Quantitative Detection of Human Immunodeficiency Virus Type 1 (HIV-1) RNA by PCR and Use as a Prognostic Marker and for Evaluating Antiretroviral Therapy

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The amount of human immunodeficiency virus type 1 (HIV-1) genomic RNA in sera is considered to be one of the most direct markers for patient prognosis and monitoring the efficacy of antiretroviral therapy. Quantitative detection of HIV-1 RNA was performed by the dilution endpoint method and competitive PCR. Conditions for detecting one copy of HIV-1 control DNA were examined to decide the dilution endpoint exactly. PCR of the gag region by SK145/SK39 primer pair and seminested PCR by SK145/SK101 primer pair gave the best result. Conditions for competitive PCR of HIV-1 cDNA, which was reverse transcribed from HIV-1 control RNA, were also studied using a SK38/SK39 primer pair in the presence of HIV-PCR MIMIC as a competitor DNA. The detection limit of HIV-1 control DNA by competitive PCR was 10 copies but that of HIV-1 control RNA was 50 copies. Time-course quantitation of HIV-1 RNA in frozen-stored sera from an AIDS patient was carried out and traced back to 1993. The amount of serum HIV-1 RNA markedly decreased when the treatment was changed but increased again before deterioration of his clinical status. It is considered that the quantitation of serum HIV-1 RNA is useful for the prognosis of HIV-infection and the evaluation of the antiretroviral therapy.

Key words serum human immunodeficiency virus type 1 (HIV-1) RNA; quantitative detection; competitive PCR; antiretroviral therapy evaluation; disease prognosis

Recently, human immunodeficiency virus type 1 (HIV-1) proviral DNA or HIV-1 genomic RNA was detected and quantified by PCR.1) We have qualitatively detected HIV-1 proviral DNA in sera from HIV-1 infected hemophiliacs by PCR and reported that the onset of HIV-1 infection was delayed in patients in whom proviral DNA was difficult to detect.2) Determination of the amount of HIV-1 genomic RNA provides a more sensitive and accurate method for measuring viral load in HIV-infected patients.1) When the PCR method is used, the degree of amplification varies among individual tubes. Therefore, HIV-1 RNA was quantified by a semiquantitative PCR assay using a serial dilution endpoint method and by a quantitative assay using the competitive PCR method.3) In this study, we also quantified HIV-1 genomic RNA in frozen-stored sera from one of the HIV-infected hemophiliacs and examined the relationship between HIV-1 RNA levels and clinical symptoms together with the efficacy of antiretroviral treatment.

MATERIALS AND METHODS

Samples HIV-1 control DNA (Positive Control, Roche Molecular Systems, Branchburg, NJ, U.S.A.) and HIV-1 control RNA (supplied by the Research Institute for Microbial Diseases, Osaka University) were used to determine the conditions for detection. Frozen-stored sera (−80°C) from HIV-1 infected hemophiliacs under medical treatment were used.

PCR Amplification and Detection of HIV-1 Control DNA PCR was performed using Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) according to the manufacturer’s instructions. An Omegnics Temperature Cycler (Hybaid, Middlesex, England) was used as the amplifier. Initial PCR was performed at 95, 52 and 72°C for 1 min each for 45 cycles in total. Seminested PCR was performed for 35 cycles under the same conditions using 5 μl of each product amplified on the initial PCR. Ten μl of each amplified product was blotted onto a nylon membrane under aspiration. Thereafter, the membrane was washed and fixed with ultraviolet light. Amplified control DNA on the nylon membrane was hybridized with a DIG-labeled probe at 54°C for 6 h, the emitted chemiluminescence was detected using a DIG Luminescent Detection Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and detected by exposing an X-ray film for 10 to 15 min.

Reverse Transcription (RT)-PCR of HIV-1 Control RNA Reverse transcriptase RAV-2 (Takara Shuzo, Kyoto, Japan) was used for reverse transcription of HIV-1 control RNA to cDNA in the presence of a primer pair on initial PCR according to the manufacturer’s instructions. DNA was amplified and detected as described above.

Semi-quantification of Serum HIV-1 RNA by the 10-Fold Serial Dilution Method Serum HIV-1 RNA was extracted and purified by the method of Chomczynski and Sacchi4) from HIV-1 particles obtained by ultracentrifugation at 100000×g for 90 min. HIV-1 RNA was then serially diluted 10-fold to 10−9. Thereafter, reverse transcription and PCR amplification were performed using the combination of primers that showed the highest detection efficiency. A DIG-labeled probe was used for detection. We used the SK145/SK39 primer pair5) for reverse transcription of HIV-1 RNA and initial PCR, and obtained satisfactory results. Seminested PCR was performed using the SK145/SK101 primer pair.5) Thereafter, spots were detected using the SK102 DIG-labeled probe,5) and the dilution limit was determined.

Competitive PCR of HIV-1 Control DNA HIV-1 control DNA was amplified by PCR in the presence of

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competitor DNA (HIV-PCR MIMIC Quantitation System, Clontech Lab., Palo Alto, CA, U.S.A.) serially diluted 10-fold using the SK38/SK39 primer pair.\textsuperscript{5,60} PCR was performed as described above for 45 cycles. After agarose/ethidium bromide gel electrophoresis, the DNA concentration in each band was compared and quantified using a Densitograph (model AE-6900M, Atto Corporation, Tokyo, Japan).

**Quantification of Serum HIV-1 RNA by the Competitive PCR Method** Polyinosinic acid (30\(\mu\)g per 100\(\mu\)l serum) was added as a carrier. Serum HIV-1 RNA was extracted and purified using RNAzol B (Tel-Test, Friendswood, TX, U.S.A.). Reverse transcription of the RNA was performed using a GeneAmp RNA PCR KIT (Roche Molecular Systems, Branchburg, NJ, U.S.A.). The HIV-1 cDNA thus obtained was amplified by PCR in the presence of competitor DNA and quantified as mentioned above. Since the efficiency of extraction and reverse transcription depends on the amount of serum HIV-1 RNA, we had to make corrections for these factors at every measurement.

All experiments were carried out at least twice, and if the results were inconsistent, further experiments were done.

**RESULTS AND DISCUSSION**

**Conditions for Detecting One Copy of HIV-1 Control DNA** For the dilution endpoint method, conditions for detecting 1 copy of HIV-1 DNA are critical. Initial PCR was performed using the primer pairs at the gag region shown in Fig. 1. Amplified DNA was detected using a DIG-labeled probe. As shown in Fig. 2, the detection sensitivity by probe SK102 was higher than that by probe SK19. Pale-black spots of amplified products from 1 copy HIV-1 control DNA were successfully detected using all 3 kinds of primer pairs (SK145/SK39, SK100/SK39 and SK145/SK101) on the initial PCR. In addition, seminested PCR using a change in one of the primers (SK145/SK101 and SK100/SK101) or additional PCR using the same primer pair (SK145/SK101) was performed. Deep-black spots of amplified products from 1 copy HIV-1 control DNA were clearly detected using all 3 kinds of primer pairs. As shown in Fig. 2, if the probe SK19 was used, pale-black spots originating from 2 copies of HIV-1 control DNA were detected when the primer pair SK145/SK39 or SK100/SK39 was used for the initial PCR. However, seminested PCR clearly detected amplified products from 1 copy of HIV-1 control DNA. Additional PCR using the same primer pair (SK38/SK39) facilitated detection of 5 copies of DNA. PCR was also performed at the env (SK68/SK69)\textsuperscript{50} and pol (P5/P6)\textsuperscript{51} regions, but more than 5 copies of HIV-1 control DNA were required on the initial PCR (results not shown).

Based on these results, we decided that the reverse transcription and initial PCR of HIV-1 RNA should be carried out using the SK145/SK39 primer pair followed by the seminested PCR using the SK145/SK101 primer pair and hybridization with a DIG-labeled SK102 probe.

**Detection of HIV-1 Control RNA** The detection limit of HIV-1 control RNA was examined under the conditions described above. As shown in Fig. 3, reverse transcription and initial PCR using the SK145/SK39 primer pair failed to reveal any spots even when 500 copies of HIV-1 control RNA were used. However, seminested PCR using SK145/SK101 successfully detected 10 copies of HIV-1 control RNA. As mentioned later, the efficiency of extraction and reverse transcription depends on the amount of serum HIV-1 RNA. Based on these findings, we determined that a 10-fold dilution should be performed after the reverse transcription and then the initial and seminested PCR should be performed using diluted samples.

**Semi-quantitative Detection of HIV-1 RNA in Serum from a Patient** HIV-1 RNA was semi-quantitatively detected in 100\(\mu\)l frozen-stored sera collected from one of the hemophiliacs positive for HIV-1 antibody and shown in

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**Fig. 1.** Relative Position of Primers and Probes in the gag Region of HIV-1 Genome
Figures below the line represent the numbers of bases in the amplified chains.

**Fig. 2.** PCR Amplification and Detection of HIV-1 Control DNA by Different Primer Pairs

Initial PCR was performed at 95, 52 and 72°C for 1 min each for 45 cycles. The second seminested PCR was performed for 35 cycles using 5 \(\mu\)l of the initial PCR product. Ten \(\mu\)l of each product was blotted onto a nylon membrane and detected by DIG-labeled SK102 probe.
Fig. 6 (●). In sera collected in February and June 1993, reverse transcribed HIV-1 cDNAs were detected at the 100-fold dilution level. However, in serum collected in November 1993, HIV-1 cDNA was detected at the 102-fold dilution level, suggesting a decrease in viral load. Semi-quantification using the 10-fold serial dilution method allows us to determine serum HIV-1 RNA levels only on a logarithmic scale. A smaller dilution step increases the number of samples to be amplified by PCR, and may be inadequate for quantification of a large number of clinical specimens. Therefore, quantitative detection was carried out using the competitive PCR method which enables us to detect serum HIV-1 RNA in absolute amounts.

Competitive PCR of HIV-1 Control DNA As shown in Fig. 4, different amounts of HIV-1 competitor DNA (238 bp, HIV-PCR MIMIC, 5 to 10000 copies) competed against a fixed amount of HIV-1 control DNA (115 bp, 100 copies). The density of both DNA bands showed almost a 1:1 ratio at lane 6 which contained 100 copies of HIV-1 competitor DNA.

Efficiency of Extraction and Reverse Transcription of HIV-1 Control RNA Various amounts of HIV-1 control RNA (50 to 10000 copies) in sera were extracted and reverse transcribed. The density of amplified HIV-1 cDNA on the gel was compared with that of the same amount of HIV-1 control DNA. The correlation between the amount of HIV-1 RNA in serum and the efficiency of extraction and reverse transcription is shown in Fig. 5.

Fig. 3. Reverse Transcription and PCR Amplification of HIV-1 Control RNA
HIV-1 control RNA was reverse transcribed into cDNA and amplified using a SK145/SK39 primer pair. The second PCR was performed using a SK145/SK101 primer pair as described in Fig. 2.

![HIV-1 RNA](image)

M 1 2 3 4 5 6 7 8 9 10

238 bp

115 bp

Fig. 4. Competitive PCR of HIV-1 Control DNA
HIV-1 control DNA (115 bp) and different amounts of competitor DNA (238 bp) were amplified in the same tube using a SK39/SK39 primer pair for 45 cycles. Lane 1 does not contain any DNA. Lane 2 contains 100 copies HIV-1 control DNA. Lanes 3 to 10 contain 100 copies HIV-1 control DNA and 5, 10, 50, 100, 500, 1000, 5000 and 10000 copies competitor DNA. M is a ladder size-marker.

![Graph](image)

Fig. 5. Efficiency of Extraction and Reverse Transcription of HIV-1 Control RNA

Different amounts (50 to 10000 copies) of HIV-1 control RNA in sera were extracted, reverse transcribed and amplified. The density of amplified cDNA was compared with that of the same amount of HIV-1 control DNA. Figures indicate the number of repetitions.

Fig. 6. Semiquantitative and Quantitative Detection of Serum HIV-1 RNA
HIV-1 RNA in 100 µl serum was quantified by the 10-fold serial dilution endpoint method (●) or competitive PCR (○). The results are expressed by 10° dilution of 100 µl serum or by the number of copies in 100 µl serum.
The low efficiency with small amounts of HIV-1 RNA was due to a low extraction efficiency (52% at 50 copies and 85% at 10000 copies). The reverse transcription efficiency was about 80% for 50 to 1000 copies of HIV-1 control RNA (data not shown). It is necessary to make a correction for the copy numbers of HIV-1 RNA in serum from the patient at every measurement.

Quantitative Detection of HIV-1 RNA in Serum from a Patient HIV-1 RNA in frozen-stored sera from February 1994 to February 1997 were quantitatively detected by competitive PCR and corrected for extraction and reverse transcription against 50 to 5000 copies of HIV-1 control RNA. As shown in Fig. 6 (C), the amount of serum HIV-1 RNA was stable from 70 to 110 copies/100 µl in 1994 and then gradually increased to 1100 copies/100 µl in June 1995 before deterioration of the patient’s clinical status. After a change of treatment, the amount of serum HIV-1 RNA decreased and showed the lowest load during his clinical course, 10 copies/100 µl serum, in December 1995. Since the detection limit of HIV-1 RNA by competitive PCR in this experiment was 50 copies, we used 500 µl serum for HIV-1 RNA extraction in this case and calculated this for 100 µl base.

The amount of HIV-1 RNA in plasma is now being used as a marker for monitoring the efficacy of anti-retroviral treatment and predicting the prognosis of patients with HIV infection. Commercial kits such as the Amplicor HIV Monitor (Roche Diagnostic Systems, Basel, Switzerland) can be used, but the detection limit is 200 copies of RNA. Although the serum HIV-1 RNA level is lower than the plasma level, the present results show that accurate quantitation of HIV-1 RNA from sera by competitive PCR is able to detect 50 copies of RNA. The amount of HIV-1 RNA per 100 µl serum equivalent can be reduced to 10 copies in practical terms by supplying 500 µl serum. This high detection sensitivity is considered to be useful for monitoring low HIV-1 RNA levels in long-term nonprogressors with HIV infection whose serum HIV-1 RNA content is usually less than 20 copies/100 µl.

Differences in the efficiency of extraction and reverse transcription resulted from the amounts of serum HIV-1 RNA is a problem requiring further study. Construction of HIV-1 competitor RNA, and co-extraction and reverse transcription of serum HIV-1 RNA with the competitor RNA will help solve this problem. There is no evidence in this experiment about the stability of the frozen-stored serum HIV-1 RNA. Since we have stored sample sera at -80°C after dividing them into 500 µl aliquots and used them without additional freezing and thawing, it would seem that HIV-1 particles in the serum are stable. In this connection, Fong et al. reported that the titer of serum hepatitis C viral RNA was not reduced in samples following immediate storage at -20°C, but was reduced by one log-fold following storage at 4°C or at room temperature for 5 d and in samples subjected to five freeze-thaw cycles.

Time-course detection of the amount of HIV-1 RNA in sera from patients in different stages is now in progress.

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REFERENCES