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Short communication

Studies on the prevalence of human papillomavirus in pregnant women in Japan

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Abstract

Aims: In order to evaluate the prevalence of human papillomavirus (HPV) in the pregnant population in Japan.
Methods: We examined cervical swabs of 1,183 pregnant women for HPV DNA using a PCR-RFLP method during October, 2000 and February, 2001. The overall prevalence of HPV in 1,183 pregnant women was analyzed and stratified by age.
Results: The overall prevalence of HPV in pregnant women was 12.5% (148 of 1,183 cases). The prevalence in pregnant women younger than 25 years (22.6%, 28 of 124 cases) was significantly higher compared with that in pregnant women ≥25 years (11.3%, 120 of 1057 cases, $P<0.0005$).
Conclusions: The data indicate a significantly higher prevalence of HPV infection in younger pregnant Japanese women.

Keywords: Human papillomavirus; infection; pregnancy; prevalence.

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Introduction

In recent years, estimates of the prevalence of HPV infection at the uterine cervix in the general population have varied widely, making comparisons difficult. The most important factor explaining the variation is the small sample size in the study population. In this context, we examined a significantly large sample of pregnant women in the Japanese population in order to clarify the prevalence of HPV in the pregnant population.

Materials and methods

During October, 2000 and February, 2001, 1,183 pregnant women were enrolled in this study. Informed consent was obtained at the obstetric outpatient clinics of the hospitals, to which the Cooperative Study Group members belong. The inclusion criteria were any pregnant women, without age or gestational week restriction, who consecutively visited the hospitals during the study period. The hospitals were major general hospitals in each district of Japan, and the pregnant women who visited these hospitals did not belong to a high risk group of sexually transmitted diseases. A sample for HPV DNA analysis was taken using Cytopick (cell sampling device from the uterine cervix, Lion Co. Ltd., Tokyo, Japan) from the uterine cervix by an obstetrician. The specimens were assayed using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) by the method of Yoshikawa et al. [15]. Briefly, the genomic DNA extracted from the cells was amplified by consensus primers for the L1 region of HPV. After amplification, the DNAs were digested by restriction enzymes. The subtypes of HPV were determined by the patterns of restriction fragment polymorphism. The prevalence of HPV was stratified by age, and the distribution of each HPV subtype was analyzed.

Table 1 Positive Rate of HPV at the Uterine Cervix in Pregnant Women in Japan in Each Age Range.

Age	No. of cases	No. of positive cases	Positive rate (%)
~19	9	4	44.4
20–24	115	24	20.9
25–29	429	53	12.4
30–34	432	50	11.6
35–39	173	13	7.5
40~	25	4	16.0
Total	1183	148	12.5

n.b.: There is a statistically significant diffrence of positive rate between pregnant women group under 25 y.o. and equal to or older than 25 y.o. ($P<0.0005$, by Chi-square analysis).

Table 2 Positive Rate of HPV at the Uterine Cervix in Pregnant Women in Japan According to Gestational Week.

Gestational weeks	No. of cases	No. of positive cases	Positve rate (%)
~11W	89	9	10.1
12–15W	138	17	12.3
16–19W	110	25	22.7
20–23W	110	11	10.0
24–27W	126	20	15.9
28–31W	176	20	11.4
32–35W	237	22	9.3
36W~	197	24	12.2
Total	1183	148	12.5

n.b.: No significant difference was observed according to gestational week.

Results

Of 1,183 pregnant women, HPV was detected in 148 cases, for a prevalence of 12.5%. The prevalence was 44.0, 20.9, 12.4, 11.6, 7.5, and 16% in pregnant women <20, 20–24, 25–29, 30–35, 35–39, >39 years old, respectively (Table 1). The prevalence in pregnant women younger than 25 years was 22.6% (28 of 124 cases), significantly higher compared with pregnant women ≥ 25 years (11.3%, 120 of 1059 cases). ($P<0.0005$, by Chi-square test).

Table 2 shows the prevalence of HPV for each gestational week. The prevalence ranged from 9.3% (at 32–35 weeks) to 22.7% (at 16–19 weeks), but no significant difference was observed among gestational weeks.

In Table 3, the distribution of HPV subtypes is shown. The distribution of high-risk viruses for cancer, namely HPV types 16, 18, 31, 35, 45, 51, 52, and 56 was 9.9%, 3.9%, 0.7%, 2.0%, 0%, 2.6%, 17.8%, and 2.6%, respectively.

Discussion

Epidemiologic observation suggested that cervical cancer is associated with infection by sexually transmissible agents. A variety of molecular epidemiologic studies suggested that human papillomaviruses (HPVs) may be an important sexually transmissible agent related to the development of neoplastic changes in the cervix [1]. An increasing incidence of cervical intraepithelial neoplasia (CIN) among young women has been noticed in recent years [9]. For this reason, pregnancy might be a unique opportunity to detect HPV infection for those women who do not take part in a screening program for cervical carcinoma. Although a number of investigators reported the prevalence of HPVs in pregnant women, this varies from study to study [2, 6–8, 14], probably due to the samll number of cases in the study populations. In our study, we examined 1,183 pregnant women, and the prevalence of HPV was 12.5%, which is a relatively reliable figure as to the prevalence of HPV in the pregnant population in Japan. De Roda Husman et al. reported that the prevalence of HPV was 9.6% in 709 pregnant women, which was similar to our results [3].

None of the HPV prevalence differed significantly among the first, second and third-trimester. This finding is inconsistent with other observations demonstrating a higher prevalence of HPV in more advanced pregnancy [5, 10]. The discrepancy between our findings and other reports remains unclear. The prevalence in pregnant women younger than 25 years (22.6%, 28 of 124 cases) was significantly higher compared with that in pregnant women ≥ 25 years. It is suggested that further investigation should be conducted concerning the prevalence of HPV infection in younger pregnant women.

One must always take into consideration the effect of pregnancy, per se, on the infection of HPV. As pregnant women are considered to be immunosuppressed, a state which facilitates survival of 'semi-allogeneic' fetus, a possibility exists that new HPV infection or reactivation

Table 3 Number and Rate of Each HPV Genotype.

HPV genotype	Number of each genotype	Rate (%)
HPV16	15	9.9
HPV18	6	3.9
HPV31	1	0.7
HPV33	2	1.3
HPV35	3	2.0
HPV39	4	2.6
HPV51	4	2.6
HPV52	27	17.8
HPV53	5	3.3
HPV54	2	1.3
HPV56	4	2.6
HPV58	10	6.6
HPV59	3	2.0
HPV61	3	2.0
HPV66	3	2.0
HPV68	4	2.6
Unknown	45	29.6
Total	152	100.0

n.b.: Two genotypes were observed in 4 cases.

of HPV might occur during pregnancy. Although such effect of pregnancy on HPV infection is controversial [4, 7, 12], the comparative study of the prevalence of HPV between the pregnant and non-pregnant state of same population is mandatory.

Another major concern in such study is the outcome of the neonates born to the mother with positive HPV, i.e., a possibility exists that respiratory papillomatosis may occur in the neonates. Although this complication is reported to be very rare [11, 13], a follow up study of neonates born to HPV positive mother is also crucial.

Acknowledgements

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References

[1] CDC: Human papillomavirus infection. MMWR 47 (1998) 88

[2] Chang-Claude J, A Schneider, E Smith, M Blettner, J Wahrendorf, L Turek: Longitudinal study of the effects of pregnancy and other factors on detection of HPV. Gynecol Oncol 60 (1996) 355

[3] De Roda Husman AM, JM Walboomers, E Hopman, OP Bleker, TM Helmerhorst, L Rozendaal, FJ Voorhorst, CJ Meijer: HPV prevalence in cytomorphologically normal cervical scrapes of pregnant women as determined by PCR: the age-related pattern. J Med Virol 46 (1995) 97

[4] Fife KH, BP Katz, EJ Brizendine, DR Brown: Cervical human papillomavirus deoxyribonucleic acid persists throughout pregnancy and decreases in the postpartum period. Am J Obstet Gynecol 180 (1999) 1110

[5] Gopalkrishna V, NS Murthy, JK Sharma, M Roy, DK Das, UK Luthra: Increased human papillomavirus infection with the increasing number of pregnancies in Indian women. J Infect Dis 171 (1995) 254

[6] Kemp EA, AM Hakenewerth, SL Laurent, PE Gravitt, J Stoerker: Human papillomavirus prevalence in pregnancy. Obstet Gynecol 79 (1992) 649

[7] Morrison EAB, MD Gammon, GL Goldberg, SH Vermund, RD Burk: Pregnancy and cervical infection with human papillomaviruses. Int J Gynecol Obstet 54 (1996) 125

[8] Peng TC, C Searle, KV Shah, JT Repke, TR Johnson: Prevalence of human papillomavirsus infections in term pregnancy. Am J Perinat 7 (1990) 189

[9] Penna C, MG Fallani, M Maggiorelli, E Zipoli, A Cardelli, M Marchionni: High-grade cervical intraepithelial neoplasia (CIN) in pregnancy: clinicotherapeutic management. Tumori 84 (1998) 567

[10] Rando RF, W Lindheim, L Hasty, TV Sedlacek, M Woodland, C Eder: Increased frequency of detection of human papillomavirus deoxyribonucleic acid in exfoliated cervical cells during pregnancy. Am J Obstet Gynecol 161 (1989) 50

[11] Shah K, H Kashima, BF Polk, F Shah, H Abbey, A Abramson: Rarity of cesarean delivery in cases of juvenile-onset respiratory papillomatosis. Obstet Gynecol 68 (1986) 795

[12] Tenti P, R Zappatore, P Migliora, A Spinillo, U Maccarini, M De Benedittis, N Vesentini, G Marchitelli, E Silini, L Carnevali: Latent human papillomavirus infection in pregnant women at term: a case-control study. J Infect Dis 176 (1997) 277

[13] Tenti P, R Zappatore, R Migliora, A Spinillo, C Belloni, L Carnevali: Perinatal transmission of human papillomavirus from gravidas with latent infections. Obstet Gynecol 93 (1999) 475

[14] Veress G, MT Csiky, J Konya, J Czegledy, L Gergely: Follow-up of human papillomavirus (HPV) DNA and local anti-HPV antibodies in cytologically normal pregnant women. Med Microbiol Immunol 185 (1996) 139

[15] Yoshikawa H, T Kawana, K Kitagawa, M Mizuno, H Yoshikura, A Iwamoto: Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. Jpn J Cancer Res 82 (1991) 524

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Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted reproduction technique using spermatozoa free from HIV-1

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Background: Use of antiretroviral drugs has reduced the mortality rate for HIV infection and many HIV-discordant couples wish to have children. It is possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen.

Methods: An improved swim-up method was used to collect HIV-free spermatozoa from the semen of HIV-positive males. Diluted semen was layered over a Percoll solution with a continuous density gradient of 30–98%, and then centrifuged. The bottom layer was collected by cutting the end from the tube and the sperm suspension was collected using the swim-up method. Spermatozoa were tested by nested polymerase chain reaction (PCR) for HIV-1 RNA and DNA, with a detection limit of one copy. Spermatozoa were used for assisted reproduction in 43 couples.

Results: HIV-1 RNA and proviral DNA were not detected by nested-PCR assay in all 73 of the collected spermatozoa samples from 52 patients. The HIV-1-negative sperm was used for *in vitro* fertilization in 12 couples and for intracytoplasmic sperm injection in 31 couples. No detection of HIV-1 RNA or proviral DNA in the culture medium of the fertilized eggs was confirmed again before embryo transfer. Of the 43 female partners, 20 conceived and 27 babies were born. HIV antibodies, HIV RNA and proviral DNA were negative in all of the females and babies.

Conclusions: HIV-negative spermatozoa could be obtained from semen of HIV-positive men. The method involves no risk of HIV transmission to female partners and their children.
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Keywords: assisted reproductive techniques, HIV-1, sperm, HIV-free spermatozoa

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Introduction

Since the mid-1990s, the use of HAART has spread, and the AIDS mortality rate has decreased by more than 80% in the industrialized world [1]. HIV infection/AIDS is becoming a controllable chronic infection and HIV-infected individuals are now living longer. Many HIV-positive people are getting married and wishing to have children.

Semprini *et al.* [2] reported that they had conducted artificial inseminations in more than 2000 HIV-discordant couples (HIV-positive male and HIV-negative female) using their swim-up method, and that no HIV transmission was observed. However, their method may be suboptimal because it has not been proven to remove HIV RNA completely, and they did not measure proviral DNA in infected cells in the semen. Zhang *et al.* [3] reported that HIV may be present as proviral DNA in seminal cells in HIV-infected men who have achieved undetectable levels of viral RNA in plasma with HAART, and this HIV could be capable of sexual transmission. It has not been determined whether HIV is attached to spermatozoa or whether spermatozoa can be infected with HIV [4,5]. Therefore, contraception is recommended for HIV-discordant couples, even if HIV RNA is undetectable in plasma [3].

Authorities in different countries have different opinions concerning the use of assisted reproductive technology using spermatozoa collected by the swim-up method [6–8]. However, it would be possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen. This study examines an improved swim-up method for isolating HIV-free sperm and its use in assisted reproductive methods.

Methods

This clinical study was approved by the ethics committees of Niigata University, Ogikubo Hospital, Keio University and Kyorin University. All of the couples visited the Hematology Department of Ogikubo Hospital and received counselling and explanations of the clinical study. Informed consent was obtained from all participating couples. Semen was obtained by masturbation, and then tested for sperm concentration, motility and deformity.

Percoll preparation

An isotonic solution of Percoll (Amersham Life Science, Tokyo, Japan) was made by dissolving 980 g Percoll in 10.0 ml 2.0 mol/l Hepes-NaOH, pH 7.4, 10.0 ml human serum albumin (25%w/v), 0.05 g fosfomycin and 0.05 g cefarotin. The resulting 98% Percoll solution was sterilized with a Millipore filter (0.45 μm pore size).

Semen pretreatments

The procedure is shown in Fig. 1. Ejaculates were diluted twice with Hanks solution, followed by standing in a test tube for 10 min to precipitate filterable micro-calculus, then filtered through an ART filter (20 μm clearance; ART filter, Nipro, Osaka, Japan) to remove fibers, micro-calculus and mucinous debris. The upper phase of sperm suspension was loaded onto 6 ml Percoll linear gradient from 98% to 30% in a separable fine-neck tube (Nipro) and centrifuged at 400 × *g* for 30 min. The separable fine-neck tube was made of glass, and its bottom was squeezed to minimize the volume of sediment. To recover the sperm precipitated in the bottom tip, the top of the tube was plugged with a rubber cap, and the middle of the squeezed bottom was snapped off with an ampoule cutter.

Motile sperm were separated by the modified swim-up method. A fine glass capillary was inserted in 2 ml of the medium in a vial, then a needle tip was introduced to the bottom through the inner capillary. The motile sperm were allowed to swim up at 37°C in an incubator with 5% CO_2–air. After 60 min, 1 ml of upper layer was collected, containing the sperm that had swum up.

The sperm suspension was divided into two portions. One was used for HIV assessment, and the other was cryopreserved with KS-II medium [9] in a liquid nitrogen container.

Standard HIV-1 materials

MOLT-4 cells infected with HIV_{LAI} and its culture supernatant were used as standards for HIV-1-infected cells and virus stock, respectively. RNA purified from virus stock and the pNL4-3 plasmid [10] were the standards for HIV-1 RNA and DNA, respectively. The concentrations of the standard HIV-1 DNA and RNA were determined by spectrophotometry and the null-class equation of the Poisson distribution of the reverse transcriptase (RT)-nested polymerase chain reaction (PCR). Cells were counted using a Burker–Turk hemocytometer (Emergo, Landsmeer, the Netherlands). The virion concentration was considered to be half the virus RNA concentration.

Detection of HIV-1 RNA and DNA

The samples of sperm suspension, culture medium or plasma were centrifuged at 35 500 × *g* for 1 h at 4°C. RNA and DNA were extracted from the precipitate using QIAamp UltraSens Virus Kit (Qiagen, Tokyo, Japan). One fourth of the eluate was tested in quadruplicate by RT-nested PCR as follows. The RT reaction was performed by incubation at 42°C for 10 min in a

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20 μl solution consisting of 1× PCR buffer II (10 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl; Perkin Elmer Life Sciences, Yokohama, Japan), 3 mmol/l $MgCl_2$, 0.2 mmol/l each dNTP, 0.1 μmol/l primer GA1R (5′-CCCAGGATTATCCATCTTTTATAG-3′, 1595–1572 [10]), 4 U RNasin (Promega, Tokyo) and 20 U SuperScript II (Invitrogen, Tokyo, Japan). The whole RT product was subjected to a first-round PCR in a 50 μl solution consisting of 1× PCR buffer II, 4 mmol/l $MgCl_2$, 0.2 mmol/l each dNTP, 0.2 μmol/l primers GA1F (5′-TGTTAAAAGAGACCATCAATGAGG-3′, 1388–1411) and GA1R and 0.5 U AmpliTaq (Perkin-Elmer). Then, 1 μl of the first-round PCR product was used in the second-round PCR in a 50 μl solution containing primers GA2F (5′-GGCCAGATGAGA-GAACCAAGG-3′, 1465–1485) and GA2R (5′-CATCCTATTTGTTCCTGAAGGGTAC-3′, 1535–1511) and the other components in first-round PCR. The primers were located in *gag* p24. The thermal profile of PCR in GeneAmp PCR System 9700 (Applied Biosystems, Tokyo, Japan) was 94°C for 2 min; three cycles of 94°C for 5 s,48°C for 10 s and 72°C for 15 s; 22 cycles of 94°C for 5 s, 60°C for 10 s and 72°C for 15 s; with a final cycle of 72°C for 1 min and then the mixture kept at 4°C. The PCR products were electrophoresed through a 2.0% agarose gel in the presence of 0.5 μg/ml ethidium bromide and photographed under ultraviolet illumination. Throughout the procedure, the medium used for washed sperm or fertilized eggs was the negative control and this medium with 10 virions added was the positive control. The whole process took approximately 5 h. For samples of peripheral blood mononuclear cells (PBMC), DNA was extracted using QIAamp DNA Kit (Qiagen) and 0.5 μg of the DNA was tested in triplicate by the PCR procedures omitting reverse transcription. Competitive RT-nested PCR was performed as previously described [11].

Infectivity of HIV-1 during incubation

After incubation at 37°C under 5% CO_2 for various periods, the virus stock was added to 5×10^6 stimulated donor PBMC in 1 ml RPMI 1640 medium supplemented with 30% immobilized fetal calf serum and 70 U/ml human recombinant interleukin 2 (Shionogi, Osaka, Japan), and further incubated for 5 days. The culture supernatants were tested for p24 concentration with VIDAS HIV P24 II (BioMérieux, Tokyo, Japan).

Clinical study

If the HIV-1 testing for virion RNA and proviral DNA was negative, the other portion of frozen sperm was thawed for use in assisted reproduction. Mature eggs were obtained by means of ovulation-inducing drugs, and then placed in a dish containing 3 ml RPMI culture medium (20% albumin). The HIV-1–negative sperm solution was introduced to eggs by means of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), and the dish containing treated eggs was incubated at 37°C under 5% CO_2 for 48 h. Before embryo transfer, the culture medium for the fertilized egg was collected and tested for the presence of HIV-1 again. Only when HIV-1 RNA and DNA assays by nested PCR were negative was embryo transfer conducted. All the female partners who underwent assisted reproductive therapy, even those who did not conceive successfully, were tested for HIV antibodies, HIV-1 RNA and proviral DNA in the blood at 1 and 3 months after the assisted reproductive technique and after delivery. The babies were tested for HIV RNA and proviral DNA in umbilical cord blood at birth and in blood until 6 months after birth.

Results

Sensitivity of the HIV-1 RNA/DNA test

The procedure to detect a single copy of either HIV-1 virion RNA or proviral DNA in sperm suspensions (the HIV-1 RNA/DNA test) was developed by selecting and improving techniques in three main steps (collection of HIV-1 virions and infected cells by centrifugation, extraction of viral RNA and DNA with silica-gel-membrane technology, and the detection of the viral RNA and DNA by nested PCR) to achieve zero apparent loss in recovery at each step. First, the exact virion concentration of the standard HIV_{LAI} virus stock was determined by direct RT-nested PCR at endpoint dilution by using the null-class equation of the Poisson distribution. Then, one virion of HIV_{LAI}, on average, was added to 1 ml Sydney IVF medium (Cook, Tokyo, Japan) and the whole procedure was initiated. When one fourth of the eluate from an extraction column was examined (replicated four times) with RT-nested PCR, 12 of

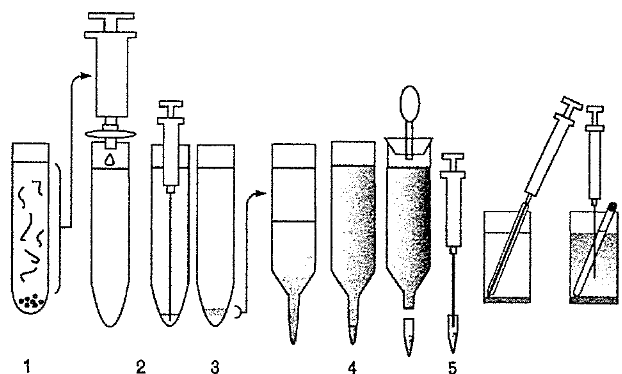

Fig. 1. Revised swim-up method to remove HIV completely. (1) The semen is diluted and debris allowed to precipitate. (2) The suspension is filtered, 0.1 ml Percoll added to the bottom, and the tube is centrifuged. (3)The sperm sediment is layered onto a linear gradient of Percoll (30–98%). (4) After centrifugation, the sediment is recovered by cutting the tube. (5) The sperm suspension is introduced into the bottom of the culture medium using a microtube. (6) The sperm that swim up are recovered.

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20 samples exhibited at least one band in four reactions (Fig. 2). Next, a single MOLT-4 cell chronically infected with HIV_{LAI} was added to 1 ml Sydney IVF medium and subjected to the HIV-1 RNA/DNA test without reverse transcription. RT-nested PCR showed that 6 of 10 samples exhibited positive reactions. The ratios of positive reactions for virions (60%) and infected cells (60%) were in close agreement with that predicted from the Poisson distribution (63%), providing evidence that the protocol has the ability to detect RNA/DNA in a single virion as well as in a single infected cell when present in as much as 1 ml of IVF medium. To study the influence of the presence of sperm in the medium on the sensitivity of the test, two sets of five samples containing 0.5, 1, 2, 4 and 8×10^6/ml spermatozoa in Sydney IVF medium were tested; one set was mixed with 50 virions and the other set with 100 infected cells. The numbers of virion RNA and proviral DNA from sperm-containing samples that were determined by competitive PCR varied in the range 75–112 copies (note two RNA copies/virion) and 96–122 copies, respectively, in a manner that was not dependent on the sperm quantity. These results strongly suggest that the protocol can detect a single virion or infected cell even in the presence of up to 8×10^6 spermatozoa per sample.

Removal of HIV-1 virions and infected cells from mixed semen by sperm-washing

To assess the efficiency of sperm-washing procedures with Percoll density gradient centrifugation and swim-up for removal of HIV-1 from semen, HIV-1 virions or HIV-1–infected cells were added to healthy donor semen. When 2×10^7 virions HIV-1 were mixed with 1.6 ml healthy donor semen containing 6.3×10^7 spermatozoa/ml, 63 copies of HIV-1 RNA were detected after centrifugation but no HIV-1 RNA was detected after swim-up. When 5×10^5 HIV-1–infected cells were mixed with 1.6 ml of healthy donor semen containing 6.3×10^7 spermatozoa/ml, no HIV-1 DNA was detected after either centrifugation or swim-up. The sperm suspension collected after swim-up was 1.0 ml in volume and contained 50 000 spermatozoa of 100% motility.

Decay of infectivity of HIV-1 during incubation

A virus solution of HIV_{LAI} was incubated in culture medium for various periods and the p24 production ability was quantified in stimulated PBMC to evaluate the stability of HIV-1 *in vitro* with regard to infectivity. Infectivity decreased semiexponentially with a half-life of approximately 13 h.

Results of the clinical study

A total of 52 HIV-1-positive individuals participated in the clinical study (Table 1); 29 were haemophiliacs and 23 had become infected through sexual contact. The median age was 33 years (range, 27–44) in the 16 untreated individuals, 34 years (range, 28–41) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 32 years (range, 20–51) in patients receiving HAART and with viral load < 50 copies/ml. Median plasma viral load was 17 500 copies/ml (range, 70–100 000) in the untreated group and 1500 copies/ml (range, 54–31 000) in patients receiving treatment and with a viral load $\geq$ 50 copies/ml.

Among 48 patients whose partner had assisted reproductive therapy, the median plasma viral load was 17 500 copies/ml (range, 70–100 000) in 15 patients in the untreated group, 4800 copies/ml (range, 54–31 000) in 10 patients receiving antiretroviral treatment and with viral load $\geq$ 50 copies/ml, and < 50 copies/ml in 23 patients taking HAART. Median CD4 cell count was 365 cells/μl (range, 66–1071) in the untreated group, 457 cells/μl (range, 60–652) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 399 cells/μl (range, 41–792) in patients receiving HAART and with viral load < 50 copies/ml. The median sperm count of the HIV-positive males was 47×10^6/ml (range, 0–82) in the untreated group, 41×10^6/ml (range, 0–65) in patients receiving antiretroviral drugs and with viral load $\geq$ 50 copies/ml, and 35×10^6/ml (range, 0–120) in patients receiving HAART and with viral load < 50 copies/ml.

Azoospermia occurred in four patients, who were excluded from this study.

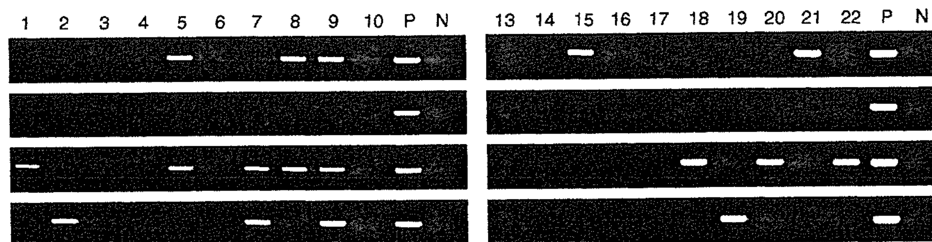

Fig. 2. Reverse transcriptase nested polymerase chain reaction (RT-nested PCR) capable of detecting a single copy of HIV-1 RNA. One virion of $HIV\text{-}1_{LAI}$, on average, was added to 20 sets of 1 ml Sydney IVF medium and then tested with the RT-nested PCR. When one fourth of the eluate from an extraction column was examined (in quadruplicate) with RT-nested PCR, 12 of 20 sets (lanes 1–10 and 13–22) exhibited at least one band in four reactions. Lanes P, positive control using 10 copies of $HIV\text{-}1_{LAI}$ RNA; lanes N, negative control with no HIV-1 RNA.

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Table 1. Characteristics of male patients with HIV infection.

		Men treated with HAART	
	Untreated men ($n = 16$)	Viral load $\geq$ 50 copies/ml ($n = 13$)	Viral load $<$ 50 copies/ml ($n = 23$)
Median age [years (range)]	33 (27–44)	34 (28–41)	32 (20–51)
Route of infection			
Haemophilia	10	8	11
Sexually transmitted	6	5	12
Median HIV viral load in serum [copies/ml (range)]	17 500 (70–100 000)	1500 (54–31 000)	$<$ 50
CD4 cell count [cells/µl (range)]	365 (66–1071)	457 (60–652)	399 (41–792)
Sperm concentration [$\times 10^6$/ml (range)]	47 (0–82)	41 (0–65)	35 (0–120)
Azoospermia (No.)	1	2	1

In all patients, the median motility rate was 28%, and the median incidence of morphologically normal spermatozoa was 12%. The median concentration of spermatozoa in patients (excluding the four patients with azoospermia) was 42×10^6/ml (range, 3–120) and 52×10^6/ml (range, 0–170) spermatozoa were collected after the Percoll centrifugation. The median motility rate was 28% and 45% before and after the Percoll procedure. Following the swim-up method, there were 1.5×10^6/ml (range, 0–11) collected spermatozoa, and the motility rate was 100%. Spermatozoa could be collected by the swim-up method in 73 semen samples from the 48 patients. No HIV-1 RNA or proviral DNA was detected in any sperm suspensions collected after the swim-up procedure. The HIV-1-negative sperm was used for IVF in 12 couples and for ICSI in 31 couples. HIV-1 RNA or proviral DNA could not be deptected in the culture medium of the fertilized eggs before embryo transfer. Of the 43 female partners, 20 conceived and 27 babies were born. HIV antibodies, HIV RNA and proviral DNA were negative in all of the females and babies.

Discussion

This study demonstrated that it is possible to detect a single copy of HIV-1 RNA or proviral DNA, and that HIV-negative spermatozoa can be obtained from the semen of HIV-positive males with the careful use of density gradient centrifugation and the swim-up technique. There has been no HIV-1 transmission in any of the female partners who underwent IVF or ICSI, nor in any of the babies.

Some studies have indicated that HIV can bind and enter into spermatozoa [4,5,12,13]. However, CD4 is not expressed on the surface of spermatocytes or spermatozoa [14,15]. Brogi *et al.* [4] have reported that HIV can attach to the surface glycoprotein of spermatozoa. In children at birth, the infection route is considered to be mother to child [16], and there is no case report of a child or embryo who has been infected with HIV via spermatozoa. It has also not been proven that a spermatid could be infected with HIV during spermatogenesis. This study showed that spermatozoa collected by the swim-up method were neither infected with HIV-1 nor had HIV-1 attached to them.

Semen contains spermatozoa, seminal plasma, white blood cells, microbes, metallic crystals and fibres of underwear. If components with higher density than spermatozoa are in a sample at centrifugation, those components may bring viruses and infected cells down to the bottom sperm fraction. Therefore, in our technique, we left diluted semen undisturbed to settle heavy components, and then took the sperm-containing upper fraction. If the sperm fraction (the bottom layer) following Percoll centrifugation is pipetted through the other denser layers, as is commonly done, HIV may contaminate the sperm fraction via the tube wall. In this study, we sealed the top of the tube after centrifugation and collected the sperm fraction by cutting off the bottom layer, which prevented contamination from the higher layers.

Gomibuchi *et al.* [17] reported that their method could not reduce HIV-1 RNA in semen to $<$ 100 copies/ml in 55.6% of patients. Kuji *et al.* [18] have reported that the use of endotoxin-free Pureseption for semen processing had a lower elimination rate for HIV than the Percoll method. Some groups have used a swim-up technique in which the spermatozoa collected after centrifugation with a separating solution were washed with a culture medium and layered below the medium, followed by swim-up. Because the difference in the specific gravity of the sperm suspension and that of the culture medium is small, HIV and mononuclear cells may easily diffuse to the top layer during the swim-up method [17]. The actual procedures of the swim-up method, such as semen-washing techniques, the materials used in centrifugation, the concentrations of separating solutions, and the methods used to collect the bottom layer (sperm fraction) vary among researchers [17,19,20]. Therefore, it is

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considered that the HIV elimination rate will also vary. Our improved swim-up method provides a safer procedure for use in assisted reproductive techniques.

Semprini *et al.* [2] have reported that HIV transmission has not occurred in over 2000 patients who underwent artificial insemination using their method. Their successful results may be explained by the fact that infectious HIV is less than 1/10 000 of all HIV virions [11,21,22] and that removal of the HIV-producing mononuclear cells by the swim-up method is a major factor in reducing infection risk. We have reported that a female was infected with HIV-1 after six artificial insemination procedures using sperm prepared only by centrifugation in another hospital [23]. Artificial insemination should not be performed when inadequate HIV elimination methods are used or when the absence of HIV is not confirmed by highly sensitive tests.

Most HIV-infected patients in this study had low sperm counts and sperm motility rates, and provided a small number of spermatozoa after the swim-up method. As we try to achieve higher virus elimination rates, the number of collected spermatozoa becomes small. Ohl *et al.* [24] reported no pregnancies after artificial insemination using sperm obtained by the swim-up method. If it takes too long for PCR procedures, or if spermatozoa are frozen, the fertilization ability of the spermatozoa may be decreased and the probability of pregnancy may be low. It is difficult to confirm rapidly the removal of HIV-1 RNA and DNA in spermatozoa actually used for artificial insemination. CD4 and chemokine receptors are not expressed on eggs [25] and, therefore, eggs cannot become infected with HIV in the sperm suspensions collected using the swim-up method even if HIV is present in the suspension. If the suspensions are contaminated with a small amount of HIV, the infectivity of the HIV would still decrease to below 1/10 after a 2 day incubation. In addition, in IVF or ICSI, it is possible to confirm the absence of HIV-1 in the culture medium of fertilized eggs before embryo transfer. Therefore, we conducted IVF or ICSI using frozen spermatozoa that had been confirmed negative for HIV-1.

In conclusion, we have demonstrated that it is possible to collect spermatozoa with evidence of the absence of HIV-1 RNA and proviral DNA from semen of HIV-infected males. Whatever method is used for assisted reproductive technique and for removal of HIV from semen to reduce the risk of secondary transmission, it is essential to confirm the absence of HIV-1 RNA and proviral DNA in the sperm preparation used for the assisted reproductive technique with the most sensitive tests possible.

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References

1. Hammer SM, Squires KE, Hughes MD, Grimes JM, Demeter LM, Currier JS, *et al.* **A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team.** *N Engl J Med* 1997; **337**:725–733.
2. Semprini AE, Fiore S, Pardi G. **Reproductive counselling for HIV-discordant couples.** *Lancet* 1997; **349**:1401–1402.
3. Zhang H, Dornadula G, Beumont M, Livornese L Jr, van Uitert B, Henning K, Pomerantz RJ. **Human immunodeficiency virus type 1 in the semen of men receiving highly active antiretroviral therapy.** *N Engl J Med* 1998; **339**:1803–1809.
4. Brogi A, Presentini R, Moretti E, Strazza M, Piomboni P, Costantino-Ceccarini E. **New insights into the interaction between the gp120 and the HIV receptor in human sperm (human. sperm/gp120/galactoglycerolipid/antigalactosylceramide/seminolipid/spermatogonia).** *J Reprod Immunol* 1998; **41**:213–231.
5. Bagasra O, Farzadegan H, Seshamma T, Oakes JW, Saah A, Pomerantz RJ. **Detection of HIV-1 proviral DNA in sperm from HIV-1-infected men.** *AIDS* 1994; **8**:1669–1674.
6. Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J. **Assisted reproduction in HIV and HCV infected men of serodiscordant couples.** *Arch Androl* 2004; **50**:105–111.
7. Weigel MM, Gentili M, Beichert M, Friese K, Sonnenberg-Schwan U. **Reproductive assistance to HIV-discordant couples: the German approach.** *Eur J Med Res* 2001; **6**: 259–262.
8. Centers for Disease Control and Prevention. **Epidemiologic Notes and Reports HIV-1 Infection and Artificial Insemination with Processed Semen.** *MMWR* 1990; **39**:249, 255–256.
9. Kaneko S, Kobayashi T, Lee HK, Won WK, Oda T, Izumi Y, *et al.* **Cryogenic preservation of low-quality human semen.** *Arch Androl* 1990; **24**:81–86.
10. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, *et al.* **Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.** *J Virol* 1986; **59**:284–291.
11. Kato S, Hiraishi Y, Nishimura N, Sugita T, Tomihama MM, Takano T. **A plaque hybridization assay for quantifying and cloning infectious human immunodeficiency virus type 1 virions.** *J Virol Meth* 1998; **72**:1–7.
12. Nuovo GJ, Becker J, Simsir A, Margiotta M, Khalife G, Shevchuk M. **HIV-1 nucleic acids localize to the spermatogonia and their progeny. A study by polymerase chain reaction in situ hybridization.** *Am J Pathol* 1994; **144**:1142–1148.
13. Baccetti B, Benedetto A, Burrini AG, Collodel G, Ceccarini EC, Crisa N, *et al.* **HIV-particles in spermatozoa of patients with AIDS and their transfer into the oocyte.** *J Cell Biol* 1994; **127**:903–914.
14. Gil T, Castilla JA, Hortas ML, Molina J, Redondo M, Samaniego F, *et al.* **CD4+ cells in human ejaculates.** *Hum Reprod* 1995; **10**:2923–2927.
15. Kim LU, Johnson MR, Barton S, Nelson MR, Sontag G, Smith JR, *et al.* **Evaluation of sperm washing as a potential method of reducing HIV transmission in HIV-discordant couples wishing to have children.** *AIDS* 1999; **13**:645–651.
16. Peckham C, Gibb D. **Mother-to-child transmission of the human immunodeficiency virus.** *N Engl J Med* 1995; **333**:298–302.
17. Gomibuchi H, Nagamatsu A, Minoura S, Nonoyama M, Tachikawa N, Oka S. **Intrauterine insemination with processed semen of HIV positive husband: study ofsemen processing.** *J AIDS Res* 2001; **3**:6–9.
18. Kuji N, Yoshii T, Kato S, Asada H, Sueoka K, Yoshimura Y. **Sedimentation kinetics of HIV-1 in two gradient media.** *58th Annual Meeting of American Society for Reproductive Medicine.* Seattle, 2002, pp. 12–17.
19. Meseguer M, Garrido N, Gimeno C, Remohi J, Simon C, Pellicer A. **Comparison of polymerase chain reaction-dependent methods for determining the presence of human immunodeficiency virus and hepatitis C virus in washed sperm.** *Fertil Steril* 2002; **78**:1199–1202.

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20. Hanabusa H, Kuji N, Kato S, Tagami H, Kaneko S, Tanaka H, *et al.* **An evaluation of semen processing methods for eliminating HIV-1.** *AIDS* 2000; **14**:1611–1616.
21. Layne SP, Merges MJ, Dembo M, Spouge JL, Conley SR, Moore JP, *et al.* **Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus.** *Virology* 1992; **189**:695–714.
22. McKeating JA, McKnight A, Moore JP. **Differential loss of envelope glycoprotein gp120 from virions of human immunodeficiency virus type 1 isolates: effects on infectivity and neutralization.** *J Virol* 1991; **65**:852–860.
23. Hanabusa H, Kato S, Kaneko S, Suzuki M, Takakuwa K, Kuji N, *et al.* **Clinical outcome of artificial reproductive technology using HIV-1 free sperm and the problems.** *17th Congress of the Japanese Society for AIDS Research.* Kobe, November 2003 [abstract 148].
24. Ohl J, Partisani M, Wittemer C, Schmitt MP, Cranz C, Stoll-Keller F, *et al.* **Assisted reproduction techniques for HIV serodiscordant couples: 18 months of experience.** *Hum Reprod* 2003; **18**:1244–1249.
25. Baccetti B, Benedetto A, Collodel G, Crisa N, di Caro A, Garbuglia AR, *et al.* **Failure of HIV-1 to infect human oocytes directly.** *J Acquir Immune Defic Syndr* 1999; **21**:355–361.

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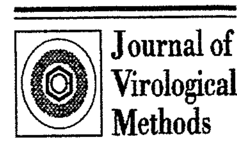

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A simple competitive RT-PCR assay for quantitation of HIV-1 subtype B and non-B RNA in plasma

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Abstract

An easy, inexpensive competitive RT-PCR assay for HIV-1 RNA quantitation was constructed. A 138-bp sequence in the HIV-1 *gag* p24 region was selected as the target and co-amplified with competitor RNA containing an internal 44-bp deletion. Quantitation of serial dilutions of control RNA samples prepared from the LAI isolate demonstrated a good linearity ($R^2 = 0.991$) within the range between 10 and 250 copies/sample. The detection limit of the assay was determined to be 3.8 copies/sample by Probit analysis and corresponded to 110 copies/ml in plasma. The intra-assay CV value was 9.1%, and the inter-assay value was 25.9%. Both were comparable to those obtained with commercially available HIV-1 RNA quantitation kits. The correlation efficient for the results obtained in 47 plasma samples from HIV-1-infected individuals (subtype A in 1, subtype B in 25, subtype C in 4, subtype F in 1, and CRF01_AE in 16) with the competitive RT-PCR and Cobas Amplicor HIV-1 Monitor test v1.5 was 0.956 for subtype B and 0.947 for subtype non-B. The assay devised is a good alternative for monitoring antiretroviral therapy in resource-poor countries.
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Keywords: HIV-1; Monitoring; Competitive RT-PCR; Viral load

1. Introduction

The pandemic of human immunodeficiency virus type 1 (HIV-1) infection has been the greatest threat to human health worldwide since its emergence two decades ago, and today about 40 million people are living with HIV/AIDS (Simon et al., 2006). As has been the pattern with respect to many other infectious diseases, the regions of the world experiencing the most significant spread of HIV-1 infection are poor. Ninety five percent of individuals infected with HIV-1 are living in developing countries with few resources, such as the countries of sub-Saharan Africa and Southeast Asia. According to the World Health Organization (WHO), nearly 6 million HIV/AIDS patients are in urgent need of antiretroviral therapy (ART), yet only 5% have access to the necessary drugs, and the remaining 95% are hopelessly waiting to die (WHO, 2004). To address this problem, WHO has launched the so-called "3 by 5" initiative (Jong-wook, 2003), which sought to treat 3 million people by the end of the year 2005, a goal made more likely by agreements enabling the use of generic antiretroviral drugs. The project appears to be operating effectively, and more patients than ever are being treated with antiretrovirals. Ironically, the introduction of antiretrovirals to countries with few resources has raised another issue: how best to monitor patients' response to treatment. In most developing countries where the "3 by 5" initiative has been implemented, it has been difficult to routinely monitor patients' CD4-positive T-cell counts or, what is worse, their plasma viral RNA concentrations (Russell, 2004). The plasma viral load is an important surrogate marker that is used to make prognoses, evaluate response to treatment, and estimate the probability of emergence of drug resistance (Kalish et al., 1999; Kempf et al., 1998; Leriche-Guerin et al., 1997; McDermott et al., 2005; Piliero, 2003). Three types of viral load quantitation kits are available commercially: the Cobas Amplicor HIV-1 Monitor test (Roche Diagnostics, Branchburg, NJ) (DiDomenico et al., 1996), the Quantiplex HIV-1 RNA Assay (Bayer, Mannheim,

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Germany) (Collins et al., 1997; Pachl et al., 1995), and the NucliSens EasyQ HIV-1 assay (bioMérieux, Boxtel, The Netherlands) (Kievits et al., 1991; de Baar et al., 1999; Yao et al., 2005). All three commercial kits have high sensitivity and reproducibility, and have been strictly validated. One significant drawback, however, is their high price. The cost per sample between 50 and 80 US dollars is too expensive to use for routine monitoring in resource-poor countries.

Competitive reverse transcription (RT)-PCR was the first method to quantitate HIV-1 RNA in plasma and used to show that viral load in plasma can be used as an important surrogate marker to assess the disease progression and the efficacy of ART (Piatak et al., 1993). Competitive RT-PCR involves co-amplification of a competitive RNA template that uses the same primers as for the target cDNA but that can be distinguished from the target after amplification by introducing a deletion, insertion, or a new restriction enzyme site. In conventional competitive RT-PCR, the amplified products of the target sequence and competitor are quantitated on agarose gel electrophoretograms, which is easy and inexpensive to perform, but has never been developed as a commercial assay. Several in-house competitive RT-PCR methods have been reported (Ernest et al., 2001; Johanson et al., 2001), but they have not been fully evaluated for quantitation of HIV-1 subtype non-B RNA in plasma samples. This paper describe a simplified, less expensive protocol for plasma viral load quantitation that is also applicable to HIV-1 subtype non-B.

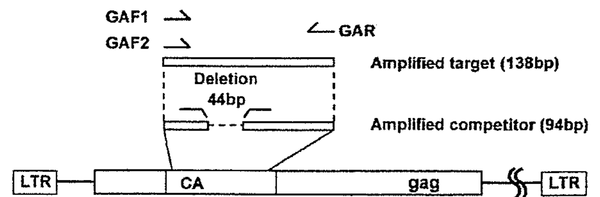
2. Materials and methods

2.1. Subjects

Blood samples were obtained from 47 HIV-1 seropositive individuals (subtype A in 1, subtype B in 25, subtype C in 4, subtype F in 1, and CRF01_AE in 16). The subtype was determined by phylogenetic analysis of sequences in RT and PR regions in *pol* and the *env* V3 region (Kato et al., 2003). The patients provided written informed consent to the study, and the study was approved by the local ethics committee.

2.2. Preparation of competitor RNA and control HIV-1 RNA

A template DNA consisting of an HIV-1 *gag* sequence (positions 1372–1509) with a 44-bp deletion (1418–1461) (Fig. 1) and a T7 promoter (TAATACGACTCACTATAGGGAGA) at the 5′ end was constructed from HIV-1 DNA clone NL4-3 by the recombinant PCR technique (Higuchi, 1990). Competitor RNA was synthesized from the template DNA with an *in vitro* transcription system and T7 RNA polymerase (Promega, Madison, WI). QIAamp UltraSens Virus Kit (QIAGEN, Hilden, Germany) was used to extract HIV-1 LAI RNA from the culture supernatant of peripheral blood mononuclear cells infected with the LAI isolate, and the extracted LAI RNA was stored at −20 °C until used as an external control for HIV-1 quantitation. The exact HIV-1 RNA concentration of the LAI RNA solution was determined by spectrophotometry and Poisson distribution analysis of the positive scores of nested RT-PCR at the endpoint dilution (Kato et al., 2006).



Primer	Sequence	Position
GAF1	5′-CAAGCAGCCATGCAAATGTTAA-3′	1372-1393
GAF2	5′-CAGGCAGCAATGCAAATGTTAA-3′	1372-1393
GAR	5′-AGTAGTTCCTGCTATGTCACTTCCC-3′	1509-1485

Fig. 1. Construction of the competitive RT-PCR assay: target genome and primer design. An HIV-1 *gag* p24 region was selected as the target for the competitive RT-PCR. Both the target and the competitor RNA molecules were co-amplified with a primer mixture of GAR, GAF1, and GAF2. The amplicons of the target and the competitor were distinguished by a 44-bp deletion. The primer sequences of GAR, GAF1, and GAF2 are shown. The nucleotide numbers are based on the HIV-1 NL4-3 sequence (GenBank accession no. M19921).

2.3. Competitive RT-PCR assay for viral RNA quantitation

Primer binding sites were selected in the most conserved sequences in the p24 region of the *gag* gene among all subtypes and CRFs of HIV-1 (Fig. 1) according to the HIV sequence database (Los Alamos database—http://hiv-web.lanl.gov/content/hiv-db/mainpage.html). Two oligonucleotides that differ in nucleotide at positions 1374 and 1380, GAF1 and GAF2, were used together as upstream primers to minimize mismatches with HIV-1 sequences reported in the database (Fig. 1). The size of amplicons of the target RNA and competitor RNA was expected to be 138 and 94 bp, respectively.

RNA was extracted from 140 μl of patient plasma with a QIAamp Viral RNA kit (QIAGEN) with elution in 80 μl. A 20 μl volume of 1, 1/10, and 1/100 dilutions of the eluate were used for subsequent competitive RT-PCR. Three different copy numbers of LAI RNA (10, 50, and 250) were assayed in duplicates as external controls.

RT-PCR was carried out in a 50 μl solution containing primers (0.2 μM GAR, 0.1 μM GAF1, and 0.1 μM GAF2), competitor RNA equivalent to 50 copies, and 1 × SuperScript III RT/Platinum *Taq* Mix (Invitrogen, Carlsbad, CA) with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal profile of RT-PCR was: 10 min at 50 °C for RT; 2 min at 94 °C for initial denaturation; 5 s at 94 °C, 10 s at 48 °C, and 15 s at 72 °C for 5 cycles; 5 s at 94 °C, 10 s at 62 °C, and 15 s at 72 °C for 35 cycles; and 1 min at 72 °C for extended elongation. Introduction of the elongation step at 48 °C was intended to minimize the effect of mismatches between primers and naturally occurring HIV-1 variants.

The plasma samples were also assayed with COBAS Amplicor HIV-1 Monitor Test v1.5 (Roche Diagnostics).

2.4. Electrophoresis and evaluation

A 20 μl sample of PCR products was electrophoresed in a 2% agarose gel at 160 V for 40 min. The gel image was photographed with a Polaroid camera under UV illu-

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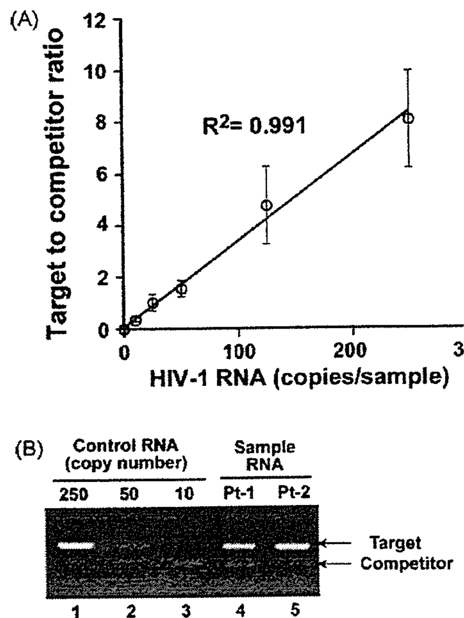

Fig. 2. Linearity and a typical agarose electrophoretogram of the results of competitive RT-PCR. (A) Linear relationship between target/competitor band ratios and initial HIV-1 RNA copy numbers. Each measurement was performed in five replicates. Means and standard deviations are indicated by circles and bars, respectively. (B) Lanes 1–3 are control LAI RNA at levels of 250, 50, and 10 copies/sample, respectively. Lanes 4 and 5 are two clinical samples (Pt-1 and Pt-2). Target/competitor band ratios of the clinical samples (2.66 and 4.10) were translated into copy numbers (160 and 240 copies per sample) using a calibration curve obtained from lanes 1–3.

mination and scanned with a CanoScan FB-636U scanner (Canon, Tokyo, Japan). The band intensity of targets and competitors was evaluated with the NIH Image program (http://rsb.info.nih.gov/nih-image/Default.html). A calibration curve was obtained from the ratios between the bands of the external controls and competitors with MS Excel software (Microsoft, Seattle, WA). The copy numbers of the samples were determined by fitting their ratios to the calibration curve.

3. Results

3.1. Competitive RT-PCR system

Five different copies (10, 25, 50, 125, and 250) of control HIV-1 LAI RNA were amplified together with a fixed dose of competitor RNA, and the ratios between the band intensity of target RNA and the band intensity of competitor RNA were plotted against the input copy numbers of target RNA (Fig. 2A). Good linearity was obtained, with a high correlation coefficient of 0.995.

A photograph of a typical electrophoretogram of competitive RT-PCR for RNA samples from plasma of HIV-1-infected individuals is shown in Fig. 2B. DNA fragments of two different sizes were observed: a 138-bp fragment amplified from target HIV-1 RNA and a 94-bp fragment amplified from competitor RNA. In practical runs, a calibration curve was obtained from co-amplification of each of three different numbers of copies (250, 50, and 10) of LAI RNA with competitor RNA. Fig. 2B shows

Table 1
Detection limit of competitive RT-PCR by limiting dilution assay

HIV-1 RNA (copies/sample)	Target amplification	
	+	−
20	10	0
10	10	0
5	7	3
2.5	2	8

Control LAI RNA samples of two-fold serial dilutions were assayed in 10 replicates, and the detection limit was calculated by Probit analysis.

that ratios of target and competitor RNA bands for patients 1 and 2 were calculated as 2.66 and 4.10, respectively, and applying them to a calibration curve revealed HIV-1 RNA copy numbers per milliliter of 4500 and 6600, respectively.

3.2. Detection limit and reproducibility of the competitive RT-PCR

The lower detection limit of the assay was assessed by testing multiple replicates of dilutions of the HIV-1 LAI RNA (subtype B) that had been quantitated by using the Poisson distribution analysis. Dilutions equal to 560, 280, 140, and 70 copies/ml were tested in 10 replicates at each dilution (Table 1). A Probit analysis showed that the 50% detection limit was 110 copies/ml. Since samples of HIV-1 subtypes A, B, C, and F and CRF01_AE exhibited equivalent efficiency of amplification and detection as described below (Fig. 3), the detection limit for subtype non-B viruses was concluded to be at the same level. The dynamic range of 110–700,000 copies/ml was obtained by testing three serial dilutions (1, 1/10, and 1/100) of the RNA sample.

The reproducibility of the competitive RT-PCR assay was assessed by assaying five seronegative plasma samples spiked with 50 copies of HIV-1 LAI RNA in triplicate (Table 2). The

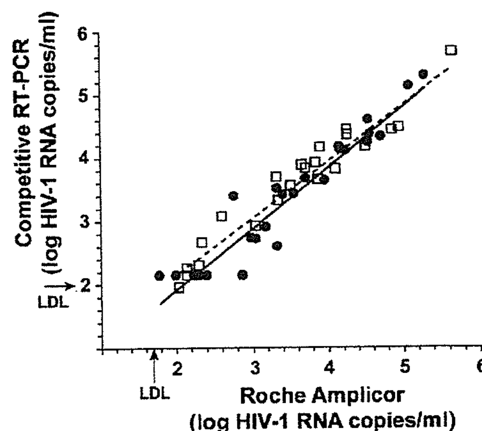

Fig. 3. Comparison between the results of competitive RT-PCR and Cobas Amplicor HIV-1 Monitor test v1.5. Plasma HIV-1 RNA levels of 47 clinical specimens containing subtype B (solid circle) and non-B (open square) are plotted. The lower detection limit (LDL) by competitive RT-PCR and Amplicor was 110 and 50 copies/ml, respectively. The correlation coefficient was 0.956 for subtype B and 0.947 for non-B. Simple regression lines for subtype B (dotted line) and non-B (solid line) are shown.

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Table 2
Reproducibility of competitive RT-PCR

Test[a]	Sample[b] (copies)					
	A	B	C	D	E	Mean ± SD
1	48.5[b]	39.4	37.2	36.8	30.9	37.2 ± 6.4
2	47.6	35.8	37.9	34.8	27.4	35.8 ± 7.3
3	41.5	47.8	40.2	36.2	33.2	40.2 ± 5.6

[a] Viral RNA extraction and competitive RT-PCR reaction were performed in triplicate in three independent experiments.
[b] Fifty copies of LAI RNA were added to the plasma from a healthy human subject in each sample tube.

inter-assay and intra-assay CV values were 25.9% and 9.1%, respectively, and comparable to those obtained with commercially available quantitation kits (16.2–87.5%) (Lin et al., 1998; Muyldermans et al., 2000).

3.3. Quantitation of clinical samples

The HIV-1 RNA concentration of 47 seropositive plasma samples of subtypes A, B, C, F, and CRF01_AE was analyzed with both Cobas Amplicor HIV-1 Monitor test v1.5 and the competitive RT-PCR assay (Fig. 3). The results showed good agreement between the two assays: the correlation coefficient for subtype B and subtype non-B was 0.956 and 0.947, respectively; and the ratio between the two measurements was 0.97 ± 0.10 (mean ± S.D.) for the subtype B samples and 1.01 ± 0.07 for the subtype non-B samples. Although the number of samples in this study was limited, these data suggest that the competitive RT-PCR allows quantitation of both subtype non-B and subtype B of the viral load.

4. Discussion

In order to establish a quantitation method for HIV-1 RNA in plasma that could be used as a routine clinical test in resource-poor countries, an attempt was made to develop an alternative to the commercial kits. The assay described above is based on a competitive RT-PCR technique and is characterized by several improvements over similar methods reported previously. First, it enabled quantitation of HIV-1 RNA in 47 plasma samples containing different HIV-1 subtypes (A, B, AE, C, F) belonging to group M. The viral loads measured with the present assay were consistent with those determined with Cobas Amplicor HIV-1 Monitor, and the ratio was 0.97 ± 0.10 (mean ± S.D.) for the subtype B samples and 1.01 ± 0.07 for the subtype non-B samples. Two competitive RT-PCR assays have been previously reported to detect or quantitate non-subtype B viruses (Emery et al., 2000; Ernest et al., 2001), but since both assays tested primary isolates containing extremely high titers instead of viruses in patients' plasma, the inhibitory effect of plasma components on RT or PCR was not examined. The target of PCR in the present study was selected as the most conserved sequence in the *gag* gene among all subtypes and CRFs of HIV-1 group M (HIV Sequence Database, Los Alamos). Furthermore, to mitigate the influence of sequence variations among subtypes on viral RNA quantitation, a mixture of two upstream primers was employed that differ at two nucleotide positions to minimize mismatches between primers and HIV-1 RNA variants, and 5 cycles with a low annealing temperature of 48 °C were introduced during the early phase of PCR.

The present assay is simple and easy to perform, and the entire procedure, from RNA extraction to analysis of electrophoresis images, takes only 4 h. The use of hot-start DNA polymerase (Platinum *Taq*) enables preparation of PCR samples at room temperature and combination of RT and PCR into one step. Because of the shortness of the amplicon (138 bp), brief reaction times were adequate for RT (10 min) and DNA elongation (15 s each) during PCR. High sensitivity for HIV-1 RNA detection was also achieved. A Probit analysis showed a 50% detection limit of 110 copies of HIV-1 RNA per ml in plasma, which is comparable with the results reported in previous studies (Piatak et al., 1993; Venturi et al., 2000), although a smaller sample volume (140 μl) was used than in those studies (2.8 and 1.8 ml, respectively).

Several commercial HIV-1 quantitation assays, such as Amplicor (Roche Diagnostics), Nuclisens (bioMérieu), and Quantiplex (Bayer), are being used as routine tests to monitor patients being treated with antiretroviral drugs in developed countries. However, they are expensive and require the use of special instruments that are often unaffordable in developing countries. The present method costs only about 10 dollars per sample and does not require any special instruments except a microcentrifuge, gel electrophoresis system, and ultraviolet transilluminator, and the simplicity of the method means that no special training program is needed for clinical technologists. These features should be advantageous in regard to adoption of the assay in resource-poor countries.

One common problem of competitive RT-PCR is the narrow dynamic range. In the current assay, 1-, 1/10- and 1/100-fold dilutions of plasma RNA samples were subjected to RT-PCR separately to cover a quantitation range from 110 to 700,000 copies/ml. Because an adequate dilution ratio cannot be predicted for each plasma sample, a fixed amount of competitor RNA (50 copies) is added to a reaction mixture rather than a plasma sample. Thus, this protocol does not correct measurements of viral loads in terms of RNA extraction efficiency, although the recovery of RNA by QIAamp Viral RNA kits (QIAGEN) was found to be almost constant at 77% (Table 2).

In conclusion, the competitive RT-PCR assay described in this study is a good candidate for an HIV-1 viral load assay for use in monitoring ART in resource-poor settings.

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References

Collins, M.L., Irvine, B., Tyner, D., Fine, E., Zayati, C., Chang, C., Horn, T., Ahle, D., Detmer, J., Shen, L.P., Kolberg, J., Bushnell, S., Urdea, M.S., Ho, D.D., 1997. A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. Nucleic Acids Res. 25 (15), 2979–2984.

de Baar, M.P., van der Schoot, A.M., Goudsmit, J., Jacobs, F., Ehren, R., van der Horn, K.H., Oudshoorn, P., de Wolf, F., de Ronde, A., 1999. Design and evaluation of a human immunodeficiency virus type 1 RNA assay using nucleic acid sequence-based amplification technology able to quantify both group M and O viruses by using the long terminal repeat as target. J. Clin. Microbiol. 37 (6), 1813–1818.

DiDomenico, N., Link, H., Knobel, R., Caratsch, T., Weschler, W., Loewy, Z.G., Rosenstraus, M., 1996. COBAS AMPLICOR: fully automated RNA and DNA amplification and detection system for routine diagnostic PCR. Clin. Chem. 42 (12), 1915–1923.

Emery, S., Bodrug, S., Richardson, B.A., Giachetti, C., Bott, M.A., Panteleeff, D., Jagodzinski, L.L., Michael, N.L., Nduati, R., Bwayo, J., Kreiss, J.K., Overbaugh, J., 2000. valuation of performance of the Gen-Probe human immunodeficiency virus type 1 viral load assay using primary subtype A, C, and D isolates from Kenya. J. Virol. Meth. 38 (7), 2688–2695.

Ernest, I., Alexandre, I., Zammatteo, N., Herman, M., Houbion, A., De Leener, F., Fransen, K., van der Groen, G., Remacle, J., 2001. Quantitative assay for group M (subtype A-H) and group O HIV-1 RNA detection in plasma. J. Virol. Meth. 93 (1–2), 1–14.

Higuchi, R., 1990. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), Recombinant PCR. In PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 177–183.

Johanson, J., Abravaya, K., Caminiti, W., Erickson, D., Flanders, R., Leckie, G., Marshall, E., Mullen, C., Ohhashi, Y., Perry, R., Ricci, J., Salituro, J., Smith, A., Tang, N., Vi, M., Robinson, J., 2001. A new ultrasensitive assay for quantitation of HIV-1 RNA in plasma. J. Virol. Meth. 95 (1–2), 81–92.

Jong-wook, L., 2003. Global health improvement and WHO: shaping the future. Lancet 362 (9401), 2083–2088.

Kalish, L.A., McIntosh, K., Read, J.S., Diaz, C., Landesman, S.H., Pitt, J., Rich, K.C., Shearer, W.T., Davenny, K., Lew, J.F., 1999. Evaluation of human immunodeficiency virus (HIV) type 1 load, CD4 T cell level, and clinical class as time-fixed and time-varying markers of disease progression in HIV-1-infected children. J Infect Dis. 180 (5), 1514–1520.

Kato, S., Saito, R., Hiraishi, Y., Kitamura, N., Matsumoto, T., Hanabusa, H., Kamakura, M., Ikeda, Y., Negishi, M., 2003. Differential prevalence of HIV type 1 subtype B and CRF01_AE among different sexual transmission groups in Tokyo, Japan, as revealed by subtype-specific PCR. AIDS Res. Hum. Retroviruses 19 (11), 1057–1063.

Kato, S., Hanabusa, H., Kaneko, S., Takakuwa, K., Suzuki, M., Kuji, N., Jinno, M., Tanaka, R., Kojima, K., Iwashita, M., Yoshimura, Y., Tanaka, K., 2006. Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted reproduction technique using spermatozoa free from HIV-1. AIDS 20 (7), 967–973.

Kempf, D.J., Rode, R.A., Xu, Y., Sun, E., Heath-Chiozzi, M.E., Valdes, J., Japour, A.J., Danner, S., Boucher, C., Molla, A., Leonard, J.M., 1998. The duration of viral suppression during protease inhibitor therapy for HIV-1 infection is predicted by plasma HIV-1 RNA at the nadir. AIDS 12 (5), F9–F14.

Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R., Lens, P., 1991. NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J. Virol. Meth. 35 (3), 273–286.

Leriche-Guerin, K., Trabaud, M.A., Cotte, L., Bissuel, F., Deruelle, E., Rougier, P., Trepo, C., 1997. Correlation between antiretroviral resistance mutations, biological parameters, and clinical evolution in zidovudine-treated patients infected with human immunodeficiency virus type 1. Eur. J. Clin. Microbiol. Infect Dis. 16 (9), 660–668.

Lin, H.J., Pedneault, L., Hollinger, F.B., 1998. Intra-assay performance characteristics of five assays for quantification of human immunodeficiency virus type 1 RNA in plasma. J. Clin. Microbiol. 36 (3), 835–839.

McDermott, A.Y., Terrin, N., Wanke, C., Skinner, S., Tchetgen, E., Shevitz, A.H., 2005. CD4+ cell count, viral load, and highly active antiretroviral therapy use are independent predictors of body composition alterations in HIV-infected adults: a longitudinal study. Clin. Infect. Dis. 41 (11), 1662–1670.

Muyldermans, G., Debaisieux, L., Fransen, K., Marissens, D., Miller, K., Vaira, D., Vandamme, A.M., Vandenbroucke, A.T., Verhofstede, C., Schuurman, R., Zissis, G., Lauwers, S., 2000. Blinded, multicenter quality control study for the quantification of human immunodeficiency virus type 1 RNA in plasma by the Belgian AIDS reference laboratories. Clin. Microbiol. Infect. 6 (4), 213–217.

Pachl, C., Todd, J.A., Kern, D.G., Sheridan, P.J., Fong, S.J., Stempien, M., Hoo, B., Besemer, D., Yeghiazarian, T., Irvine, B., et al., 1995. Rapid and precise quantification of HIV-1 RNA in plasma using a branched DNA signal amplification assay. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 8 (5), 446–454.

Piatak Jr., M., Saag, M.S., Yang, L.C., Clark, S.J., Kappes, J.C., Luk, K.C., Hahn, B.H., Shaw, G.M., Lifson, J.D., 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science 259 (5102), 1749–1754.

Piliero, P.J., 2003. Early factors in successful anti-HIV treatment. J Int Assoc Physicians AIDS Care (Chic Ill) 2 (1), 10–20.

Russell, S., 2004. The economic burden of illness for households in developing countries: a review of studies focusing on malaria, tuberculosis, and human immunodeficiency virus/acquired immunodeficiency syndrome. Am. J. Trop. Med. Hyg. 71 (2 Suppl.), 147–155.

Simon, V., Ho, D.D., Kalim, Q.A., 2006. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet 368 (9534), 489–550.

Venturi, G., Ferruzzi, R., Romano, L., Catucci, M., Valensin, P.E., Zazzi, M., 2000. Ultrasensitive in-house reverse transcription-competitive PCR for quantitation of HIV-1 RNA in plasma. J. Virol. Meth. 87 (1–2), 91–97.

World Health Organization, 2004. The world health report 2004-changing history. Geneva, Switzerland.

Yao, J., Liu, Z., Ko, L.S., Pan, G., Jiang, Y., 2005. Quantitative detection of HIV-1 RNA using NucliSens EasyQ HIV-1 assay. J. Virol. Meth. 129, 40–46.

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原　著

Real-time PCR を用いた HIV-1 RNA 測定キットの基礎的検討

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Key words: AIDS, human immunodeficiency virus (HIV), PCR, viral infection

要　旨

第 2 世代の HIV-1 RNA 定量法として新しく開発された，コバス TaqMan HIV-1「マニュアル」の性能評価のため，希釈直線性，検出限界，再現性および干渉物質の影響を検討した．また，6 種類のサブタイプの分離株を用いてアンプリコア法と測定値を比較した．希釈直線性の検討の結果，測定値の得られた 1.67×10^2～1.73×10^6 copies/mL の範囲において良好な直線性が得られた（$r^2=0.991$）．また，検出限界の検討の結果，検出限界は 40 copies/mL であり，本キットはアンプリコア法よりも高感度で広い測定範囲をもつことが確認された．再現性は，実験内変動係数が 27.4～50.8%，実験間変動係数が 29.3～81.5% であった．アンプリコア法とは良好な相関性を示したが（$r^2=0.960$），アンプリコア法での値と比較して，すべてのサブタイプで測定値が有意に高くなることが認められた（平均 3.1 倍，$p=0.002$）．特にサブタイプ C の試料ではその傾向が強かった（7.1 倍）．アンプリコア法から TaqMan マニュアル法に移行する際には，この点に留意する必要があると思われる．

〔感染症誌　81：1～5, 2007〕

序　文

血中 HIV-1　RNA の定量は，患者の病期進行や抗 HIV 薬による治療効果を判定するための重要な指標の一つとなっている[1)]．現在，血中 HIV-1　RNA の定量には，ロシュ社が開発したアンプリコア HIV-1 モニター version 1.5（以下，アンプリコア法）が広く臨床で用いられている[2)]．しかし，この方法で低濃度の検体を測定する場合，ウイルスを遠心によって濃縮する必要がある．最近，Real-time PCR を原理とする，測定域の広いコバス TaqMan HIV-1「マニュアル」[3)]（以下，TaqMan マニュアル法）が新しく開発された．そこで我々は，このキットの性能を評価するために，希釈直線性，検出限界，再現性，サブタイプ依存性および干渉物質の影響を検討し，既存のキットであるアンプリコア法と性能を比較した．

材料と方法

1.　TaqMan マニュアル法

HIV-1　RNA 定量は，同キットの添付文書に従って以下のように行った．まず，キットに含まれる High Pure　System（以下 HPS）を用いて血漿検体 500µL から RNA を抽出した．HPS の RNA 抽出原理は，キット付属の定量標準 RNA（以下 QS）を含むカオトロピックイオン溶液によりウイルスを溶解し，シリカ・マトリックスを用いて全 RNA を精製するものである．この精製 RNA をキットの増幅試薬に添加し，専用機器である COBAS TaqMan 48 Analyzer を用いて HIV-1　RNA の定量を行った．この定量法は基本的に TaqMan プローブを用いた Real-time PCR であり，測定結果は増幅曲線のエルボー値（*Ct*）をもとに専用ソフトウエアで自動算出される．検討に際し，コバス TaqMan HIV-1「マニュアル」のキットはロシュ社より提供を受けた．

2.　材料

希釈直線性，再現性および干渉物質の影響の実験には，HIV-1IIIB 株培養上清を用いた．サブタイプ依存性の検討には，サブタイプ A，B，C，AE，F および G に属するそれぞれ 1 株の臨床分離株の培養上清を用いた．これらの分離株は，神奈川県衛生研究所へ HIV 検査依頼があり，研究への同意の得られた患者血液より分離した株であり，サブタイプ A，B，AE，G が

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平成19年 1 月20日

日本人，サブタイプC，Fは外国籍患者より分離された．サブタイプはenv V3領域，gag p24領域について塩基配列を決定後，Clustal Xを用いて決定した．

検出限界の実験は，ポアソン分布式によって濃度を決定した[4)]III_B株RNA溶液を用いた．培養上清の希釈は，抗体検査とNAT検査の両方で陰性が確認された健常人プール血漿を用いて行った．RNA溶液の希釈は，RNaseフリーのPBSを用いて行った．

3．方法

1）希釈直線性の検討

III_B株培養上清のp24濃度をVIDAS HIV p24（ビオメリュー社）で測定し，p24濃度が1,000pg/mLになるように調製した試料を母液とし，10倍ずつ6段階希釈を行った．測定は各濃度で3回ずつ行った．

2）検出限界の検討

ポアソン分布法で定量したIII_B株由来のRNA液を10，20，40および100 copies/mLに希釈した．測定は各濃度で10回ずつ行った．

3）再現性の検討

III_B株培養上清をp24濃度が0.01，1および100pg/mLになるように希釈し，各濃度について5点同時測定を5回ずつ行い，実験内変動係数と実験間変動係数を算出した．

4）陰性検体の測定

HIV抗体陰性かつNAT検査陰性の血漿51検体を1回ずつ測定した．

5）干渉物質の影響

ビリルビンF，ビリルビンC，溶血ヘモグロビンおよび乳糜（干渉チェック・Aプラス，国際試薬）のそれぞれが測定値に与える影響を調べた．各試薬で検討した濃度は，ビリルビンFが18.4mg/dL，ビリルビンCが19.7mg/dL，溶血ヘモグロビンが490mg/dL，乳糜が1,470度（ホルマジン濁度）とし，5回測定を行って，無添加検体の測定値と比較した．

6）希釈直線性のサブタイプ依存性

6種類のサブタイプ（A，B，C，AE，F，G）の分離株培養上清を，p24抗原濃度0.01，1および10pg/mLに調製した．測定は各濃度で3回ずつ行った．

7）TaqManマニュアル法とアンプリコア法の比較

上と同じ6種類のサブタイプの検体について，アンプリコア法による測定を各濃度で2回ずつ行い，TaqManマニュアル法の値と比較した．

4．統計解析）

希釈直線性の検討，およびTaqManマニュアル法とアンプリコア法の比較は，測定値の逆数の重みをつけた回帰分析によって行った．再現性の検討は分散分析によって行った．干渉物質の影響の検討はスチューデントのt検定を用いて行った．

Fig. 1 Linearity test of COBAS TaqMan. Data supports good linearity from 1.67×10^2 to 1.73×10^6 HIV RNA copies/mL. Linearity was tested using reference HIV strain IIIB diluted with HIV-negative plasma.

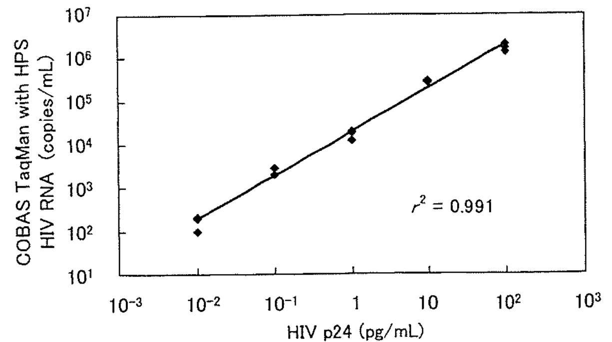


Table 1 Minimum detection of COBAS TaqMan. Sensitivity was 40 copies/mL at a 100% hit rate. The HIV RNA copy number was determined by applying results of RT-nested PCR at endpoint dilution to the null class equation of the Poisson distribution [copy number per reaction = -logP (0) ; P (0), the probability of negative reactions].

HIV RNA (copies/mL)	n	positive	negative	%
10	10	6	4	60
20	10	7	3	70
40	10	10	0	100
100	10	10	0	100

成 績

1．希釈直線性の検討

p24濃度0.001～1,000pg/mLのウイルス試料を測定した結果，0.001pg/mLの試料ではRNAが検出されず，1,000pg/mLの試料は測定限界以上（$>1 \times 10^7$ copies/mL）であった．測定値の得られた0.01～100pg/mLの各濃度に対して測定値（1.67×10^2～1.73×10^6 copies/mL）をプロットしたものをFig. 1に示す．単回帰分析を行った結果，決定係数r^2は0.991，回帰係数は24,900 copies/pgであり，定量可能範囲内において測定値の直線性は良好であった．

2．検出限界の検討

低濃度のHIV-1 RNA試料（10，20，40および100 copies/mL）を用いて検出限界を検討した．100 copies/mLの試料と40 copies/mLの試料は，10回の測定すべてでHIV-1 RNAが検出されたが，20 copies/mLの試料は10回のうち7回，10 copies/mLの検体は10回のうち6回で検出された（Table 1）．

3．再現性の検討

実験内変動係数は，p24濃度0.01pg/mLで50.8%，

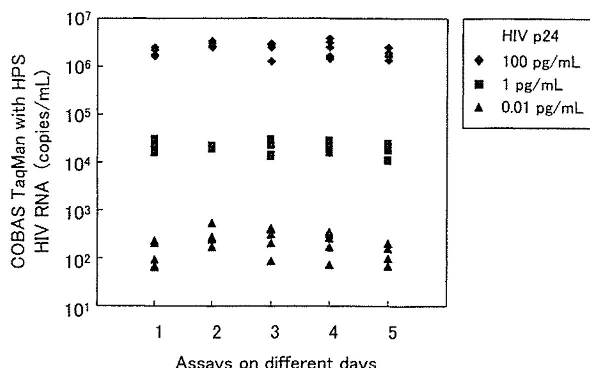


Fig. 2 The precision of COBAS TaqMan was tested using reference HIV strain ⅢB diluted with HIV-negativ plasma. Individual data points are shown. Intraexperimental CV at 100, 1, and 0.01pg/mL ware 27.4%, 27.6%, and 50.8%. Interexperimental CV were 41.4%, 29.3%, and 81.5%.

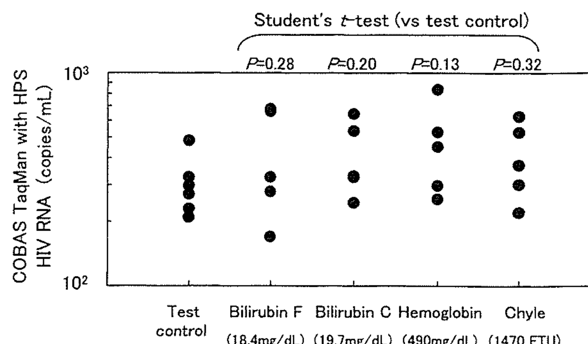


Fig. 3 Interference test of COBAS TaqMan with high bilirubin F, bilirubin C, hemoglobin and chyle. These interference materials did not adversely affect the performance of COBAS TaqMan ($p > 0.05$).

1pg/mL で 27.6%，100pg/mL で 27.4% であった．また，実験間変動係数は，0.01pg/mL で 81.5%，1pg/mL で 29.3%，100pg/mL で 41.4% であった(Fig. 2)．

4. 陰性検体の測定

HIV 抗体と NAT の両方が陰性の血漿 51 検体を測定した結果，すべての検体で HIV-1 RNA は検出されなかった．

5. 干渉物質の影響

無添加検体の平均値が 301.7 copies/mL であったのに対して，ビリルビン F 添加検体の平均値は 419.8 copies/mL，ビリルビン C 添加検体は 413.6 copies/mL，溶血ヘモグロビン添加検体は 469.8 copies/mL，乳糜添加検体は 407.4 copies/mL であった (Fig. 3)．4 種類の干渉物質を添加したいずれの検体も，無添加検体に比べて測定値に有意差はみられなかった (p = 0.13〜0.32)．

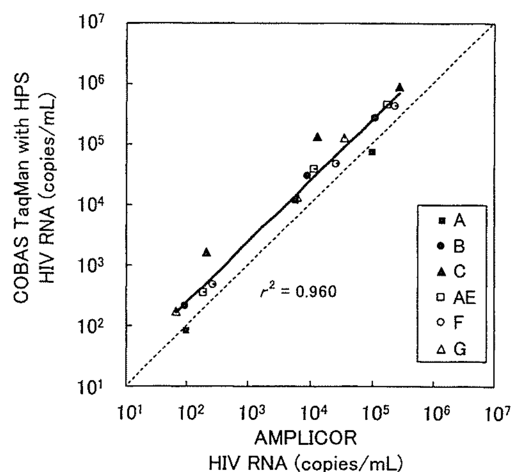
6. 希釈直線性のサブタイプ依存性

p24 抗原濃度を 0.01，1，10pg/mL に調製した 6 種類のサブタイプの分離株の HIV-1 RNA 濃度を測定し，サブタイプごとに各濃度の測定値を単回帰分析した．その結果，決定因子 r^2 は 0.976〜0.999 であり，すべてのサブタイプで良好な直線性が得られた．

7. TaqMan マニュアル法とアンプリコア法の比較

既存の HIV-1 RNA 定量法であるアンプリコア法と比較するため，サブタイプ依存性の検討で用いた 6 種類のサブタイプの分離株について，アンプリコア法でも同様に測定を行った．その結果，アンプリコア法の測定値も，サブタイプに依存しない良好な希釈直線性を示した (r^2=0.970〜0.999)．そこで，すべてのサブタイプ分離株の試料について，アンプリコア法と TaqMan マニュアル法の測定値の関係を調べると

Fig. 4 Correlation of viral loads determined by COBAS TaqMan and AMPLICOR. Correlation was tested using 6 cultured isolates (subtypes A, B, C, AE, F, and G) diluted with HIV-negative plasma. COBAS TaqMan showed an excellent titer correlation with AMPLICOR (r^2 = 0.960), but titers determined by COBAS TaqMan were 3.1 times higher than those by AMPLICOR.



(Fig. 4)，両者の間には良好な相関性が得られたが (r^2=0.960)，回帰係数は 3.1 となり，1 より有意に高かった (p =0.002)．測定値の比をサブタイプ試料ごとに分散分析によって解析すると，サブタイプ C の検体の測定値の比 (7.1) は，他のサブタイプの測定値の比 (1.3〜2.8) よりも有意に高かった (P =0.007) (Fig. 5)．

TaqMan マニュアル法とアンプリコア法の測定値の正確さを検討するために，ポアソン分布法での定量値が 100 copies/mL のパネルを二つの方法で測定した．その結果，前者の測定値は 310±190 copies/mL,

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Fig. 5 Ratios of viral load results for COBAS TaqMan and AMPLICOR. The mean titer obtained with COBAS TaqMan was 3.1 times higher than that with AMPLICOR, and 7.1 times higher in a sample of subtype C.

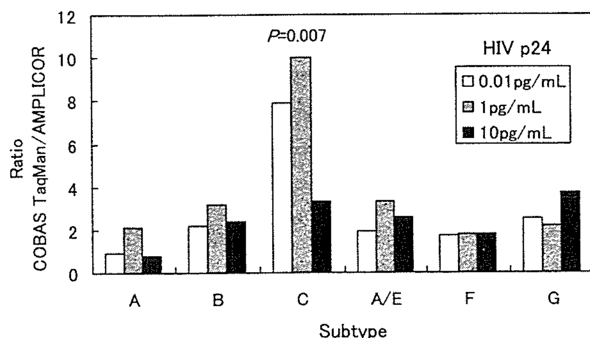
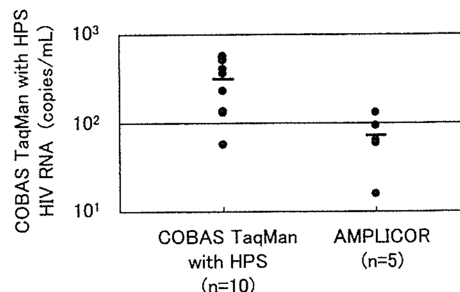


Fig. 6 Accuracy of COBAS TaqMan and AMPLICOR was tested using 100 copies/mL HIV RNA sample solution (subtype B). Mean ± SD of measured values for COBAS TaqMan and AMPLICOR ware 306 ± 190 copies/mL and 72 ± 42 copies/mL. Results of COBAS TaqMan were about 3 times higher than the concentration of HIV RNA determined by the Poisson distribution.



後者の測定値は72±42 copies/mLであった（Fig. 6）.

考察

アンプリコア法に代わるHIV-1 RNA測定キットとして開発されたTaqManマニュアル法は，測定域が40～10^7 copies/mLであり，AMPLICORの50～7.5×10^5 copies/mLに比べて広い範囲の定量が可能とされている．我々の結果でも，検出限界が40 copies/mLであり，1.67×10^2～1.73×10^6 copies/mLの範囲で良好な希釈直線性があることが確かめられた．測定値は6種類のサブタイプすべてで良好な希釈直線性を示し，再現性に関しては，実験内変動係数が27.4%～50.8%，実験間変動係数が29.3%～81.5%であり，既存のアンプリコア法と同程度であった[5]．また，干渉物質の影響は，血漿に存在する可能性のある4種の物質，ビリルビンF，ビリルビンC，溶血ヘモグロビンおよび乳糜でまったく認められなかった．

アンプリコア法との比較では，r^2=0.960と良好な相関を示したが，TaqManマニュアル法の測定値がアンプリコアでの値よりも有意に3倍程度高くなることが認められた．このような傾向は患者血清でもみられることが報告されている[6]．測定濃度の全領域にわたって測定値がアンプリコア法より高めに出ていることから，その原因として，定量基準として用いられているQSに起因している可能性が高いと考えられる．2つの方法で用いられているQSは一次構造の異なる別なRNA分子である．2つの方法ではそれぞれのQSを基準として定量するため，これらQSの基準値に違いがあった場合，それをもとに算出される定量値にも有意差が生じてしまう．別な原因としては，TaqManプローブを用いるTaqManマニュアル法と，PCR産物をプレートハイブリダイゼーションにより定量するアンプリコア法では，プライマー，プローブ，反応条件などが異なるために，QSおよび検体中のHIV-1 RNAに対するPCRの増幅効率や検出効率に違いが生じ，その結果，定量値が異なってしまった可能性が考えられる．

TaqManマニュアル法は，既存のアンプリコア法と比較して広い測定範囲をもつことが確認された．アンプリコア法では，検体中のHIV-1 RNA濃度によって超遠心機を必要とする高感度法と標準法を使い分ける必要があったが，TaqManマニュアル法は，すべての測定範囲において同一の方法を用いることができ，作業効率やデータの連続性において優れている．しかし，本検討において，TaqManマニュアル法はアンプリコア法よりも有意に高い値を示すことが認められたことから，アンプリコア法からTaqManマニュアル法への移行の際，測定値が3倍程度高くなることが予想される．HIV感染症「治療の手引き」第9版では，CD4陽性リンパ球数が200～350/mm^3であり，かつ血中ウイルス量が100,000 copies/mL以上の場合，抗HIV療法の開始を推奨している．治療開始の参考値としてTaqManマニュアル法の値を用いる場合は，以上の点に留意する必要がある．

文献

1) O'Brien WA, Hartigan PM, Martin D, Esinhart J, Hill A, Benoit S, *et al.*: Changes in plasma HIV-1 RNA and CD4+ lymphocyte counts and the risk of progression to AIDS. Veterans Affairs Cooperative Study Group on AIDS. N Engl J Med 1996 ; 334 : 426—31.
2) Mulder J, McKinney N, Christopherson C, Sninsky J, Greenfield L, Kwok S : Rapid and simple PCR assay for quantitation of human immu-

nodeficiency virus type 1 RNA in plasma : application to acute retroviral infection. J Clin Microbiol 1994 ; 32 : 292—300.

3) Katsoulidou A, Petrodaskalaki M, Sypsa V, Papachristou E, Anastassopoulou CG, Gargalianos P, *et al.* : Evaluation of the clinical sensitivity for the quantification of human immunodeficiency virus type 1 RNA in plasma : Comparison of the new COBAS TaqMan HIV-1 with three current HIV-RNA assays-LCx HIV RNA quantitative, VERSANT HIV-1 RNA 3.0 (bDNA) and COBAS AMPLICOR HIV-1 Monitor v1.5. J Virol Methods 2006 ; 131 : 168—74.

4) Kato S, Hanabusa H, Kaneko S, Takakuwa K, Suzuki M, Kuji N, *et al.* : Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method : Assisted reproduction technique using spermatozoa free from HIV-1. AIDS 2006 ; 20 : 967—73.

5) 川田かおる，満田年宏，近藤真規子，齋藤隆行，今井光信，伊藤　章：PCR による HIV-1　RNA 定量法の基礎的検討—測定値の再現性と阻害物質の影響．感染症誌　1998 : 72 : 1041—5.

6) 菊池　嘉，福武勝幸，天野景裕，白阪琢磨，山本善彦，今井光信，他：リアルタイム PCR 法による HIV-1　RNA 定量キット COBAS　TaqMan HIV-1 Test（High Pure System）の検討．第 18 回日本エイズ学会　静岡　2004 12.

Evaluation of a New Real-time PCR Assay Kit for Quantification of Human Immunodeficiency Virus RNA in Plasma

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We evaluated the use of COBAS TaqMan HIV-1 for highly pure system (COBAS TaqMan) recently developed as a second-generation quantification of human immunodeficiency virus type 1 (HIV-1) RNA. The linearity, sensitivity, reproducibility, and effects of possibly interfering materials were examined and results compared to those of AMPLICOR HIV-1 MONITOR Test version 1.5 (AMPLICOR) using 6 virus isolates that were all different subtypes. Excellent linearity was obtained at 1.67×10^2–1.73×10^6 copies/mL ($r^2 = 0.991$). Sensitivity was 40 copies/mL at a 100% hit rate. Intraexperimental CVs were 27.4–50.8%, and interexperimental CVs were 29.3–81.5%. Although COBAS TaqMan showed an excellent titer correlation with AMPLICOR ($r^2 = 0.960$), mean titer obtained with COBAS TaqMan was 3.1 times higher than that with AMPLICOR ($p = 0.002$), and 7.1 times higher in a sample of subtype C, suggesting discrepancies in HIV-1 RNA quantification between the two kits. This point should be noted when AMPLICOR is replaced by COBAS TaqMan.

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