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A METHOD FOR THE QUANTITATION OF INFECTIOUS HIV-1 RNA IN PATIENT SERUM USING THE POLYMERASE CHAIN REACTION

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) RNA was detected and quantified in the serum of HIV-1 seropositive individuals with the acquired immunodeficiency syndrome (AIDS) and AIDS related complex (ARC) using the polymerase chain reaction(PCR) and a nonisotopic enzyme-linked affinity assay. In HIV-1 infected patients who were not receiving therapy, serum HIV RNA was detected in 0/10 asymptomatic, 10/14 ARC, and 13/15 AIDS patients with copy numbers ranging from 10^7 to 10^6/200ul of serum based on a log-linear relationship between absorbance and known copy number of gag gene RNA. Linear regression analysis demonstrated a correlation between infectious titer in 26 patient sera co-cultured with donor peripheral blood mononuclear cells (PBMC) and PCR product absorbance (r=0.76). The RNA detected in patient serum is from infectious particles because signal could be reduced by sedimentation, or rCD4 binding. Signal did not come from HIV DNA. Quantitation of infectious HIV-1 RNA in cell free serum by PCR may be a useful marker for disease progression or monitoring antiviral therapy.

INTRODUCTION

The current pandemic of HIV-1 infection poses difficult diagnostic and therapeutic dilemas, in part because of the lack of a method to directly quantitate infectious virus in patients. Recently, quantitative plasma cultures have been shown to correlate with disease state and, to a lesser extent with 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 by the variation in the ability of clinical isolates of HIV to replicate in culture.

PCR techniques have been widely applied to the detection of HIV proviral DNA in seropositive patients[3,4], infants born to seropositive mothers[5], seronegative subjects at risk for HIV infection[6,7], indeterminate western blots[8], and subsets of peripheral blood mononuclear cells used to quantitate the relative number of cells containing HIV proviral DNA in circulation[9]. In addition HIV RNA has been detected in PBMC[10,11] and plasma[12,13] by reverse transcription followed by PCR of extracted RNA.

The products of the PCR reaction are usually detected by gel electrophoresis and ethidium bromide staining, and their identity confirmed by liquid or filter hybridization to complementary DNA probes[14]. Methods to quantitate the PCR product using biotinylated[15], radioactively labelled[16], enzyme-linked[17], and fluorophore labelled[18] probes have been described. Additionally, PCR product may be quantitated by biotinylation and avidin-affinity based collection, either with isotopically labelled probes[19], or enzyme labelled probes[20].

In this report, we describe a method to detect and quantitate HIV RNA in patient serum using a technique that includes extraction of RNA, reverse transcription, PCR and quantitation of product with a non-isotopic enzyme-linked affinity assay. This

study shows that infectious virus in the serum can be detected and quantitated by molecular techniques in symptomatic patients with disease due to HIV. Quantitation of circulating virus could provide a new, rapid method to assess disease progression and to evaluate the efficacy of antiviral therapy in patients with AIDS.

PATIENTS AND METHODS

After informed consent was obtained, whole blood samples were obtained by venipuncture. Serum was separated within one hour and stored at -70 C until further use. Serum was collected from 15 seronegative healthy controls and 39 HIV-1 infected patients. The HIV infected patients were from CDC class 2, 4a, 4c, and 4d as defined by 1986 CDC criteria[21].

HIV RNA in 200ul of serum was extracted and reverse trancscribed by methods previously described[22,23]. Oligomers used for PCR included SK 38, SK 39, SK 19, SK 145, SK 68 and SK 69, all of whose sequences have been published previously[24]. Synthesis of oligonucleotides were done on a nucleic acid synthesizer. Biotinylation of SK 38 and horseradish peroxidase (HRP) labelling of SK19 were prepared as described[25]. The complimentary DNA was amplified in a 100ul reaction volume containing 50mM KCl, 20mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$, .02% gelatin, 1mM dNTP (Pharmacia), 50 pmoles of each primer, and 2.5U of AmpliTaq® DNA polymerase (Perkin Elmer-Cetus). The reaction was overlaid with 50ul of light weight mineral oil (Sigma). 30 cycles of amplification were done in a DNA thermal cycler

(Perkin Elmer-Cetus) with the following program: 95 C 30 sec, 55 C 30 sec, 72 C 1 min for 30 cycles followed by a 10 min extension at 72 C.

To construct a standard, SK 145 and SK39 were extended and modified to produce linker primers with EcoRI and KpnI restriction sites added to each primer respectively. HIV_{mn} DNA was amplified with this primer pair to yield a 300bp gag gene product containing the desired restriction sites. PCR DNA and plasmid pSP72 (Promega) were digested separately with EcoRI and KpnI (New England Biolabs), then ligated under standard conditions in a 1:4 molar ration of pSP72 to insert respectively. Transformation of DH5alpha competent cells (BRL) with the resulting ligated plasmid was carried out according to the suppliers protocol. A clone was obtained which successfully took the 300bp insert. The insert was then sequenced using the Sequenase^{R} kit (United States Biochemical) 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.

Avidinated 2.54um polystyrene beads (Eastman Kodak) were mixed 1:1 with a blocking solution containing 5x Denhardt's solution, 0.5% gelatin, and 50ug/ml sheared herring sperm DNA and incubated overnight at 4 C. 5ul of PCR product and 45ul of 5x SSPE were heated to 95 C for 5 min and then cooled on ice to denature the sample. 1 pmole of SK 19-HRP and Denhardt's solution to a final concentration of 5x

were added to the denatured PCR product and hybridized for 1 hour at 42 C. 100ul of blocked avidinated beads (approximately 150 pmoles of avidin) was added to each well of a 1.2um loprodyne membrane bottom plate (Pall Biosupport). The plate was placed on a vacuum filtration holder (Millipore). The beads were washed with PBS by suspension and filtering. The vacuum was released and the hybridized PCR product was added to a well containing beads and a capture reaction was allowed to take place for 20 minutes. The bead-target-oligomer probe complex was then washed with PBS. A color development solution [containing O-phenylenediamine (Sigma)] was added to each well and a 10 min reaction was allowed to take place. The reaction was stopped with 2N H_2SO_4. A clear polystyrene microtiter plate (CoStar) was placed below the membrane plate in the filtration holder and the reaction solution was vacuum-filtered into the microtiter plate. The absorbance is read at 490 nm on a plate reader (Dynatek).

Fresh PBMC from seronegative blood donors were stimulated in RPMI-1640 medium containing 5ug of phytohemagglutinin (PHA) per milliliter and 20% fetal calf serum for 3 days. Patient serum was serially diluted in 24 well culture plates(Costar) and co-cultured with 1 x 10^6 3 day old PHA stimulated donor cells/well in duplicate according to the procedure described by Ho, et al.[1]. Virus replication was detected by testing culture supernatants at days 7, 14, 21, and 28 for HIV p24 antigen production with a p24 antigen-capture kit (Abbott) according to the protocol from the supplier.

RESULTS

Reconstruction experiments were done using HIV_{IIIB} stock mixed with seronegative donor serum. A dilution series of virus in serum was made prior to viral RNA extraction. Gag gene cRNA from a plasmid vector served as an external standard of known copy number. Reverse transcription and amplification of known amounts of gag gene cRNA and infectious HIV_{IIIB} viral RNA alone yielded a log-linear relationship between absorbance and 10^2 and 10^5 copies of gag cRNA and 10^1 and 10^4 $TCID_{50}$ of virus respectively (fig. 1). Based on observed absorbance, there is at least a log difference between infectious copy number of our HIV_{IIIB} virus stock and absolute copy number of RNA. When identical copy numbers of plasmid HIV DNA and cRNA are compared, there is a greater absorbance for each DNA dilution suggesting inefficiency of reverse transcription (data not shown). The assay can detect 10 copies of DNA or 100 copies of RNA.

Serum from 15 seronegative healthy controls and 39 HIV-1 antibody positive patients were then examined. The negative absorbance cutoff(0.135) was defined by taking the mean optical density of 15 seronegative sera (0.084 ± 0.17) plus three standard deviations. Ten HIV-1 antibody patients who were asymptomatic were also negative. 10/14 ARC and 13/15 AIDS patients had detectable signal with copy numbers based on a gag RNA standard curve ranging from 10^2 to 10^5/200ul of serum (fig.2). Patients considered to have negative signal were also assessed by PCR with a primer pair from the envelop gene (SK 68 and SK69) and still had no detectable product (data not

shown).

To test the reproducability of this assay, 11 patient sera, 9 HIV positive and 2 seronegative controls were subjected to separate extraction, reverse transcription, and amplification on the same day. Linear regression analysis demonstrated good correlation of mean optical density values from separate extractions ($r=0.983$) and reverse transcription ($r=0.943$) done on the same day. When the same serum sample was extracted on different days, correlation of mean optical density was somewhat less ($r=0.83$).

To verify whether any HIV proviral DNA was present in the serum sample after RNA extraction, serum that had undergone extraction for RNA was directly amplified without a reverse transcription step. None of the HIV infected patient sera that were tested demonstrated any absorbance above the negative cutoff. Signal from patient sera could be obtained by ultracentrifugation and after concentration through binding to rCD4 linked to sepharose beads, indicating signal resulted from viral particles with intact gp120 which could bind CD4 (Data not shown). Finally 200ul of patient serum was cultured quantitatively. Figure 3 shows the correlation between infectious titer and PCR absorbance in 26 patients. Three patients were culture positive and negative by PCR. Five patients are PCR positive and culture negative. 18 patients, seven negative by both assays and 11 positive by both assays are also represented. Linear regression analysis of 26 patient sera demonstrated a correlation between the

infectious titer and PCR product absorbance (r=0.76). 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. Sixteen of the 39 patients were p24 antigen positive (p24 > 70pcg/ml) and 14/16 were PCR positive as compared to 10/23 p24 antigen negative patients who were PCR positive ($p < .001$, Fisher's exact test). Among the sixteen p24 antigen positive patients, there was no correlation between quantitative p24 antigen level and PCR signal. Patients with less than 400 CD4 cells/mm^3 were more likely to be PCR positive, 22/29 compared to patients with >400 CD4 cells/mm^3 2/10 ($p < .003$, Fisher's exact test).

DISCUSSION

We have demonstrated that infectious HIV RNA can be detected and quantitated in patient serum by PCR and a nonisotopic enzyme-linked affinity assay over a four log range. Serum PCR for HIV-1 is reasonable rapid and can be performed as described in two days. The RNA detected in patient serum is from viral particles as evidenced by sedimentation, CD4 binding and culture.

It has become increasingly important to find serologic or virologic markers as monitors of HIV-1 disease progression and antiviral therapy. CD4 count and serum p24 antigen[26]; B-2 microglobulin and serum neopterin[27]; plasma and PBMC HIV-1 culture[1]; and PCR for PBMC HIV DNA[28] have all been explored as possible markers of HIV disease and antiviral therapy. Quantitative plasma HIV culture was shown to have

good correlation with disease progression[1,2] and to monitor antiviral therapy[1]. However this procedure is very laborious and requires 3-4 weeks to complete.

Serum PCR for HIV RNA may not be as sensitive as culture at very low copy number but demonstrated a correlation between copy number derived from absorbance and infectious titer, when samples were negative by both assays or when $\geq$50 copies are present. Of note, when signal from infectious HIV and gag gene cRNA are compared, infectious virus signal is higher at each dilution indicating more copies of viral RNA are present which may not necessarily be infectious. When absorbance from known copies of plasmid HIV DNA and cRNA are compared, DNA has greater signal at each dilution and a log greater sensitivity implying that inefficiency exists with reverse transcription.

The assay is reproducible when the same serum sample is run on the same day. The reproducibility decreases somewhat for the same sample run on different days. This probably results from the differences in efficiency of extractions done on separate days and the three separate enzymatic reactions that are necessary to complete the assay. When a cRNA standard is run in parallel through all the steps (except extraction) and subjected to all the same variables as unknowns; copy number from unknown samples can be derived from the log-linear relationship of gag cRNA copy number and absorbance.

We could not detect RNA in the serum of 6/29 ARC or AIDS patients and none of the asymptomatic patients. This was confirmed by amplification with primers from another gene sequence. There were also three patients who were culture positive and PCR negative. These cultures were positive at titers that are below the current sensitivity of this assay. Sensitivity may be improved by ultracentrifugation, increasing the volume of serum sampled, the amount of PCR product used for hybridization in the enzyme affinity assay or increasing the cycle number. There may be differences in the amount of circulating infectious vs. absolute copy number of HIV RNA or neutralizing antibody which could account for samples which are PCR positive and culture negative. Samples which are culture positive and PCR negative occur at low titer and reflect the current level of sensitivity of our assay.

In conclusion, the amount of infectious HIV in patient serum can be detected and quantitated by PCR. Serum HIV PCR correlates with the presence of serum p24 antigen, CD4 counts < 400/mm^3 and infectious titer and may be a useful marker for disease progression or monitoring antiviral therapy. Further studies will be necessary to validate this approach.

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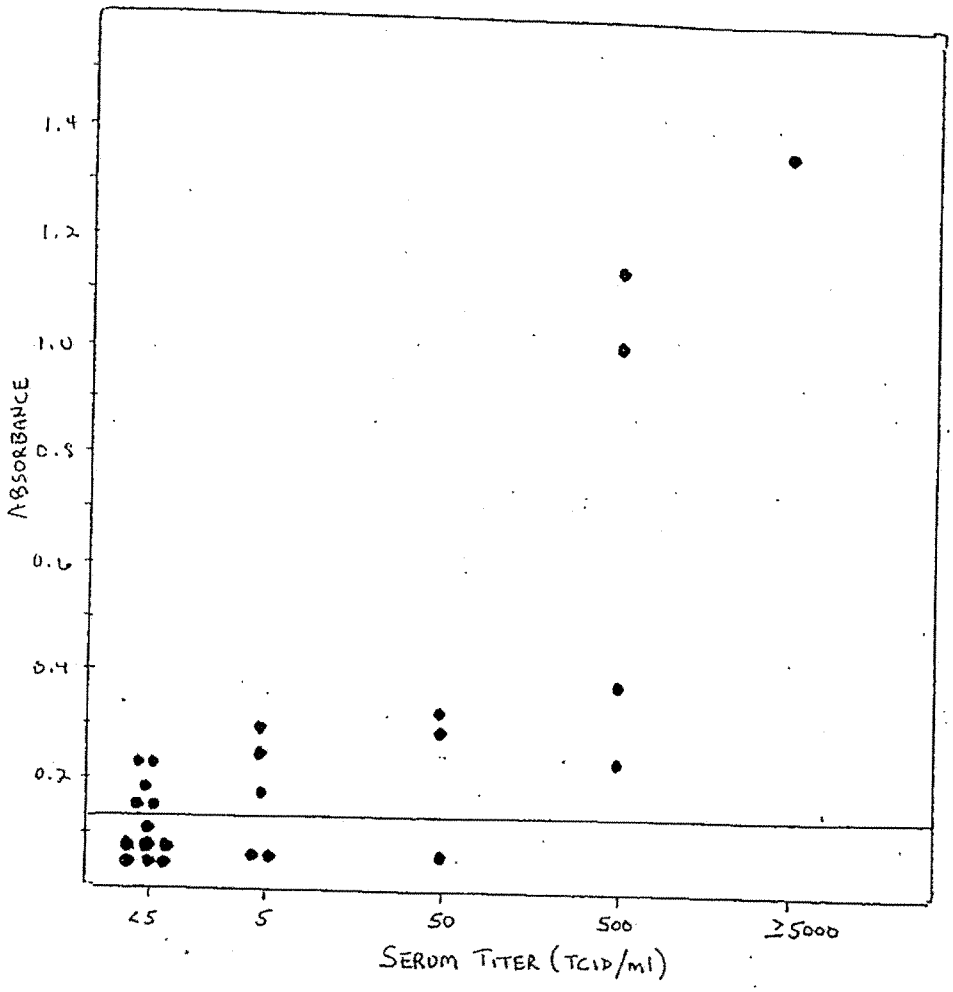
Figure 1.

PCR Quantitation of cRNA gag construct and infectious HIV RNA copy number by enzyme-linked affinity assay.

Figure 3.

Quantitative Serum HIV RNA PCR signal in relation to Serum HIV-1 Infectious titer in 26 patients with HIV-1 Infection.



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