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Quantification by additive RT-PCR of HIV-1 RNA plasma levels in different stages of HIV-1 infection

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In this study, virion-associated RNA was measured in plasma from twenty six patients in various stages of HIV-1 disease by the additive RT-PCR method. Plasma viral RNA levels were inversely correlated (r = -0.72894) with total $CD4^+$ cell counts and directly (r = 0.86964) with serum titre β_2-microglobulin in chronically infected patients. This additive RT-PCR is based on a mathematical logistic adjustment of the standard curve and the use of an internal standard identical to the target molecule, which represents a control system for the efficiency of RT-PCR and allows a continuous assessment of the accuracy based on the recovery.

Key Words: Quantitative PCR, HIV-disease.

The natural history and pathogenesis of human immunodeficiency virus type-1 (HIV-1) infection are closely linked to the replication of the virus *in vivo* (6, 19, 27). The evaluation of the effectiveness of antiviral therapy in patients requires an accurate quantitative measurement of HIV-1 levels. Since it has been shown that cell-free infectious virus in plasma is an indicator of HIV-1 replication (28), different methods such as in vitro culture techniques (13, 16), p24 antigen assays (18), and polymerase chain reaction (PCR)-based methods (1, 14, 23) have been used so far to measure virus load in HIV-infected patients. Plasma culture

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techniques are laborious, time-consuming, subjected to selection of viral strains, and have a generally limited sensitivity in asymptomatic individuals (15). P24 antigen assays show a weak sensitivity (18, 25). PCR is a powerful tool for assessing the presence of small amounts of nucleic acids and its sensitivity exceeds plasma culture and p24 antigen assays (16).

Since the inception of PCR, three different types of PCR-assays (qualitative, semiquantitative and quantitative) have been intensively used to study nucleic acid sequences. Initially, PCR was used as a qualitative assay for the detection of HIV-1 (13). Afterwards, a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) assay was developed to measure plasma HIV-1 RNA levels (14, 29). In this case, the amount of viral RNA is calculated by comparing the signal intensity of the amplimer to a standard curve constructed by amplifying known amounts of viral RNA. Although this method can provide important information on the relative number of HIV-1 RNA copies, it does not take into account the differences between the efficiency of the reverse transcription and DNA amplification. Due to the extreme sensitivity of PCR, it is difficult to control the test-to-test variation. In order to avoid this variability a quantitative competitive PCR (QC-PCR) was developed. A RNA template matched to the target sequence of interest, but different from it by virtue of an introduced internal deletion or restriction point, is used in a competitive titration of the reverse transcription and PCR steps, providing a stringent internal control (4, 20, 24). The number of HIV-1 RNA copies in patient samples is determined by the point of equivalence of the signal intensity between the wild-type RNA and serial dilutions of the modified RNA. This QC-PCR procedure has a greater accuracy than previous methods, but it is well known that RT-PCR efficiency and PCR product signals change when an internal standard of different size or sequence to the wild type is used (26). Another limitation is that each sample generates 5 or 6 dilution tubes, making the assay technically demanding when a large number of samples are studied.

By definition, the best internal standard to quantitate RNA is a template identical to the target molecule to be studied, since this ensures an amplification efficiency identical to that of the target RNA. An alternative approach to RNA quantitation, additive RT-PCR has been described (8, 11, 12, 22). In this method a non-modified internal standard identical to the target molecule, which simplifies and improves the methodology, and a logistic fitting of the standard curve as an alternative to the classical linear-exponential adjustment are used. This quantitative additive RT-PCR was applied to measure HIV-1 RNA levels in the plasma from patients in various stages of HIV-1 disease.

Materials and Methods

Subjects.– Fifteen uninfected control subjects and twenty six HIV-1 infected patients at different stages of HIV disease, 22 chronically HIV-1 infected (10 asymptomatics: A1, A2; and 12 symptomatics: B2, B3, C3) (5) and 4 patients with primary HIV-1 infection in acute phase, were included in this study. HIV-1 RNA levels and immunological parameters were studied in all of them.

Sero-immunological parameters.– The HIV antibody status of serum samples was tested using HIV-1 enzyme immunoassay (EIA) (Abbot Laboratories, Illinois) and confirmed by HIV-1 western blot (Diagnostics Pasteur, Marnes-la-

Coquete). $CD4^+$ lymphocytes were numbered by using a FACScan Flow Cytometer (Becton Dickinson, Rutherford, NJ). The HIV-1 p24 antigen was determined by Diagnostic Pasteur EIA and the serum b_2-microglobulin was measured by using Immunolite chemiluminescent EIA (Diagnostic Products Corporation, Los Angeles, Ca).

RNA isolation.- HIV-1 RNA from patient plasma samples was isolated essentially by following the Chomczynski single-step method (7). Total RNA from 2 ml of plasma was extracted using 10 ml denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7, 0.5 % sarkosyl, and 0.1 M 2-ME), 1 ml 2 M sodium acetate, pH 4, 10 ml phenol, and 2 ml of 49:1 chloroform/isoamyl alcohol. Then RNA was precipitated with 100 % isopropanol at -20 °C overnight. Finally, pellets were washed with 75 % ethanol, dried and dissolved in 20 ml of RNase-free water with 40 U of rRNasin® (Promega, Madison, WI) and frozen at -80 °C in two aliquots of 10 ml each, since 10 ml (equivalent to 1 ml of plasma) is used per RT-PCR assay.

Virus.– $LAV\text{-}1_{BRU}$ (2) isolates were propagated on CEM cells, a leukemic T cell line. To obtain HIV-1 stock solution, cell-free supernatant was harvested during the exponential phase of growth and frozen in aliquots at -80 °C.

Standard HIV-1 RNA.– In order to obtain specific RNA as an internal standard identical to the target molecule to be studied, a HIV-1 RNA stock solution was isolated from 100 ml cell-free supernatant as previously described (7). The pellet was dried and dissolved in 30 ml of RNase-free water with 60 U of rRNasin® (Promega). The standard HIV-1 RNA was quantified by free solution capillary electrophoresis (FSCE) (9) to know the precise number of copies. Briefly, FSCE with an ultraviolet (UV) detector (Europhor, Prime Vision IV, Toulouse) was developed using a fused silica capillary with 50 mm internal diameter, 100 cm in length (60 cm to the detector) and borate hydrochloric acid buffer, pH 8 (Merck, Darmstadt). The sample was introduced into the capillary by high vacuum for 1 s. Separation within the capillary was performed under constant voltage at 250 V/cm.

Reverse transcription and polymerase chain reaction.– Isolated RNAs were assayed by RT-PCR in a thermal cycler 480 (Perkin-Elmer Cetus, Norwalk). The assay consisted of: 1) serial dilutions of the standard HIV-1 RNA from 10^1 to 10^8 fg to construct the standard curve; 2) duplicated patient samples containing 10 ml of plasma total RNA each. 10^3 fg of the control RNA was added to one of these aliquots; 3) negative controls; and 4) ten tubes containing 10^3 fg of control RNA were included as an interassay control. Reverse transcription was carried out for 1 h at 42 °C in 20 ml reaction volume, containing 1 mM deoxynucleoside triphosphates (dNTPs) (Promega), 120 U of Moloney murine leukemia virus reverse transcriptase (Promega), 2.5 mM of random hexamers (Promega), 10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1 % Triton X-100, 1.5 mM $MgCl_2$ (Promega). Then the samples were denatured at 99 °C for 10 min.

Reverse transcribed samples were subjected to amplification with the addition of 10 pmol of both primers: sense gag-908: 5′-GGAGCTAGAACGATTCGCAGTTA-3′ and antisense gag-1540: 5′-TGGGATAGGTGGATTATTTGTCA-3′, and 5 U Taq DNA polymerase (Promega) in 80 ml of 1x PCR buffer (Promega). The oligomer primers were selected by using the "Oligo" program

(MedProbe, Norway). The amplification protocol was: 30 s at 95 °C, 30 s at 52 °C and 60 s at 72 °C, 40 cycles, then 7 min at 72 °C. A 655 bp band was obtained as expected.

Detection and quantitation of PCR products.– Fifteen ml aliquots of each RT-PCR product was quantified by measuring the intensity of fluorescence with a Hitachi F-2000 fluorometer (Hitachi, Japan) using Hoechst reagent 33258 (Polysciences Inc., Warrington) as dye (lexc: 365 nm and lem: 460 nm). Another 15 ml aliquot of each RT-PCR product were electrophoresed on an agarose gel and stained with ethidium bromide. A standard curve was generated by plotting the input copy number of each concentration of the standard RNA versus the fluorescence units.

Statistical methods.– Significance tests of the relationship between $CD4^+$ cell counts and HIV-1 RNA levels in plasma, and between serum titre β_2-microglobuline and RNA levels were performed by linear regression analysis.

Results

Sero-immunological data.– All sero-immunological data were analysed at different stages of HIV disease in 26 patients. Twenty two chronically HIV-1 infected patients (10 asymptomatic and 12 symptomatic) are shown in table I and four patients with primary HIV-1 infection in acute phase in table II.

RNA standard curve.– The standard HIV-1 RNA concentration was quantified by FSCE (fig. 1) by integrating the peak area. Serial dilutions of this standard HIV-1 RNA were amplified at different cycles providing the optimal cycle (40 cycles) and the right amounts (10^1 to 10^8 fg, which correspond with 0.9×10^3 -0.9×10^{10} virions) to be used as a standard curve (fig. 2). 10^3 fg of this standard RNA was selected as the suitable amount for additions and it was added to one aliquot of each patient sample for reverse transcription and further amplification by PCR.

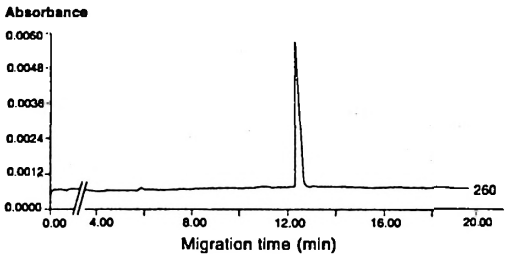

Fig. 1. *Capillary Electropherogram showing standard HIV-1 RNA at 100 ng/ml.*
A unique and specific peak was obtained at 12.5 min. FSCE conditions were as follows: Injection: vacuum for 1 s. Capillary: 50 mm x 100 cm (60 cm to detector). Buffer: borate hydrochloric acid, pH 8. Separation voltage 25 kV (30 mA), temperature 25 °C, UV detection at 260 nm.

A logistic adjustment was applied to the standard curve (fig. 2), defined by the equation $y = [(a-d)/(1+(x/c)^b)] + d$, in which a = maximal value of the signal, b = slope parameter, c = value at inflexion point, and d = minimal value of the signal (Sigmaplot, Jandel Scientific Co, Ca). This equation that describes the curve was used to calculate the concentration of RNA in plasma samples by matching the unit of fluorescence of each sample against the standard curve. Circulating HIV-1 RNA was expressed as either the number of copies per ml of plasma or virions per ml of plasma, since 1 fg RNA $\approx 1.8 \times 10^2$ copies $\approx 0.9 \times 10^2$ virions $\approx 10^2$ virions.

HIV-1 RNA levels.– A quantification of HIV-1 RNA in plasma from individuals at different stages of HIV-1-infection was performed. Results were extrapolated

Table I. *Virological and immunological parameters in 22 chronically HIV-1 infected patients.* The data shown are the median and the intervals of the values for each group.

Patient's number	CDC clinical stage[a]	$CD4^+$ T cells x 10^6/l	β_2-micro-globulin (mg/l)	HIV-1 RNA copies x 10^3 per ml plasma[b]	HIV-1 RNA Additive expected value[c]	HIV-1 RNA Additive real value[d]	Recovery (%)
4	A1	584.5 (536-634)	2 (1.98-2.58)	2930 (82-577)	473 (262-757)	483 (238-787)	100 (91-105)
6	A2	397 (360-490)	2.16 (1.60-2.99)	234 (82-605)	414 (262-785)	411 (272-714)	94 (91-104)
3	B2	227 (215-289)	3.54 (3.15-3.85)	1470 (549-7300)	1650 (729-7480)	1716 (722-69600)	99 (93-104)
4	B3	98 (72-169)	5.96 (4.60-10.12)	18500 (2160-22300)	18700 (2340-22500)	18300 (2200-22900)	98 (91-107)
5	C3	26 (518-180)	17.29 (3.34-20.00)	20800 (1720-36500)	21000 (1900-36700)	21400 (1770-39300)	102 (89-107)

[a]Clinical stage based on the 1993 Center for Disease Control Classification System (5).
[b]Copies number of RNA in plasma patients are referred to relative fluorescence intensity units.
[c]Copies x 10^3 per ml. Recovery expected of 100 % when the internal control (10^3 fg) was added.
[d]Copies x 10^3 per ml. Real values obtained when the internal control was added.

Table II. *Virological and Immunological parameters in 4 patients with primary HIV-1 infection in acute phase.*

Patient	p24 Ag (pg/ml)	$CD4^+$ T cells x 10^6/l	HIV-1 RNA copies x 10^5 per ml plasma[a]	HIV-1 RNA Additive expected value[b]	HIV-1 RNA Additive real value[c]	Recovery (%)
23	3657	756	3740	3742	3854	103
24	3350	789	3060	3062	2786	91
25	2380	909	2130	2132	1983	93
26	3020	1072	2340	2342	2319	99

[a]Copies number of RNA in plasma patients are referred to relative fluorescence intensity units.
[b]Copies x 10^5 per ml plasma. Expected recovery of 100 % when the internal control (10^3 fg) was added.
[c]Copies x 10^5 per ml plasma. Real values obtained when the internal control was added.

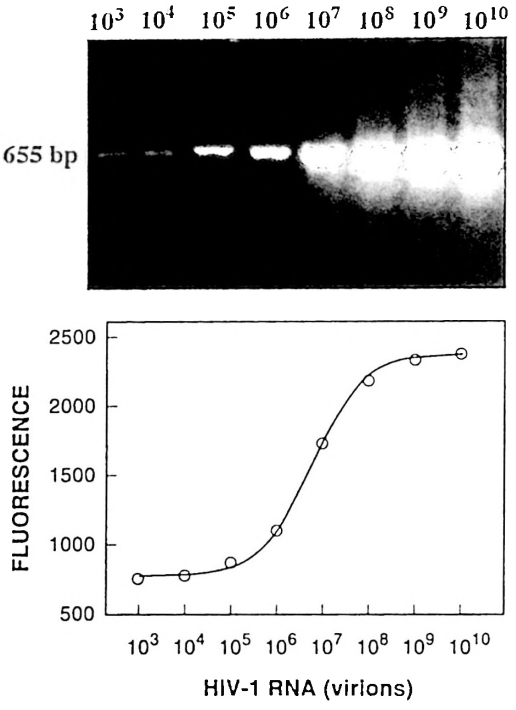

Fig. 2. *Standard curve of HIV-1 RNA.*
The upper panel shows the amplification products from serial dilutions of the standard HIV-1 RNA (10^3 to 10^{10} virions, which correspond with 10^1-10^8 fg) electrophoresed on an agarose gel. The lower panel shows the standard curve of the HIV-1 RNA calculated with the Sigmaplot program (fluorescence units versus amounts of viral RNA: 10^3-10^{10} virions).

in the standard curve (fig. 2), based on a logistic adjustment defined above, in which a = 2363; b = -0.7889; c = 58313 and d = 776.7. Although there is a general agreement on the choice of cycles/concentrations in the linear-exponential zone to build up the standard curve, the logistic adjustment was used as an alternative fitting, since this adjustment gives more accurate results when a wide range of concentrations are used, mathematical error being also minimized (11, 22).

Circulating HIV-1 RNA was expressed as the number of copies per ml of plasma or virions per ml of plasma. Virion-associated RNA was measurable in plasma by additive RT-PCR from all of the 26 HIV-1 subjects tested. Plasma viral RNA levels ranged from 8.2×10^4 to 3.74×10^8 copies per ml (tables I, II). In acute HIV-1 infection the average circulating RNA level was 281,750,000 copies per ml. In symptomatic chronic patients was 14,458,250 copies per ml and 295,700 copies per ml in asymptomatic chronic patients it. Positive signals were not observed in any of the fifteen uninfected control subjects, and without the reverse transcriptase step no HIV DNA was detectable in any of the RNA samples from plasma patients.

Table III. *Correlations among plasma viral RNA levels, CD4⁺ lymphocyte counts and β_2 microglobulin serum titers.*
Patiens with primary HIV-1 infection were excluded from this analysis All correlations shown are significant at the $p < 0.05$ level.

	CD4⁺ cells	β_2-microglobulin	HIV-1 RNA
Asymptomatics (N=10)			
CD4⁺ cells	–	–0.19068	–0.11906
β_2-microglobulin		–	0.70444
HIV-1 RNA			–
Symptomatics (N=12)			
CD4⁺ cells	–	–0.75411	–0.65981
β_2-microglobulin		–	0.80299
HIV-1 RNA			–

Correlation between HIV-1 RNA levels and immunological data.– Virus levels measured by Additive RT-PCR were inversely correlated ($r = -0.72894$; $p < 0.05$) with total $CD4^+$ cell counts and directly correlated ($r = 0.86964$, $p < 0.05$) with serum titre β_2-microglobulin for the entire group of chronically infected patients. The absolute number of $CD4^+$ lymphocytes was inversely correlated ($r = -0.70644$; $p < 0.05$) with serum titre of β_2-microglobulin. When data were analysed from subgroups of patients, asymptomatic and symptomatic, significant differences between these subgroups were found with the best correlation showing in symptomatic patients (table III). This demostrates a higher correlation between immunological parameters and viral load with the disease progression. Patients with primary HIV-1 infection, in whom correlation among viral load, CD4 counts and β_2-microglobulin were not expected, were excluded from previous studies. However, correlation analysis between two different methods to quantitate viral load, p24 antigen and additive RT-PCR, was performed in patients with primary HIV-1 infection, obtaining a significant correlation ($r = 0.92130$; $p < 0.05$). The p24 antigen from chronic patients was not included in this analysis because it was not present at measurable levels in many patients.

Precision and accuracy of additive RT-PCR assay.– To determine the intra- and interassay variability, which examines method precision, the HIV-1 RNA standard was assayed. The coefficient of variation (CV) was 4.2 % when ten duplicated samples from the same RT-PCR assay containing 10^3 fg of the standard RNA were run. The CV was 9.4 % when these ten duplicated samples were analyzed in four different RT-PCR assays.

The accuracy of the method is shown by the recovery mean of 97.96 % (tables II, III). The use of an internal standard identical to the target sequence represents a control system for the efficiency of RT-PCR and allows a continuous evaluation of the accuracy based on the recovery.

Discussion

Considerable efforts have been made to find the optimal method to measure viral load in plasma from all HIV-1 infected patients. The development of an accurate and precise method is important when monitoring therapy and making clinical management decisions. Although target amplification by PCR is an extremely sensitive method, the use of PCR for routine quantitative analysis has been questioned due to the exponential nature of the amplification process (10). This inherent feature makes difficult to obtain accurate and reliable results because of the variable efficiency of the reverse transcription and DNA polymerase steps. A minor change in the amplification efficiency will result in a large change in the magnitude of a PCR signal. Different competitive RT-PCR assays using an internal standard with a similar sequence to the target have been developed (4, 20, 24). The use of this internal standard can minimize the problem, but RT-PCR efficiency changes when an internal standard of different size or sequence to the wild-type is used (26). In contrast, an internal standard identical to the target sequence to quantitate HIV-1 RNA by an additive RT-PCR method (22) was used. The RNA molecule used as internal standard is a wild-type HIV-1 RNA. This RNA was obtained from culture infected CEM cells and it has theoretically an amplification efficiency identical to HIV-1 RNA isolated from clinical samples. The method is based on the

quantification of the signal of the amplified RNA from the clinical samples plus the internal standard which is compared to the signal of the clinical samples and the internal standard separately amplified. Duplicated samples with and without the internal standard can be used to calculate the recovery (8, 11, 12, 22).

Plasma HIV-1 RNA levels determined by additive RT-PCR differed among the different clinical stages. The highest levels of RNA are obtained in patients with primary HIV-1 infection and after this initial burst of viremia, viral titers decline dramatically often as much as 100-1000-fold over the next few weeks (17, 21). An ≈ 950-fold reduction is observed in viral load over chronic asymptomatic patients. In chronic symptomatic patients, viral titers increased again ≈ 50-fold over asymptomatic patients. These data agree with those described by other authors using quantitative competitive RT-PCR (4, 19). Additive RT-PCR is more simple than classical competitive PCRs because no standards with different concentrations are used for each sample. While in QC RT-PCR, quantification is possible when wild-type and mutant signals are equal, results are extrapolated in this method to a standard curve based on a logistic equation since the real kinetic of the PCR process with Taq polymerase is better fitted with a logistic than a linear-exponential growth (11, 22). Similar results have been shown by other authors, who have studied activity versus increasing concentrations of the template (3).

In summary, this assay only requires two reaction tubes per patient and the amount of HIV-1 RNA is calculated by using an equation that describes the standard curve. It has the advantage of controlling the efficiency of RT and DNA amplification in each sample by using an internal standard identical to the target sequence. The method only requires to analyze each patient sample by duplicate, being very useful when monitoring HIV-1 RNA levels in a large number of samples. Additional studies with more subjects are clearly indicated to more extensively address this additive RT-PCR to quantitate plasma viral load.

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Se miden los niveles plasmáticos de RNA del VIH-1, por el método RT-PCR aditivo, en 26 pacientes en diferentes estadios de la enfermedad por VIH-1. Los niveles plasmáticos de RNA viral, en los pacientes con infección crónica, se correlacionan inversamente ($r = -0.72894$) con el recuento de células $CD4^+$ y de forma directa ($r = 0.86964$) con el título de β_2 microglobulina sérica. Este RT-PCR aditivo se basa en un ajuste matemático logístico de la curva estándar y en la utilización de un estándar interno idéntico a la molécula diana, lo cual representa un sistema de control de la eficiencia de la RT-PCR y permite una valoración continua de la exactitud basada en la recuperación.

Palabras Clave: PCR cuantitativa, Enfermedad por VIH.

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