC, and 1 AIDS) and 17 positive (1 asymptomatic, 5 ARC, and 11 AIDS) by both assays. Linear regression analysis of the results obtained from these 42 sera demonstrated a correlation between the titer of infectious virus and PCR product absorbance ( $r = .70$ ,  $P < .01$ , two-tailed  $t$  test).

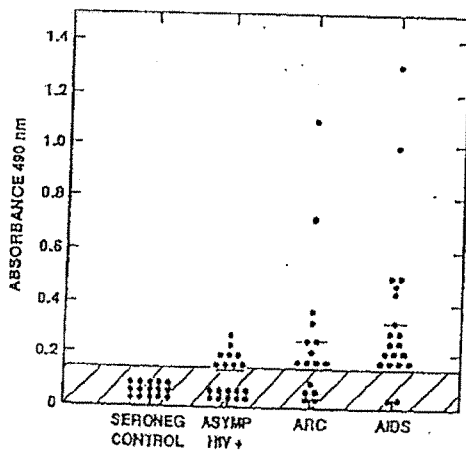


Figure 2. Quantification of human immunodeficiency virus (HIV) RNA in serum based on absorbance in 15 seronegative control subjects and 55 patients in different stages of HIV infection (asymptomatic, AIDS-related complex [ARC], and AIDS). Horizontal bar indicates mean; horizontal line, diagnostic cutoff value.

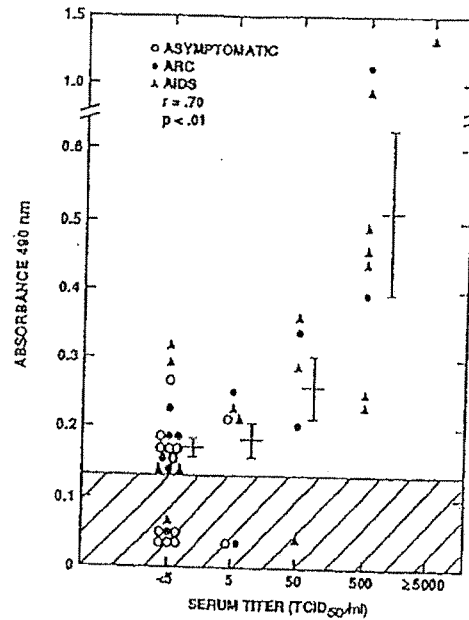


Figure 3. Quantitative serum human immunodeficiency virus (HIV) RNA polymerase chain reaction (PCR) signal in relation to serum HIV infectious titer in 42 patients in different stages of HIV infection (asymptomatic, AIDS-related complex [ARC] and AIDS). Linear regression analysis demonstrated correlation between titer of infectious virus and PCR product absorbance (two-tailed  $t$  test). Horizontal bar indicates mean and SB; horizontal line, diagnostic cutoff.

.01, two-tailed  $t$  test). Serum culture results demonstrated a strong correlation with disease stage: 2 of 13 asymptomatic subjects had positive serum culture compared with 6 of 12 ARC and 12 of 17 AIDS patients.

Surrogate markers of HIV disease progression such as CD4 count and serum p24 antigen level were considered in relationship to PCR measurement of HIV RNA. Of the 55 sera, 21 were p24 antigen-positive ( $>70$  pg/ml), of which 19 were PCR-positive; 19 of 34 p24 antigen-negative sera were PCR-positive ( $P < .007$ , Fisher's exact test). However, among the 21 p24 antigen-positive patients, there was no correlation between amount of serum p24 antigen and PCR signal. Serum from patients with  $<400$  CD4 cells/mm<sup>3</sup> was more likely to be PCR-positive: 29 of 37 compared with 9 of 18 sera from patients with  $>400$  CD4 cells/mm<sup>3</sup> ( $P < .05$ , Fisher's exact test).

Discussion

These results demonstrate that HIV RNA in serum can be detected and quantitated by reverse transcription, PCR, and

a nonisotopic enzyme-linked affinity assay. The technique described can be done in 2 days and can detect as few as 10 TCID<sub>50</sub> of HIV<sub>1</sub> stock in a small volume of serum, 10 copies of HIV DNA, or 100 copies of cRNA. The observed absorbance values of *gag* gene cRNA copies and infectious HIV<sub>1</sub> stock demonstrated at least a 10-fold increase in RNA copies in the virus stock compared with the cRNA, suggesting an RNA copy-to-infectious HIV<sub>1</sub> ratio of 10-100:1. On the basis of reconstruction experiments, quantification of viral RNA may be achieved over a range of 10<sup>3</sup> to 5 × 10<sup>4</sup> copies of input RNA, with a linear range for cRNA of 10<sup>3</sup> to 5 × 10<sup>4</sup>. Although an absorbance of ≥0.135 would be considered positive in this assay, the ability to quantitate copy number at absorbances of <0.2 would be difficult. This is also true for copy numbers with an absorbance of ≥1.5. Thus, only qualitative results can be reliably achieved above and below those absorbances.

The level of sensitivity achieved with respect to the ability of the enzyme-linked affinity assay to detect PCR product from 10 copies of input DNA and 100 copies of RNA is consistent with previously published results. Studies using 30 cycles of amplification of an HIV-specific sequence and hybridization with an isotopically labeled probe have reported the ability to detect as little as 3-5 copies of input DNA [12, 13]. After 30 cycles of amplification, 10 copies of input DNA could be detected in a nonisotopic enzyme immunoassay with probe hybridization [14]. PCR has also been used for quantification of input RNA. By use of an isotopic detection system and 30 cycles of amplification, 100 copies of input mRNA could be detected [15].

Overall, there was agreement between serum PCR and culture. However, three sera were culture-positive and PCR-negative. These negative PCR results were obtained in sera that had <50 TCID<sub>50</sub>/ml as determined by culture. Because the PCR technique is dependent on the efficiency of extraction of viral RNA from a small volume of serum and the relative efficiencies of the three separate enzymatic reactions, the sensitivity of serum PCR for detection of virus may be limited when a low titer of infectious virus is present in small samples. Ultracentrifugation of larger volumes to concentrate viral particles might improve the sensitivity of the assay. In addition, sensitivity can be enhanced by increasing the number of amplification cycles done or the amount of PCR product assayed in the hybridization reaction (unpublished data).

Serum PCR may increase the sensitivity of quantitative assays of cell-free virus. In 15 sera viral RNA was detected by PCR while coculture of serum was negative. This could indicate a relatively high proportion of defective or poorly infectious viruses present in some individual sera. Alternatively, this may be an indication of the relative insensitivity of quantitative culture techniques applied to serum. In two recent studies of plasma HIV cultures [1, 2], the sensitivity of this method varied from 100% to 56%. These differences may be related to the clinical stage of disease among patients stud-

ied. However, as in the current study using serum, both groups found a higher prevalence or titer of plasma virus in patients with AIDS and ARC compared with asymptomatic patients. Differences in sensitivity between studies of cell-free virus could be related to processing of specimens, culture methods, differences between serum and plasma, or the varying ability among donor lymphocytes from different individuals to support HIV replication in vitro [16]. Comparison of serum and plasma cultures in our laboratory has shown that virus can be recovered somewhat more efficiently from plasma (unpublished data), which may explain the relatively low rate of serum culture positivity (20/42) in this study.

In conclusion, virion HIV RNA was detected and quantitated in the serum of HIV-positive patients by PCR. These findings provide evidence that the amount of cell-free virus in circulation correlates with the presence of HIV infectious titer, serum p24 antigen positivity, CD4 counts <400/mm<sup>3</sup>, and the presence of HIV-related symptoms. Direct detection of cell-free HIV RNA by PCR is more rapid than quantitative culture and provides a technique independent of cell culture infectivity. Serum PCR may provide an additional marker of disease progression and drug efficacy that could improve our ability to monitor the course of HIV infection. Further studies will be necessary to validate this approach.

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## Hepatic Involvement in Patients with Human Immunodeficiency Virus Infection: Discrepancies between AIDS Patients and Those with Earlier Stages of Infection

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The effect of human immunodeficiency virus (HIV) infection on type and severity of liver disease was studied in 61 HIV-positive patients who did not have AIDS and in 45 AIDS patients. Liver biopsies revealed viral hepatitis in 12 of 18 non-AIDS patients but in only 4 of 34 AIDS patients ( $P < .0005$ , Fisher's exact test). Acute, non-A non-B, and chronic active hepatitis B were seen exclusively in the non-AIDS group; however, chronic persistent hepatitis B was seen in both groups. In 9 of 18 AIDS patients intra vitam liver histopathology established diagnoses of opportunistic infections or tumors. Tissue reaction to certain pathogens, such as hepatitis B virus, mycobacteria, and cryptococci, seems to be milder in AIDS patients than in others who are HIV positive or the expected reaction of the normal host. This is likely because of impaired cell-mediated immunity in patients with advanced HIV disease.

A high proportion of AIDS patients show evidence of liver disease through hepatomegaly or elevated serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), or alkaline phosphatase levels [1-3]. Almost all AIDS patients show macro- or microscopic changes of the liver at autopsy [4]. Some observers have suggested that the course of chronic hepatitis B virus (HBV) infection-associated liver disease might improve if a patient develops immunodeficiency due to infection with human immunodeficiency virus (HIV) [5, 6]. We investigated differences in type and severity of hepatic diseases between patients with early or late-stage HIV disease.

### Materials and Methods

**Patients.** Between 1983 and the first quartile of 1987, 106 HIV-infected patients from two hospitals in West Berlin were analyzed

retrospectively. They included inpatients and outpatients ( $n = 39$ ) at Klinikum Steglitz and inpatients only ( $n = 67$ ) at Klinikum Rudolf-Virchow.

**Laboratory tests.** Liver transaminases (ALT, AST) and alkaline phosphatase levels were recorded to evaluate disease activity and liver function. Sera were assayed for hepatitis A and B (Abbott Laboratories, North Chicago) and HIV (ELISA [Abbott], Western blot).

**Pathology.** We defined histopathologic viral hepatitis as panlobular infiltration with mononuclear cells, hepatic cell necrosis, hyperplasia of Kupffer's cells, and variable degrees of cholestasis. Chronic active hepatitis B (CAH-B) implicated the presence of "bridging" or multilobular hepatic necrosis during protracted, severe acute viral hepatitis. The presence of mononuclear cell infiltration restricted to the portal areas without hepatic cell necrosis was indicative of chronic persistent hepatitis B (CPH-B). For immunocytochemical demonstration of hepatitis B surface antigen (HBsAg) and core antigen (HBcAg) in liver cells, monoclonal antibodies were used (alkaline phosphatase-anti-alkaline phosphatase or peroxidase-anti-peroxidase [PAP after Sternberger] technique). After routine histopathology, special stainings for acid-fast bacilli (Truant auramine-rhodamine stain, Ziehl-Neelsen stain) and fungi (Grocott-Gomori methenamine-silver nitrate stain, periodic acid-Schiff) were done on several samples.

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