METHODS

Selection of patients. The patients enrolled in the present study were a subset of a well-characterized cohort of 166 patients with haemophilia who had received non-heated plasma-derived coagulation products before 1987 and had been observed regularly since 1995 at Ogikubo hospital (Tokyo, Japan). Plasma samples from patients with known HCV and HIV serological status were stored at –80 °C. Of these patients, 57 were positive and 109 were negative for anti-HIV. After exclusion of HCV-RNA-negative and interferon-treated patients, and those with a mixture or shift in HCV subtypes during the follow-up, 13 HCV-1a-RNA-positive patients (eight HIV-positive and five HIV-negative patients) were selected at random for this study. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of each institution. Every patient gave written informed consent to participate in the virological research.

Laboratory tests. Laboratory evaluation included complete blood cell count and serum transaminases [alanine aminotransferase (ALT)]. $CD4^+$ cell counts were examined by fluorescence-activated cell sorting at SRL Inc. (Tokyo, Japan). Serum HCV-RNA levels and HIV-RNA levels were measured by a commercial PCR assay (Amplicor HIV-1 Monitor and Amplicore HCV monitor; Roche Diagnostics). The detection limits of PCR for HCV-RNA and HIV-RNA were 500 IU ml^{-1} [0.5 kilo international unit (KIU) ml^{-1}] and 50 copies ml^{-1}, respectively.

HCV-RNA isolation and amplification from the core, E1 and E2 regions. Nucleic acids were extracted from serum samples using a SepaGene RV-R Nucleic Acid Extracting kit (Sanko Junyaku) in accordance with the manufacturer's protocol. Viral RNA was reverse-transcribed to cDNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and random hexamer primer (Takara Shuzo) as described previously (Ohno *et al.*, 1997).

Partial core, E1 and E2 fragments were amplified by using PCR with primers as described previously (Tanaka *et al.*, 2002). To reduce the number of artificial substitutions arising during PCR, Platinum *Pfx* DNA Polymerase (Invitrogen) with a very high fidelity was used.

Cloning and sequencing of cDNA. The amplified products were ligated into pCR-Blunt II-TOPO Vector and used to transform DH5-α high-efficiency competent cells according to the manufacturer's protocol (Invitrogen). The plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen) and the presence of the inserts confirmed by digestion with *Eco*RI. Sequencing was performed on more than 10 clones per patient at the baseline (1995–1997) and the end point (2002–2003). All clones were sequenced with Prism Big Dye (Applied Biosystems) in an ABI 3100 DNA automated sequencer.

Construction of phylogenetic trees. Nucleotide sequences of HCV were aligned by using the program CLUSTAL_X and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetic Analysis software (MEGA version 3.0; Kumar *et al.*, 2001). The MEGA algorithms were used to calculate the mean Tamura–Nei pairwise distance for all clones as well as a matrix of Tamura–Nei pairwise distances for each patient. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times.

Genetic diversity of HCV over a time course. Two approaches were used to infer the genetic diversity of HCV in each patient. In the first approach, total evolutionary distances among a heterogeneous viral population were compared between the baseline and end point for each patient in the phylogenetic tree. The phylogenetic tree of genetic diversity was constructed by using the maximum-likelihood (ML) method and the ancestral sequence was inferred at every node using the ML method (Yang *et al.*, 1995). As the evolutionary distance in each branch, the number of synonymous substitutions per synonymous site (synonymous distance) was estimated by the modified Nei–Gojobori method. Total synonymous distances were assumed to represent the genetic diversity of a heterogeneous viral population in each patient.

The other approach is the coalescence theory based on estimation of the genetic diversity. A consensus sequence based on the sequences of all HCV clones isolated from each patient was used as an outgroup to locate the position of the root in each phylogenetic tree. The topology of the phylogenetic tree was estimated by the neighbour-joining method (PHYLIP). Based on the topology, we constructed the phylogenetic tree and inferred the evolutionary rate by the ML method under the premise of the molecular clock (TipDate) (Rambaut, 2000). Based on the trees and the evolutionary rates estimated by TipDate, the coalescent analysis of genetic diversity was conducted for each patient using the Genie v3.5 software (Pybus *et al.*, 2001; Pybus & Rambaut, 2002). In brief, time t was transformed to year using the HCV molecular evolutionary rate, assuming the sample-collection time to be the present. Function $N(t)$ (effective numbers of HCV infections through time) was estimated by the ML method to infer the genetic diversity of HCV (Pybus & Rambaut, 2002). Although there are several models to infer $N(t)$, the best-fit model was different among patients. Therefore, we chose a simplified model in which the genetic diversity was assumed to be exponentially increased over time (expansion model).

Identification of positively selective regions. Positively selected regions were identified using the modified method of Suzuki & Gojobori (2001). In brief, a phylogenetic tree of sequences from HCV clones was reconstructed in each patient by the ML method. The ancestral sequence was inferred at every node in the phylogenetic tree using the ML method (Yang *et al.*, 1995). Then, synonymous and non-synonymous substitutions throughout the phylogenetic tree were estimated in each branch for each codon site. Here, to see the differences in selective pressure for HCV between the HIV-positive and -negative groups, we independently summed the total numbers of synonymous (N_s) and non-synonymous (N_n) substitutions occurring at each codon site of the HCV clones from either eight patients infected with HIV or five patients without HIV infection. The mean numbers of synonymous (C_s) and non-synonymous (C_n) sites were calculated for each codon site by the modified Nei–Gojobori method. The genetic distance of synonymous (d_S) and non-synonymous (d_N) was calculated as N_s/C_s and N_n/C_n, respectively. Although the ratio d_N/d_S is usually used for estimating selective pressure, we used $(d_N+0.5)/(d_S+0.5)$ ratio instead in the present study, because no synonymous substitution was found in several codon sites. The ratio was calculated along with the sequence by the sliding-window analysis. Each window size consisted of three codons.

RESULTS

Comparison of clinical characteristics between HCV patients with and without HIV infection

When we compared clinical data between HCV patients with HIV (HIV-positive group) and without HIV (HIV-negative group), there were no significant differences of mean age, sex, putative duration of HCV infection or mean peak ALT levels (116 vs 146) (Table 1). Changes of ALT levels also were not different between these two groups. Mean peaks of HCV-RNA levels in the HIV-positive group (2300 ± 668 KIU ml^{-1}), however, were significantly higher than those in the HIV-negative group (936 ± 423, $P=$

Table 1. Clinical characteristics among HCV patients in this study

All patients are male. HCV genotype of all patients is 1a. LC, Liver cirrhosis; ALT, alanine aminotransferase; NT, not tested; +, positive; −, negative.

ID	Age	Putative date of HCV infection	LC	HIV	AIDS	HIV-RNA (copies ml^{-1})	CD4 at baseline (μl^{-1})	HCV-RNA (KIU ml^{-1})			ALT (U l^{-1})	
								Range	Peak	Increase ×2	Range	Increase ×2
NT211	29	1982	−	+	+	130 000	20	130–2100	2100	yes	20–156	yes
GM248	39	1986	−	+	−	23 000	110	290–1200	1200	yes	34–96	yes
OT33	34	1982	−	+	−	2300	286	170–2000	2000	yes	40–43	no
HH127	33	1980–1982	−	+	−	22 000	270	330–3200	3200	yes	27–168	yes
TA92	32	1984	−	+	−	98 000	271	1300–2900	2900	yes	29–34	no
KY48	31	1980–1983	−	+	−	55 000	242	730–2700	2700	yes	66–213	yes
NK112	28	1982	+	+	+	100 000	27	2300–2600	2600	yes	16–28	no
YY321	27	1987	−	+	+	95 000	35	1200–1700	1700	no	181–186	yes
KK202	19	1987	−	−	−	NT	NT	310–1600	1600	yes	21–41	no
KN201	45	1982	+	−	−	NT	NT	300–710	710	no	38–98	no
TS246	20	1984	−	−	−	NT	NT	230–1000	1000	yes	27–37	no
SH265	20	1985	−	−	−	NT	NT	340–470	470	no	130–470	yes
ST251	26	1984	−	−	−	NT	NT	590–900	900	no	38–83	yes

0.0019), which is consistent with previous reports (Eyster *et al.*, 1994). Additionally, seven of eight patients in the HIV group had HCV-RNA elevation more than twice during follow-up, whereas only two patients in the HIV-negative group had HCV-RNA elevation.

For the eight HCV patients in the HIV-positive group, HIV-RNA and CD4 are shown in Table 1. Three of the patients had already developed AIDS and had very low CD4 counts (20, 27, 35 μl^{-1}), and the remaining five patients with HIV also had relatively low CD4 levels (110–286) at the baseline (1995–1997) before initiating highly active anti-retroviral therapy (HAART). Thereby, all HIV-infected patients studied were considered to be in an immunity-suppressed condition. Four patients with a CD4 count less than 200, including the three AIDS patients, received anti-HIV treatments.

Long-term intra-host diversity of HCV evaluated on distinct genomic regions

It has been shown previously that the genetic diversity of HCV changes in an oscillatory manner during the natural course of the infection (Devereux *et al.*, 1997). Taking into account that the genetic diversity of HCV analysed at a single time point might not accurately reflect the dynamic profile of the population over time, we have examined 26 serum samples collected from 13 patients at two distinct time points with intervals of at least 6 years (6–8 years). At least 11 HCV clones were isolated from a single patient at the baseline (1995–1997) and at the end point (2002–2003) of the follow-up. Overall, 325 HCV clones were thus isolated and analysed. All of them belonged to genotype 1a. Phylogenetic relation of the HCV clones isolated from all patients is shown in Fig. 1. Assuming that HCV is composed of a heterogeneous viral population, which is evolving throughout time in a given host (carrier), we aimed to estimate the size and heterogeneity of the population. Two different methods were used to attain this aim.

First, we directly compared the genetic diversity of a heterogeneous viral population between the two time points. To do that, we estimated totals (for all patients in each of the two groups) of intra-host synonymous distances at each of the two time points. This estimation was done independently in both E1 and E2 genomic regions (Fig. 2). The increased difference from the baseline to the end point between the HIV-positive and HIV-negative groups was tested by the regression analysis, and the genetic diversity of the HIV-positive group is significantly higher than that of the HIV-negative group (P=0.043).

Second, the coalescent analysis of genetic diversity of HCV was conducted for each patient. Further, mean curves of the effective numbers of HCV infections were compared between HIV-positive and -negative groups (Fig. 3). Although the estimated mean number was initially relatively lower in the HIV-positive group, the rapid change to exponential growth, was observed several years after HIV infection in this group, whereas in the HIV-negative group, the effective number was gradually increasing throughout the period of time. The difference of exponential growth is significant (P=0.04). Hence, the result obtained by either method indicated the HIV-positive group to have higher genetic diversity of the heterogeneous viral population than the HIV-negative group, suggesting that this was due to the exposure of HIV infection.

Putative positively selective regions in the E1 and E2 regions

Since the higher genetic diversity of HCV was observed in HIV-positive patients, we further examined genetic evi-

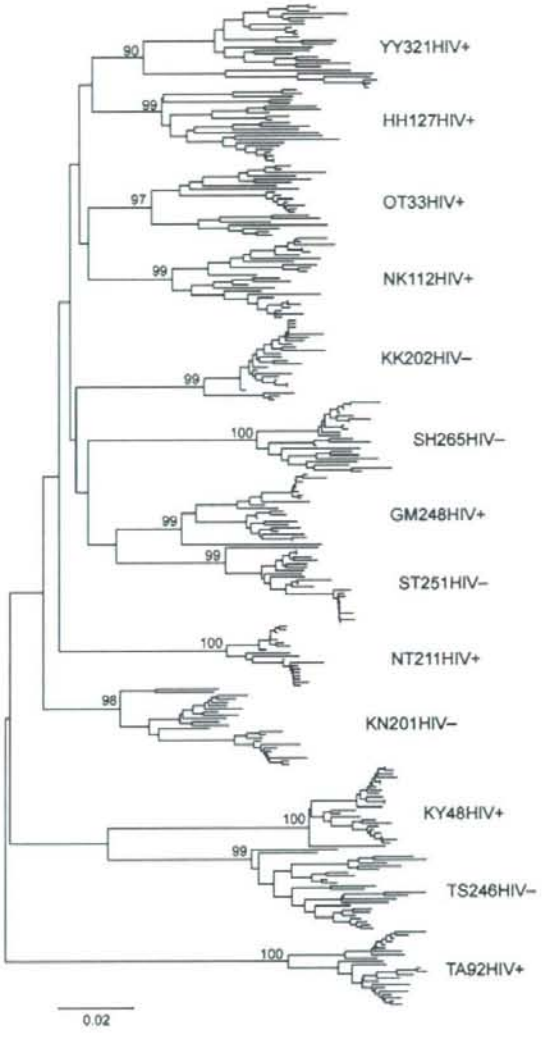

Fig. 1. A phylogenetic tree of E1 and E2 regions of the 325 HCV clones isolated from 13 patients. The significant phylogenetic cluster was observed in each of the eight HIV-positive (HIV+) and five HIV-negative (HIV−) patients. Numbers at nodes indicate bootstrap values of 1000 replications.

dence of the selective immune pressure in both groups. Selective immune pressure was estimated in each, E1 and E2, gene. Some differences were observed between the HIV-positive and -negative groups (Table 2, Fig. 4). Immune epitopes (11 aa segments in the E1 and 5 aa segments in the E2 region) that were observed only in the HIV-negative group might have relatively weak antigenicity. Some of the segments were previously recognized as HCV-specific potential immunogenic targets such as

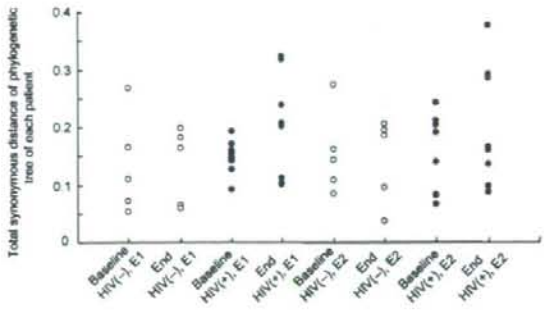

Fig. 2. Total synonymous distances of the phylogenetic tree of each time point. *y*-axis indicates total synonymous distance of the phylogenetic tree constructed by the sequences isolated at each time point. *x*-axis indicates two time points [baseline and end point (End)] and two groups [HIV-positive (HIV+) and -negative (HIV−)]. Each dot represents a patient.

cytotoxic T lymphocyte (CTL) epitopes (URL: http://hcv.lanl.gov/content/immuno/tables/ctl_summary.html), indicating that the positively selected segments estimated in the present study are associated with the immune response. On the other hand, positively selected segments around the hypervariable region (HVR1) regardless of HIV infection should have strong antigenic epitopes, suggesting little influence of the HIV coinfection on the natural immune selection targeting this region.

DISCUSSION

A previous meta-analysis showed a significantly elevated relative risk of severe liver disease in patients coinfected

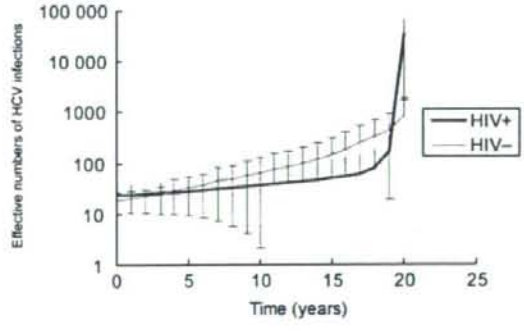

Fig. 3. The mean effective numbers of HCV infections in HIV-positive (HIV+) and -negative (HIV−) groups over the years from the baseline. Two lines indicate the dynamics of the mean effective numbers of HCV infections (*y*-axis) estimated in the E2 region and the bars indicate standard deviations. *x*-axis indicates number of years from the end point.

Table 2. Putative positively selective regions

Unknown epitopes identified only in the HIV-negative group are shown in bold.

Region	PS value*	HCV with HIV	HCV without HIV		
E1	PS>1.0	366–368	**192–195**,	**203–205**,	212–215,
			223–229,	234–237,	**245–247**,
			255–257,	295–299,	**346–349**
			353–358,	**359–361**	
E2	PS>1.0	383–412, 414–418, 434–438	382–410,	418–420,	432–436,
			443–446,	463–465,	**480–482**,
			486–488	496–500	

*PS (positive selection) $=(d_N+0.5)/(d_S+0.5)$.

with HIV and HCV (Braitstein *et al.*, 2004; Graham *et al.*, 2001). Another cohort study of HIV/HCV-coinfected patients also indicated association of low CD4 cell count, alcohol consumption rate and age at HIV/HCV coinfection with acceleration of the liver fibrosis (Benhamou *et al.*, 1999). The increased HCV replication in the HIV/HCV-coinfected patients would induce an intermediate immune response that is large enough to induce hepatic cell destruction and fibrosis but not enough to eradicate the virus from its reservoirs (Lai *et al.*, 2003; Poynard *et al.*, 2003).

In the present study, the genetic diversity of HCV was higher in the HIV-positive group compared with the HIV-negative group, which could be associated with either or both higher rate of HCV replication and HIV-associated immunosuppression, leading to less selective pressure on HCV in the HIV-positive group. The increased HCV replication by immune dysregulation in the HIV/HCV-coinfected patients might damage liver cells through apoptosis and other means such as cytokine disruption as reported previously (Puoti *et al.*, 2001). Although several

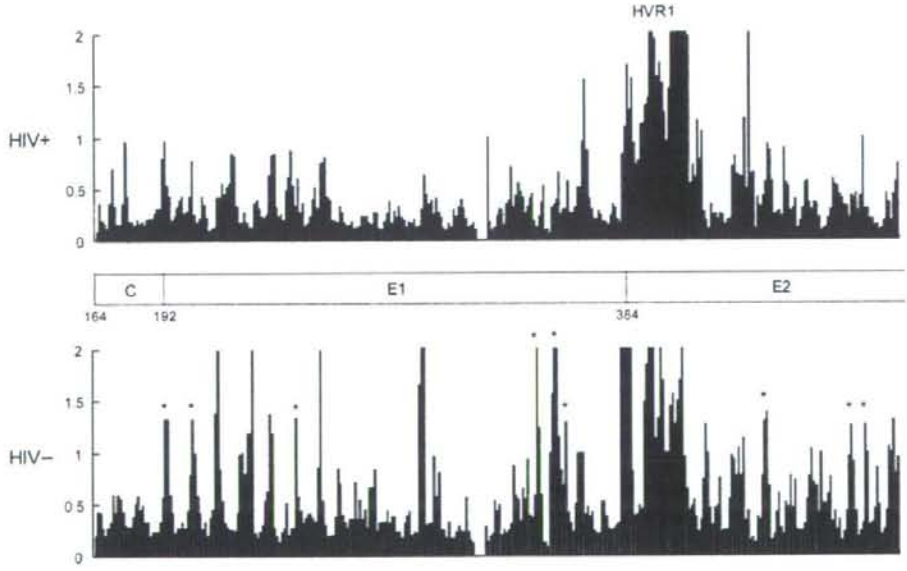

Fig. 4. Comparative histograms of the E1 and E2 regions of the HCV clones from HIV-positive (HIV+) and -negative (HIV−) patients. *y*-axis indicates $(d_N+0.5)/(d_S+0.5)$ ratio, columns exceeding value 1.0 represent the putative positively selective regions. Regions outside of any known CTL epitopes are indicated by asterisks and probably indicate unknown epitopes. *x*-axis indicates the schematic position in the HCV genes: C, core; HVR1, hypervariable region.

studies showed that HCV diversity decreases with the degree of HIV-related immunosuppression (Babik & Holodniy, 2003; Mao *et al.*, 2001; Martell *et al.*, 1992; Qin *et al.*, 2005; Roque-Afonso *et al.*, 2002; Toyoda *et al.*, 1997), this might be associated with the different parameters assessed for the genetic diversity; the conclusions in most previous studies were made by analysing total numbers of HCV clones and overall genetic distances at the amino acid level.

To assess the discrepancy with our results by coalescence-based estimation, we further examined the role of HCV-targeted immune pressure. The HCV nucleotide substitution pattern was compared between subjects with intact immune system versus those with HIV-associated immunosupression using a recently designed approach. Previous reports had used the mean of pairwise synonymous and non-synonymous distances within isolates (Ray *et al.*, 2000; Blackard *et al.*, 2004). Since most isolates do not have an independent evolutionary process, the mean may not represent overall genetic diversity of the heterogeneous viral population. To examine the non-redundant evolutionary process, recent methods have inferred the evolutionary process throughout the phylogenetic tree (Sheridan *et al.*, 2004; Suzuki & Gojobori, 2001; Hanada *et al.*, 2006). In the present study, we have applied a new approach to examine selection of HCV affected by HIV. The approach supported the theory that the diversified population of HIV-positive patients is due to less selective pressure and allows identification of specific regions indicating the presence of positive selection in HIV-negative patients compared with HIV-positive patients with immunosuppression. Although these positively selective segments, which were observed only in the HIV-negative group, might have relatively weak antigenicity, most of them were located inside potential immunogenic targets and others might be somewhat new antigen-recognition regions associated with HCV-specific immune responses. Interestingly, no influence by HIV coinfection was observed in the HVR1, which contains sequence-specific immunological B-cell epitopes that induce the production of antibodies restricted to the specific viral isolate (Kato *et al.*, 1993), indicating that the positively selected segments regardless of HIV infection should have strong antigenic epitopes. Taken together, our findings indicate that defenceless HCV clones that are extinct in usual conditions can survive in HIV-positive patients because of less immune pressure leading to HIV infection. Consequently, the genetic diversity of HCV will be greater in HIV-positive patients. In fact, the diversity of both synonymous and non-synonymous substitutions was larger in HIV-positive patients than HIV-negative patients (data not shown).

One limitation of the present study is the general lack of functional immunological data. In this study, HCV-specific CD4 ELISPOT responses were not detected in all subjects and CD8 cell counts were not measured. Therefore, we chose to use HIV load and CD4 cell counts as surrogate markers of immune suppression. Further investigations of HCV diversity in conjunction with HCV-specific cellular responses will be required when more-sensitive immunological assays are available. Another potential limitation of the study is that only two time points were sampled for each individual. However, as we inferred the evolutionary process based on the phylogenetic tree constructed using the number of clones that were isolated through the long period of follow-up (more than 6 years), we believe that the genetic diversity of the heterogeneous viral population may represent an actual evolutionary process.

In conclusion, HIV-positive patients have more diversified HCV populations than HIV-negative patients, possibly because of reduction of selective pressure from the immune system. The positively selective regions determined in this study might be antigen-recognition regions associated with HCV-specific immune responses.

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Prediction of the Efficacy of Antiviral Therapy for Hepatitis C Virus Infection by an Ultrasensitive RT-PCR Assay

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The efficacy of interferon therapy for hepatitis C virus (HCV) infection improved remarkably. However, virologic relapse occurs in a substantial proportion of patients with virologic response (defined as an HCV RNA level below 50 IU/ml at the end-of-treatment). A highly sensitive RT-nested PCR assay capable of detecting almost a single copy of HCV RNA and a real-time RT-PCR assay to quantify HCV RNA down to 120 copies per ml were developed. The RT-nested PCR assay showed that 1 IU of HCV RNA is equivalent to 12.2 copies. For 28 patients with virologic response (12 relapsers and 16 sustained virologic responders), week-4 and end-of-treatment plasma samples were retested. At week 4, HCV RNA was detected by the RT-nested PCR and qualitative COBAS Amplicor HCV version 2.0 in 8/9 (89%) and 6/9 (67%) samples from relapsers, and in 4/16 (25%) and 2/16 (13%) samples from sustained virologic responders, respectively. End-of-treatment samples with HCV-negative by the qualitative COBAS Amplicor were positive by the present assay in 4/12 (25%) of relapsing patients and 0/16 (0%) of sustained virologic responders. The viral levels detected by the present assay in the Amplicor-negative samples were 3.5–17.3 copies/ml, which is below the detection limit of COBAS Amplicor. In conclusion, the highly sensitive RT-nested PCR assay can predict sustained virologic response at week 4 and virologic relapse at the end-of-treatment more accurately than COBAS Amplicor, suggesting its usefulness in monitoring antiviral therapy for HCV infection. ***J. Med. Virol. 79:1113–1119, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; ultrasensitive RT-PCR; accurate prediction; residual HCV RNA

INTRODUCTION

Hepatitis C Virus (HCV) chronically infects over 170 million people worldwide. After acute infection, approximately 15% to 25% of infected individuals overcome the virus with resolution of the infection, while the remainders become chronically infected. Of individuals with chronic infection, 10% to 20% progress and thereafter 1% to 5% develop hepatocellular carcinoma [Cohen, 1999]. In Japan, the number of HCV-infected patients is estimated to be 3 million in a population of 120 million. It should be noted that over 90% of hemophilia patients had been infected with HCV and about 40% had been co-infected with human immunodeficiency virus from contaminated blood products in the early 1980s. They are now facing an increasing risk of developing end-stage liver disease.

Recently, treatment for chronic hepatitis C has made remarkable progress. Current protocols of combination therapy of peginterferon plus ribavirin have achieved high rates (40–63%) of sustained virologic response [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004; Torriani et al., 2004], defined as a serum HCV RNA < 50 IU/ml at week 24 after the end-of-treatment. Since it was reported that patients who did not achieve an early virologic response (EVR), defined as a serum HCV RNA < 50 IU/ml at week 12, did not respond to further therapy [Davis et al., 2003], the consensus guidelines have recommended that therapy be discontinued for patients who did not achieve EVR [European Association for the Study of the Liver, 1999; National Institutes of Health Consensus Development Conference, 2002; Strader et al., 2004]. Moreover, to avoid side effects and save treatment costs, early identification of nonresponse or relapse is increasingly required. Although rapid virologic response, defined as a serum HCV RNA < 50 IU/ml at week 4, was reported to be highly associated with sustained virologic response, virologic relapse occurred at various rates (9–27%)

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among patients who achieved rapid virologic response [Jensen et al., 2006]. This may be explained by the fact that the detection limit of 50 IU/ml is not low enough to identify the patients with strong early virologic suppression that leads to sustained virologic response.

Several studies using highly sensitive HCV assays have shown that virologic relapse can occur in patients with a low level of residual HCV RNA at the end-of-treatment, which cannot be detected by COBAS Amplicor HCV version 2.0 (Roche Molecular diagnostics, Pleasanton, CA) [Sarrazin et al., 2001; Watkins-Riedel et al., 2004; Desombere et al., 2005; Gerotto et al., 2006]. However, early virologic assessment has not been fully evaluated with such highly sensitive assays. To evaluate accurately the relationships between EVR and sustained virologic response, and between the residual HCV RNA at the end-of-treatment and virologic relapse, a highly sensitive reverse transcription (RT)-nested PCR assay that detect almost a single copy of HCV RNA and a real-time RT-PCR assay with a wide quantitation range were developed. These techniques were used to retest plasma samples from patients with relapse or sustained virologic response who had virologic responses at the end-of-treatment.

MATERIALS AND METHODS

Patients and Samples

Plasma samples were collected from 28 patients who had been treated with interferon therapy at Ogikubo Hospital (Tokyo, Japan) and achieved virologic responses at the end-of-treatment; 12 relapsers and 16 sustained virologic responders. All patients were male hemophiliac patients and had been infected with HCV through contaminated blood products. The characteristics of patients are shown in Table I. There were no significant differences in age, sex, HIV-1 co-infection or alanine aminotransferase levels between relapser and sustained virologic responders groups. Although the patients with genotype 1 infection appeared to have a higher rate of relapse, a statistical difference was not obtained ($P = 0.13$). All plasma samples were stored at −60°C until analysis.

RNA Extraction

Total RNA was extracted from 500 μl of plasma using a QIAamp UltraSens Virus Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations with some modifications. Briefly, plasma was diluted with 500 μl of phosphate buffered saline and centrifuged at 18,000 rpm (35,000*g*) for 60 min. Most of the supernatant was discarded and the residual 30 μl and precipitate were resuspended with 300 μl of Buffer AR containing 5.6 μl of carrier RNA preheated at 60°C. The mixture was incubated for 10 min at 60°C using a mixing incubator. Thereafter, RNA was purified as recommended.

Reverese Transcription-Nested PCR

Primers. Reverse transcription (RT)-nested PCR was performed using two sets of primers targeting a region of the 5′ untranslated region of HCV. Reverse transcription (RT) was carried out with primer KC12, 5′-CTCGCAAGCACCCTATCAGGCAG-3′ (nucleotides (nt) 276–299, identical to primer KY78 designed by Roche Molecular Systems) [Young et al., 1993]. The primer pair for the first-round PCR consisted of forward primer KC11, 5′-CTCCCCTGTGAGGAACTACTGTCT-3′ (nt 24–51), and reverse primer KC12. The primer pair for the second-round PCR consisted of forward primer KC13, 5′-TCCCGGGAGAGCCATAGTG-3′ (nt 115–133) and reverse primer KC14, 5′-TCCAAGAAAGGACCC-3′ (nt 176–196).

RT-nested PCR. RT was carried out in a 20-μl solution of PCR Buffer II (10 mM Tris–HCl, 50 mM KCl), 3 mM $MgCl_2$, 10 mM of DTT, 0.5 mM of each dNTP, 0.5 μM of RT primer (KC12), 0.5 U of RNase inhibitor (Promega, Madison, WI), and 2.5 U of SuperScript III (Invitrogen, Carlsbad, CA). The mixture was incubated for 5 min at 60°C, 5 min at 55°C, 5 min at 50°C, and then 5 min at 45°C. A GeneAmp PCR system 9700 thermocycler and thin-walled MicroAmp reaction tubes (Applied Biosystems, Foster City, CA) were employed for RT and PCR. The first-round PCR was carried out using the whole RT product in a final volume of 50 μl containing 1× PCR Buffer II, 2.5 mM $MgCl_2$, 200 μM

TABLE I. Patient Characteristics

Category	Virologic relapsers	Sustained virologic responders
Number of cases	12	16
Age, median (in years) (range)	35 (24–69)	32 (20–66)
Sex, male/female	12/0	16/0
Genotype[a]		
1	5 (42%)	0 (0%)
2	2 (17%)	2 (40%)
3	5 (42%)	2 (40%)
4	0 (0%)	1 (20%)
HIV co-infection		
HIV positive	6 (50%)	6 (38%)
HIV negative	6 (50%)	10 (62%)
ALT level, median (in U/L) (range)[b]	115 (29–264)	120 (59–708)

[a]Of 16 cases with sustained virologic response, genotype was determined in five cases.
[b]ALT, Alanine aminotransferase.

each dNTP, 1 μM KC11 and KC12 primers, and 1.25 U of AmpliTaq DNA polymerase. The mixture was incubated for 2 min at 97°C to facilitate denaturation, then PCR amplification was performed with 5 cycles of 5 sec at 97°C (denaturation), 15 sec at 48°C (annealing), and 15 sec at 60°C (extension), 25 cycles of 5 sec at 94°C and 15 sec at 60°C, and a final extension step of 5 min at 72°C. One microliter of the first-round PCR product was applied to the second-round PCR in a 50-μl solution containing the same components as the first-round PCR except for primers KC13 and KC14 instead of KC11 and KC12. PCR amplification was performed with preincubation of 2 min at 94°C, 5 cycles of 5 sec at 94°C, 15 sec at 48°C, and 15 sec at 60°C, 25 cycles of 5 sec at 94°C and 15 sec at 60°C, and a final extension step of 5 min at 72°C. The final PCR product was analyzed by electrophoresis in 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Evaluation of Extraction Efficiency

Extraction efficiency was evaluated by comparing the HCV RNA copy counts of seven samples (one sample of genotype 1a, two genotype 1b, one genotype 2a, one genotype 2b, one genotype 3a, and one genotype 4) determined by Poisson quantitation (described below) of HCV RNA in plasma and purified RNA obtained from plasma using the modified RNA extraction protocol.

Poisson quantitation of purified RNA. The HCV RNA concentration was determined by statistical analysis of the results of RT-nested PCR of the sample diluted to near endpoint. First, serial 10-fold dilutions of the extracted RNA solution were assayed with RT-nested PCR as described above. Then, the diluted solution that had conferred the second to last positive band was serially diluted twice and tested with RT-nested PCR. Finally, the RNA solution diluted at the ratio by which the last positive signal was given in a series of twofold dilutions was assayed in 20 replicates. Assuming that a single copy of HCV RNA is detectable, the HCV concentration can be calculated from the proportion of negative reactions (P_0) using the null class equation of the Poisson distribution [Simmonds et al., 1990]. The formula is HCV RNA concentration $= -\ln P_0 / V \times d$ (copies/μl), where V and d are the sample volume (μl) assayed and the dilution ratio, respectively. In these experiments, RNA Diluting Buffer (50% glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% Sarkosyl, 10 μg/ml poly A) was used to ensure no loss of RNA during the dilution procedures. We call the above procedure "Poisson quantitation."

Direct poisson quantitation of plasma RNA. Sample plasma was diluted with Virus Lysis Buffer (50% glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% Sarkosyl, 10 μg/ml poly A, 2 mM DTT, and 0.4 U/μl RNase inhibitor). Diluted samples were quantified by Poisson quantitation without RNA extraction. This method can quantify the absolute copy number of HCV RNA in plasma, since it is not affected by loss of RNA during the purification procedure. It was confirmed that contamination of less than 0.1 μl of plasma in one reaction did not affect quantitation of HCV RNA in this RT-PCR condition.

HCV RNA Real-Time RT-PCR

RT was carried out using a high-capacity cDNA Archive kit (Applied Biosystems) in a final volume of 10 μl containing 5 μl of RNA sample solution and 0.3 μM of KC12 primer. The RT reaction was carried out with a GeneAmp PCR system 9700 for 30 min at 48°C, followed by reverse transcriptase inactivation for 5 min at 95°C. The PCR reaction was carried out in a final volume of 50 μl containing 10 μl of the RT product, TaqMan buffer (Applied Biosystems), 5 mM $MgCl_2$, 400 μM dUTP, 200 μM dATP, dCTP, dGTP (each), 0.025 U/μl AmpliTaq Gold, 0.01 U/μl AmpErase UNG, 0.3 μM forward primer (KC13), and reverse primer (KC14). For signal detection, 0.1 μM MGB probe 5′-CTGCGGAACCGGTG-3′ (KCP) was used. The PCR amplification was performed using Sequence Detection Systems 7700 (Applied Biosystems). The temperature settings were: uracil-*N*-glycosylase incubation for 2 min at 50°C; AmpliTaq Gold activation for 10 min at 95°C; 40 cycles of 15 sec at 95°C; and 1 min at 60°C. Measurements of standard and unknown samples were done in triplicate. After amplification, real-time data acquisition and analysis were performed with the accompanying software. For the standard HCV RNA, we used an RNA solution purified from the plasma of one patient with genotype 1b, which is the most common genotype among Japanese patients and quantified by Poisson quantitation.

Detection and Quantitation of HCV RNA in Clinical Samples

From 28 HCV-infected patients who achieved a viral response with antiviral treatment (12 relapsers and 16 sustained virologic responders), 53 stored plasma samples at week 4 and at the end-of-treatment (9 week-4 samples and 12 end-of-treatment samples from relapsers, and 16 week-4 samples and 16 end-of-treatment samples from sustained virologic responders) were retested by the RT-nested PCR and real-time RT-PCR assays developed in this study. One tenth of the RNA purified from plasma samples as described above was subjected to in-house RT-nested PCR in 10 replicates. Plasma in which HCV RNA was detected in nine or more reactions was quantified by real-time RT-PCR, because a reliable HCV RNA value cannot be obtained for these samples by Poisson quantitation. The results of the RT-nested PCR and COBAS Amplicor HCV v2.0 assays were tested by Fisher's exact probability test with respect of the outcome.

RESULTS

Extraction Efficiency

The efficiency of HCV RNA extraction in the modified protocol of QIAamp UltraSens Virus kit was estimated by comparing the quantities of HCV RNA in seven plasma samples and in their extracted RNA, involving

six different HCV genotypes. The ratios ranged from 50% to 107% with a median of 89%.

Sensitivity of RT-Nested PCR

To evaluate the sensitivity of the RT-nested PCR, the World Health Organization international standard for HCV RNA (NIBSC code 96/798) was quantified by Poisson quantitation. In a total of 4 runs, HCV RNA was detected in 24 reactions of 34 replicates. This result showed that 1 IU was equivalent to 12.2 amplifiable copies (range based on SD, 9.9–15.3 copies).

Linearity, Reproducibility, and Genotype Reactivity of Real-Time RT-PCR

The data obtained for drawing the standard curves in the real-time RT-PCR assay were statistically analyzed to assess the precision within each run (intra-assay) and between runs (inter-assay). The standard curves were obtained with 10-fold dilutions of the RNA standard, ranging from 5 to 5,000 copies per reaction (equivalent to 120–120,000 copies/ml), and analyzed in triplicate in a total of four independent experiments. The results of the analysis are summarized in Table II. The standard deviation of the intra-assay on the basis of C_T values ranged from 0.05 to 0.74 cycles, even including samples with only 5 copies per reaction, and that of the inter-assay ranged from 0.70 to 1.42 cycles.

To overcome the variation of nucleotide sequences of 5′ untranslated region among HCV genotypes, we used the most conserved region for designing PCR primers. To evaluate the genotype reactivity of the real-time RT-PCR, obtained values of HCV RNA were compared with those determined by Poisson quantitation for HCV strains of various genotypes. There was very good linearity between the real-time RT-PCR and Poisson quantitation regardless of genotype (Fig. 1). The coefficient of variation ranged from 7.2% to 30.8% for all quantified points and all genotypes.

Detection and Quantitation of HCV RNA in Stored Plasma

From 28 patients who achieved virologic responses at the end-of-treatment, 53 plasma samples at week 4 or at the end-of-treatment were retested by the RT-nested PCR. The detection rates, statistical tests of week-4 and end-of-treatment samples, and the predictability of sustained virologic response by the undetectability of HCV RNA and relapse by the detection of HCV RNA are shown in Table III. In testing week-4 samples, the RT-nested PCR assay detected HCV RNA in 8 of 9 (89%) available samples from relapsers, and in 4 of 16 (25%) from sustained virologic responders ($P = 0.003$), whereas COBAS Amplicor detected 6/9 (67%) from relapsers and 2/16 (13%) from sustained virologic responders ($P = 0.01$). Among the 13 patients who were HCV RNA-negative at week 4 by the RT-nested PCR assay, 12 (92%) achieved sustained virologic response, compared to 14 of the 17 (82%) who were negative by COBAS Amplicor. In testing end-of-treatment samples, 4 of 12 (33%) Amplicor-negative samples from relapsers were HCV-positive by the RT-nested PCR assay, whereas 0 of 16 end-of-treatment samples from sustained virologic responders were HCV-positive ($P = 0.02$). Among the four patients who were HCV RNA-positive at the end-of-treatment by the RT-nested PCR assay, four (100%) had virologic relapse.

The viral load determined by Poisson quantitation in eight Amplicor-negative samples (six samples from relapsers and two samples from sustained virologic responders) were within 3.5–17.3 copies/ml (Table IV), which was below the detection limit of current highly sensitive assays such as transcription-mediated amplification.

DISCUSSION

This study demonstrates that an ultrasensitive HCV assay can identify both sustained virologic response and relapse in patients with high probabilities. As previously shown, the more sensitive the assay used for the quantitation of HCV RNA, the more predictive the measurements are of virologic relapse after discontinuation of therapy [Sarrazin et al., 2001; Desombere et al., 2005]. The currently developed highly sensitive HCV assay based on RT-nested PCR has an ability to detect almost a single copy of HCV RNA, which was achieved by a stepwise declining temperature in reverse transcription and a lower annealing temperature of 48°C for the first five PCR cycles. To assess the sensitivity of this assay, equivalency between the international unit and the amplifiable copy number of HCV RNA was determined using the WHO international standard. The international unit was first determined in 1998 based on a mean of measurements for the WHO international standard that were quantified by 22 multinational laboratories using various quantitation methods in 1998 [Saldanha et al., 1999], therefore, it is not yet clear how many HCV virions are equivalent to 1 IU.

TABLE II. C_T Values With RNA Dilutions for Standard Curve (Genotype 1b)

Copies/reaction	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Inter-assay (std dev)
5,000	22.20 (0.05)	23.60 (0.23)	22.30 (0.12)	21.98 (0.15)	0.70
500	26.54 (0.28)	27.59 (0.35)	25.90 (0.21)	25.39 (0.12)	0.88
50	30.99 (0.21)	31.35 (0.48)	29.14 (0.16)	28.55 (0.11)	1.26
5	35.46 (0.74)	35.40 (0.64)	33.27 (0.60)	32.60 (0.28)	1.42

Serially diluted samples were analyzed in triplicate in each experiment. Good consistency (intra-assay) and reproducibility (inter-assay) of the real-time PCR are shown in this table.

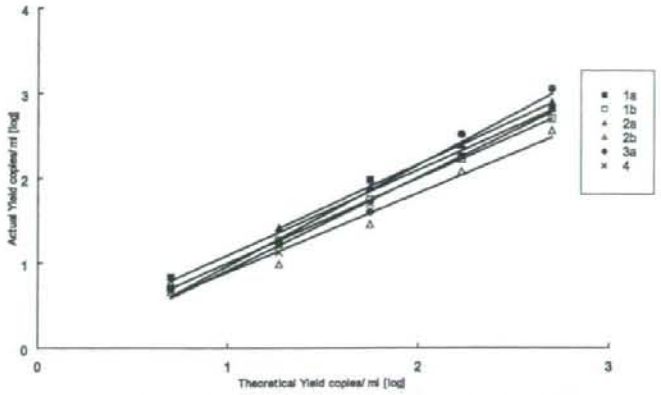

Fig. 1. Linearity of yield with RNA dilutions isolated from genotype 1a, 1b, 2a, 2b, 3a, and 4 samples. A sample from each genotype was serially diluted, and quantified by real-time RT-PCR. Actual yield copy counts were obtained from 10-fold diluted RNA samples by real-time RT-PCR, and theoretical yield copy counts were obtained from the dilution based on Poisson quantification using our RT-nested PCR.

Previously, conversion factors of international unit and amplifiable copy were determined at 2.75 and 5.2 copies/IU by Puig et al. [2002] and Gorrin et al. [2003], respectively. The result of 12.2 copies/IU in this study, which was obtained by analyzing the Poisson distribution of clear positive reactions at endpoint dilution of the WHO international standard, was considerably higher than the previous values. Taken together with a high efficiency (89%) of extraction of HCV RNA from plasma, the present assay is considered to have the ability to detect almost a single copy of HCV RNA in plasma samples.

The present qualitative RT-nested PCR assay was compared with the COBAS Amplicor HCV version 2.0 with a lower detection limit of 50 IU/ml for 53 plasma samples from 28 patients receiving antiviral therapy that were collected at week 4 or the end-of-treatment. While COBAS Amplicor detected HCV RNA in 8 samples, the present assay detected HCV RNA in 16 samples (all of 8 Amplicor-positive samples and 8 of 45 Amplicor-negative samples), providing evidence that the assay is more sensitive for the detection of HCV RNA in clinical samples than COBAS Amplicor version 2.0. Furthermore, regarding prediction of relapse or sustained virologic response after treatment, the present assay is more predictive than COBAS Amplicor at week 4 ($P = 0.003$ vs. $P = 0.02$) as well as at the end-of-treatment ($P = 0.02$ vs. $P = 1$). It is most likely that this difference is explained by a lower detection limit of the present assay. As mentioned above, the RT-nested PCR assay is approaching the ability to detect a single copy of HCV RNA. Therefore, when a 0.5 ml plasma sample is used, the sensitivity of the assay can be calculated from the equation for the Poisson distribution as 5 copies/ml at 95% detection, which is lower than 50 IU/ml for COBAS Amplicor and 25–50 copies/ml for transcription-mediated amplification assay (VERSANT HCV RNA Qualitative Assay) [Sarrazin et al., 2001].

TABLE III. Comparison of the Detection Rates of HCV RNA by Our in-House RT-Nested PCR at Week 4 and the End-of-Treatment, and COBAS Amplicor HCV Version 2.0 at Week 4

	In-house RT-nested PCR			COBAS Amplicor HCV v2.0		
	Relapse	Sustained virologic response		Relapse	Sustained virologic response	
Week 4						
Positive	8	4	Relapse/total positive = 8/12 (67%)	6	2	Relapse/total positive = 6/8 (75%)
Negative	1	12	SVR/total negative = 12/13 (92%)	3	14	SVR/total negative = 14/17 (82%)
P-value	0.003			0.02		
End-of-treatment						
Positive	4	0	Relapse/total positive = 4/4 (100%)	0	0	Relapse/total positive = 12/0
Negative	8	16	SVR/total negative = 16/24 (67%)	12	16	SVR/total negative = 16/28 (57%)
P-value	0.01			1.00		

TABLE IV. Characteristics and Quantified Viral Load of HCV RNA in Plasma Which Was HCV RNA Negative by Cobas Amplicor HCV Version 2.0 (Sensitivity, 50 IU/ml)

Patients	Outcome	Sampling	HCV RNA (copies/ml)
1	Relapse	Week 4	3.5
2	Relapse	Week 4	8.0
3	Relapse	EOT	4.9
4	Relapse	EOT	11.0
5	Relapse	EOT	3.5
6	Relapse	EOT	17.3
7	SVR	Week 4	3.5
8	SVR	Week 4	7.6

EOT, End-of-treatment.

Several clinical studies have shown that early viral assessments provide identification of nonresponders to antiviral therapies [Davis et al., 2003; Jensen et al., 2006], and the consensus guidelines of American Association of Study for Liver Disease (AASLD) [Strader et al., 2004] recommended discontinuation of therapy in patients who do not achieve EVR. Moreover, Jensen et al. [2006] suggested that therapy can be discontinued by 24 weeks in patients who achieve rapid virologic response at week 4, because there was no significant difference in rate of sustained virologic response between patients treated for 24 and 48 weeks. However, virologic relapse occurs at various rates among patients with rapid virologic response (9–27%) [Jensen et al., 2006].

The ratios of sustained virologic response among patients who were HCV negative at week 4 of treatment by the present assay and COBAS Amplicor were 12/13 (92%) and 14/17 (82%), respectively, suggesting that undetectability of HCV RNA at week 4 by the more sensitive assay is more predictive of sustained virologic response. The threshold of plasma viral levels in patients with a strong virologic suppression during the early phase that leads to sustained virologic response may be much below 50 IU/ml. The qualitative transcription-mediated amplification assay, which has a higher sensitivity than COBAS Amplicor (detection limit 25–50 copies/ml), showed a slightly higher detection rate for HCV RNA than COBAS Amplicor (18/21 (86%) by transcription-mediated amplification assay versus 16/21 (76%) by COBAS Amplicor) in the testing of week-4 samples from relapsers [Desombere et al., 2005]. Because the sensitivity of the present RT-nested PCR assay is higher than that of the transcription-mediated amplification assay (5 copies/ml vs. 25–50 copies/ml at 95% detection), the predictability of sustained virologic response by the RT-nested PCR assay may be higher than by the transcription-mediated amplification assay.

Despite of the high sensitivity in detecting a single copy, one of nine samples at week four in relapsers was HCV-negative by the present assay. There are two possible explanations for this result. First, antiviral treatment leads to a complete replication arrest in blood, but small quantities of HCV RNA may persist in the liver [Radkowski et al., 2005]. Second, macrophages and lymphocytes may serve as a replication sites for HCV RNA, resulting in failure of detection of HCV RNA in plasma samples [Laskus et al., 2000; Watkins-Riedel et al., 2004; Radkowski et al., 2005]. On the other hand, detection of HCV RNA at week 4 is not well correlated with virologic relapse; 4 of 16 (25%) and 2 of 16 (13%) sustained virologic responders were HCV positive at week 4 by the present assay and COBAS Amplicor, respectively. It may be too early to identify patients with relapse or nonresponse by the detection of HCV RNA at week 4, because an early decision to discontinue treatment at week 4 would be a serious error in patients with the potential for a sustained virologic response.

In testing end-of-treatment samples, the RT-nested PCR assay detected residual HCV RNA in 4 of 12 (33%) relapsers, and 0 of 16 (0%) sustained virologic responders ($P = 0.02$), all of whom were HCV RNA negative by COBAS Amplicor. Despite of the small number of samples, this result suggests that patients who are HCV-positive by the RT-nested PCR assay at the end-of-treatment will relapse with quite a high probability after treatment. The detection rate of 33% in end-of-treatment samples from relapsing patients by the RT-nested PCR assay is similar to that obtained with the transcription-mediated amplification assay [Sarrazin et al., 2001; Desombere et al., 2005]. By retesting end-of-treatment samples with both the RT-nested PCR assay and the transcription-mediated amplification assay, it was found that the more highly sensitive assays can detect residual HCV RNA at the end-of-treatment from relapsers more frequently, and is suggested that virologic relapse occurs on the basis of replication of remaining HCV virions from a very low level after discontinuation of the antiviral therapy.

As previously reported, the level from which strongly suppressed HCV RNA can lead to virologic relapse is thought to be below 50 IU/ml, which may cause errors in the assessment of the efficacy of antiviral therapies [Sarrazin et al., 2001]. Indeed, the transcription-mediated amplification assay detected residual HCV RNA in the Amplicor-negative plasma from relapsers [Morishima et al., 2006]. However, there has been no report to quantify the minimum level of residual HCV RNA before virologic relapse. This study showed that the quantified HCV RNA concentration of relapsers was in the range of 3.5–17.3 copies/ml (Table IV), which is below the detection limit of the transcription-mediated amplification assay as well as that of the COBAS Amplicor HCV assay.

The assay had several limitations in its data and procedures. First, this study enrolled a limited number of patients. Particularly, the number of week-4 samples from relapsers ($n = 9$) was too small to evaluate the statistical difference in the detection rates between the present RT-nested PCR assay and the COBAS Amplicor HCV assay. Second, in comparison with commercially available kits, RT-nested PCR is labor-intensive, and thus is difficult to use for a large number of samples simultaneously. Therefore, the present assay may not be

suitable for routine use in clinical trials. However, since the threshold level of residual HCV RNA capable of producing a virologic relapse is quite low, the present assay may be useful for distinguishing virologic relapse from sustained virologic response.

In conclusion, a highly sensitive assay, almost capable of detecting a single copy of HCV RNA, was developed for the accurate prediction of the efficacy of antiviral therapy in HCV infection. It has been shown that sustained virologic response can be predicted by the undetectablity of HCV RNA at week 4, and, more strongly, that relapse is associated with detection of HCV RNA at the end-of-treatment.

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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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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

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

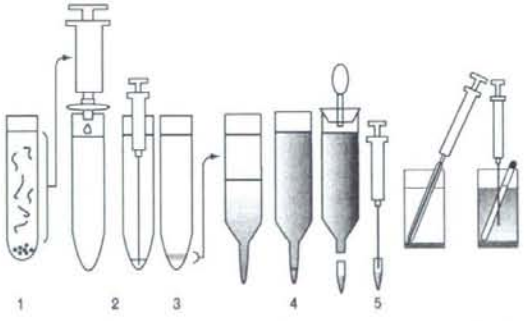

Fig. 1. Revised swim-up method to remove HIV completely. (1) The semen is diluted and debris allowed to precipitate. (2) The suspension is filtered, 0.1 ml Percoll added to the bottom, and the tube is centrifuged. (3)The sperm sediment is layered onto a linear gradient of Percoll (30–98%). (4) After 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.

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.

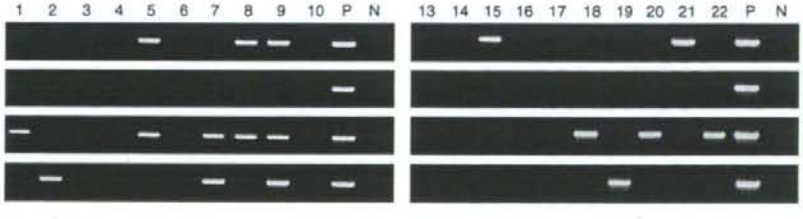

Fig. 2. Reverse transcriptase nested polymerase chain reaction (RT-nested PCR) capable of detecting a single copy of HIV-1 RNA. One virion of $HIV\text{-}1_{LAI}$, on average, was added to 20 sets of 1 ml Sydney IVF medium and then tested with the RT-nested PCR. When one fourth of the eluate from an 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.

Table 1. Characteristics of male patients with HIV infection.

		Men treated with HAART	
	Untreated men ($n = 16$)	Viral load ≥ 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

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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