

Equipment A Waters Alliance 2695 HPLC and a Micromass ZQ-2000 MS (Waters Assoc., Milford, MA, U.S.A.), controlled with MassLynx version 4.0 software, were used for detection. The analytical column was a SunFire C₁₈ column (3.5 μ m, 2.1 \times 50 mm, Waters), protected by a SunFire C₁₈ Guard Column.

Chromatographic and Mass Spectrometric Conditions The mobile phase was a mixture of 0.1 mM EDTA in 0.1% acetic acid (A), 100% acetonitrile (B) and 100% methanol (C). An isocratic mobile phase consisting of A–B–C (65 : 15 : 20) was used during the first 2 min of the run, followed by a linear gradient elution consisting of A–B–C (10 : 70 : 20) for the next 8 min. The final conditions were maintained for the final 5 min. The system was then reequilibrated for an additional 13 min using the initial conditions. The flow rate of the mobile phase was 0.2 ml/min, the column temperature was 40 °C, and the amount of injected sample was 5 μ l.

The mass spectrometer was operated in positive ion electrospray mode. The capillary sprayer voltage was 3.5 kV and the sample cone voltage was 30 V for both raltegravir and A-86093. The source temperature was 120 °C and the desolvation temperature was 350 °C. The desolvation and cone gas flow-rates were set to 600 and 50 l/h, respectively. The acquisition mass range is m/z 200–800 at 0.5 s per scan with a 0.1 s interscan delay. All mass spectra are acquired in centroid mode.

Quantitative analysis, carried out in Selected-ion recording (SIR) mode, detected raltegravir at m/z 445, and the internal standard (IS), A-86093, at m/z 748, all in the form of ions. The quantitation calculations were performed using analytical software, MassLynx version 4.0 (Waters).

Standard Solutions Stock solutions of raltegravir and A-86093 were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 384.0 μ g/ml of raltegravir and 41.0 μ g/ml of A-86093. These stock solutions were stored at –80 °C and thawed on the day of analysis. The stock solution was diluted in drug-free plasma to yield ralte-

gravir concentrations of 0.010, 0.192, 1.920, 3.840 and 7.680 μ g/ml.

Sample Preparation Two milliliters of methylene chloride/hexane (50 : 50, v/v) containing the IS (0.328 μ g/ml) and 0.3 ml of 0.2 M ammonium acetate were added to a 500 μ l plasma sample prepared from peripheral blood anticoagulated with heparin. The mixture was vortexed for 5 min and then centrifuged at 3500 *g* for 5 min. The upper layer was separated and evaporated dry. The dried material was then dissolved in 50 μ l of a mobile phase solution. Lastly, 5 μ l of the upper solution was injected into the LC-MS system. The institutional review board of National Hospital Organization Nagoya Medical Center approved this study and each subject provided written informed consent.

Validation Inter- and intraday precision values using this method were estimated by assaying control plasma containing five different concentrations of raltegravir five times on the same day and on three separate days to obtain the relative standard deviation (RSD). Accuracy was determined as the percentage of the nominal concentration. To assess the absolute recoveries of raltegravir extracted from plasma, the peak area ratios of the analytes to the internal standard were compared with those obtained from the mobile phase having the same concentration. The mean recoveries were determined in triplicate.

RESULTS

LC-MS Chromatograms Figures 2A and B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 0.192 μ g/ml of raltegravir and 0.328 μ g/ml of A-86093 (IS). Under the described chromatographic conditions, retention times were 8.2 min for raltegravir and 12.9 min for A-86093. Figures 2C and D show chromatograms obtained from a blank plasma sample. Assays performed on drug-free human plasma succeeded to show no interfering peaks during the interested intervals of the retention times. Figure 2D is the expanded figure of the

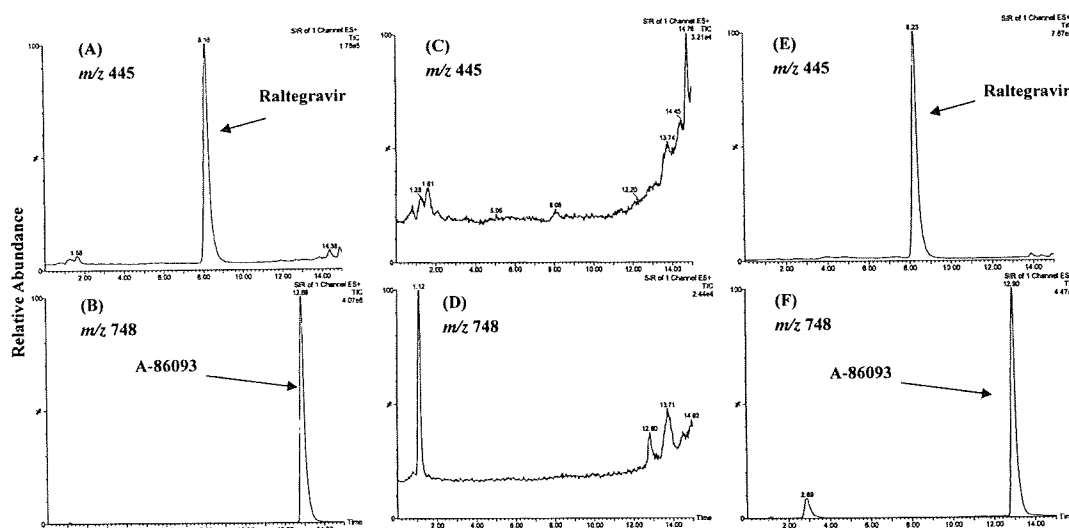


Fig. 2. Selected-ion Recording Chromatograms for Raltegravir and A-86093

(A) and (B) were obtained from a spiked plasma containing 0.192 μ g/ml of raltegravir and 0.328 μ g/ml of A-86093 (IS). (C) and (D) were obtained from a blank plasma sample. (E) and (F) were obtained from a plasma sample from an HIV-1-infected patient on raltegravir. (A), (C) and (E) were monitored with m/z 445. (B), (D) and (F) were monitored with m/z 748. (C) and (D) are the expanded figures of the baselines in (A) and (B), respectively.

Table 1. Intraday and Interday Precision and Accuracy for Raltegravir

Expected ($\mu\text{g/ml}$)	Intraday ($n=5$)		Interday ($n=15$)		Accuracy (%)	Recovery (%)
	Measured ($\mu\text{g/ml}$)	RSD (%)	Measured ($\mu\text{g/ml}$)	RSD (%)		
0.010	0.010 \pm 0.001	8.5	0.010 \pm 0.001	10.4	103.4 \pm 10.7	83.7 \pm 9.8
0.192	0.192 \pm 0.011	5.7	0.187 \pm 0.016	8.7	97.2 \pm 8.5	83.6 \pm 3.1
1.920	1.932 \pm 0.095	4.9	1.912 \pm 0.089	4.6	99.6 \pm 4.6	87.4 \pm 7.0
3.840	3.872 \pm 0.118	3.0	3.825 \pm 0.184	4.8	99.6 \pm 4.8	80.6 \pm 6.5
7.680	7.656 \pm 0.097	1.3	7.631 \pm 0.186	2.4	99.4 \pm 2.4	86.5 \pm 6.1

RSD, relative standard deviation. Means \pm S.D.

Table 2. Plasma Raltegravir Concentrations after the Oral Administration of 400 mg of Raltegravir to an HIV-1-Infected Patient

Time (h)	Raltegravir concentration ($\mu\text{g/ml}$)
0	1.24 \pm 0.03
0.5	1.78 \pm 0.02
1	5.21 \pm 0.17
2	3.14 \pm 0.05
3	2.67 \pm 0.04
6	1.46 \pm 0.02

Means \pm S.D.

baseline part of Fig. 2B. These peaks did not affect the quantification of IS. Figures 2E and F show chromatograms of a plasma sample from an HIV-1-infected patient treated with raltegravir. There were no interfering peaks affecting quantification of raltegravir in this chromatogram. Anticoagulants of heparin and EDTA did not hinder the selected-ion recording chromatograms for raltegravir and A-86093.

Validation: Linearity, Precision, Accuracy and Recovery Calibration curves of raltegravir appeared linear in the concentration range of 0.010 to 7.680 $\mu\text{g/ml}$ with a correlation of 1.000.

Precision, accuracy, and recovery of our LC-MS method are shown in Table 1. The selected concentration of raltegravir covers the expected plasma concentrations found in the patients. The RSDs calculated for raltegravir in the inter- and intraday assays ranged from 1.3 to 10.4%, which are similar to previously reported values.¹⁶⁾ Accuracies ranged from 97.2 to 103.4%. Recoveries from plasma ranged from 80.6 to 87.4%. Mean extraction recovery of the IS was 87.9%. These results indicate that this method achieves a high degree of reproducibility and accuracy.

Raltegravir Concentrations in Plasma Plasma raltegravir concentrations in an HIV-1-infected patient are shown in Table 2. The patient received oral administration of 400 mg raltegravir twice daily. The samples were collected on day 8 after the start of HAART. When raltegravir is administered by a single 400 mg dose, plasma concentrations are expected in the 0.01 to 4.71 $\mu\text{g/ml}$ range.^{16–18)} In this study, raltegravir concentrations at steady state following multiple-dose administration ranged from 1.2 to 5.2 $\mu\text{g/ml}$.

DISCUSSION

Clinical trials of raltegravir have demonstrated potent antiviral responses in both therapy naïve and experienced patients.^{11,19,20)} Moreover, raltegravir has demonstrated a clean safety profile in these studies and may not have the tox-

icity and tolerability issues as the current anti-HIV drugs. Thus, raltegravir has become an important component of combination treatment regimens and its use has been initiated for the treatment of heavily pretreated patients with a multidrug-resistant virus.

We first wanted to judge whether therapeutic drug monitoring of raltegravir is necessary. To achieve this, the development of determination method for raltegravir is essential. Until now there has been a methodological report for the determination of raltegravir using LC-MS/MS.¹⁶⁾ However, this method has several disadvantages in terms of cost performance and essential equipment; for example, the authors used isotoped labeled raltegravir and/or the setting of the LC-MS-MS equipment.

To avoid such disadvantages we decided to use an LC-MS method using an available IS (A-86093) for determining plasma protease inhibitor concentrations. The reason we chose ritonavir analogue A-86093 is the stability and better elution point of the compound on the HPLC as were reported previously.¹⁴⁾ Validation showed our method was successful in measuring plasma raltegravir with high precision and satisfactory RSD values. The raltegravir calibration curve was linear at the concentration range of 0.010 to 7.680 $\mu\text{g/ml}$, and the average accuracy ranged from 97.2 to 103.4%. Both inter- and intraday RSDs for raltegravir were less than 10.4%, which is similar to previously reported values.¹⁶⁾ Recovery of raltegravir was more than 80.6%. These results indicate our newly developed method achieves the same level of reproducibility and accuracy as the LC-MS/MS method. As plasma concentrations of raltegravir are expected in the 0.01 to 4.71 $\mu\text{g/ml}$ range when raltegravir is administered at single dose of 400 mg,^{16–18)} our method successfully covers this region with good precision and accuracy. Actually, the raltegravir concentration change was clearly demonstrated; it rose from 1.2 (0 h) to 5.2 $\mu\text{g/ml}$ (1 h), then decreased to 1.5 $\mu\text{g/ml}$ (6 h) when raltegravir was orally administered 400 mg twice daily in an HIV-1-infected patient.

Recently, Poirier *et al.* reported the HPLC method for determining plasma raltegravir concentration with fluorescence detection.²¹⁾ Our's and Poirier's methods can specifically determine the raltegravir concentration and the sensitivities seem almost equivalent. Therefore, an alternative use is possible according to the availability of the equipments.

This conventional LC-MS method can provide a routine clinical application, and permits management of drug interactions and toxicity.

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Beneficial Effect of GB Virus C Co-Infection in Human Immunodeficiency Virus Type 1-Infected Individuals

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Abstract: Several reports have documented a better prognosis for HIV-1-infected patients co-infected with GBV-C, while other reports have contradicted such findings with the result that this issue remains controversial. We attempted to clarify the complicated status of the effect of GBV-C co-infection on HIV-1-infected patients. GBV-C RNA was detected in 37 samples in 182 HIV-1-infected patients (20.3%) using RT/nested PCR. Of these, 3 were determined to be GBV-C genotype 1, 12 were genotype 2, and the remaining 22 were genotype 3. The GBV-C viral load quantified by real-time PCR ranged from 7.8×10^3 to 3.3×10^6 copies/ml. Weakly negative correlation was observed between GBV-C viral load and HIV-1 viral load in 19 HAART-naïve patients, indicating that a higher GBV-C viral load is associated with a greater suppression of HIV-1 replication. A previously published *in vitro* study suggested that GBV-C infection would induce up-regulation of RANTES, leading to suppression of HIV-1 replication. However, in our present study, the blood RANTES level was significantly lower in the GBV-C co-infected group than in the uninfected group (190–9,959 vs. 264–31,038 pg/ml, $P=0.004$). Our results suggested that a suppression of HIV-1 replication by GBV-C co-infection is not mediated by up-regulated RANTES, and thus call for another as yet unknown factor.

Key words: GBV-C, HIV-1, RANTES, Co-infection

GB virus C (GBV-C) is a single-stranded RNA virus belonging to the Flaviviridae family (10, 12, 20). It is distributed worldwide, and five major genotypes that have been recognized are prevalent in various geographical regions (15, 16, 24). GBV-C is transmitted parenterally, and a high prevalence rate of GBV-C infection is observed among hemophiliacs (22.9–38.6%), hemodialysis patients (10.2–16.4%) and intravenous drug users (IVDU) (32.1–34.9%) (2, 4, 17, 23, 27, 28). GBV-C is also transmitted through sexual contacts (5, 11). For example, 12.5% of homosexual men were determined to be infected with GBV-C in our previous report (6).

Due to its close relationship to hepatitis C virus, also a member of the Flaviviridae family, GBV-C was thought to be the cause of hepatitis in humans at the

time of discovery in 1995 (12). As it became clear that GBV-C by itself is not a causative agent of hepatitis or any other disease, however, studies shifted toward the influence of GBV-C co-infection.

In 2001, two reports that appeared in the New England Journal of Medicine showed a beneficial effect of the GBV-C co-infection in HIV-1 infection in so far as human immunodeficiency virus type 1 (HIV-1)-infected patients who are co-infected with GBV-C had an improved mortality rate (22, 26). In addition, subsequent studies by Xiang et al. and Jung et al. showed that the co-infection with GBV-C resulted in increased cytokine levels including RANTES (CC15), SDF-1, and MIP-1, whereby HIV-1 replication is inhibited (7,

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GBV-C, GB virus C; HAART, highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; IVDU, intravenous drug users; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; RANTES, regulated upon activation, normal T-cell expressed and secreted; RT, reverse transcription; VL, viral load.

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25).

In contrast, several other studies have found no beneficial effect of the GBV-C co-infection and one that showed that an increased mortality rate was observed in GBV-C co-infected individuals (8, 18, 19).

We conducted the present study to clarify the complex status of the effect of GBV-C co-infection on HIV-1-infected patients.

Materials and Methods

Patients. HIV-1-infected patients ($n=182$) who underwent medical examinations at Nagoya Medical Center, Japan, and from whom informed consent was obtained, were enrolled in the study. Of the 182, 151 were male. One hundred forty-three patients were infected with HIV-1 through sexual contacts (78 homosexuals, 53 heterosexuals, and 12 bisexuals). Other routes included via blood or blood products in 13 patients, and IVDU and accidental needle-stick in 1 patient each. One hundred forty-seven patients were Japanese, 19 were from Brazil, 5 from Thailand, 2 each from Peru, America, and Korea, and 1 each from China, Bangladesh, Uganda, Myanmar, and Ukraine.

Samples. Plasma samples of the individuals mentioned above were collected, and stored at -80°C until analyzed. In addition, approximately 1 year after the date of the sample collection, plasma samples were obtained again from the same patients to conduct a follow-up study.

Detection of GBV-C RNA. Purification of RNA from the plasma samples and the reverse transcription-polymerase chain reaction (RT-PCR) method to amplify GBV-C RNA were performed as described in our previous report (6). Briefly, RNA was extracted from the thawed samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo). The extracted RNA was used in a RT reaction followed by a first-round PCR (RT-PCR) using a Superscript One-Step RT-PCR for long templates (Invitrogen, Tokyo). Subsequently, cDNA was further amplified in a second-round PCR using a TaKaRa LA Taq (TaKaRa Shuzo, Tokyo). The same primers and the parameter settings as in our previous report were used for the RT/nested PCR. The PCR products were detected on a 1.2% agarose gel containing ethidium bromide. The expected size of the PCR product was 252 base pairs.

Determination of nucleotide sequences. PCR products of approximately 250 base pairs in size were purified from the agarose gel using a QIAquick Gel Extraction Kit (QIAGEN), and labeled using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Tokyo). The nucleotide sequences were

determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Determination of GBV-C genotypes. The obtained nucleotide sequences were compared against the reference sequences of 5 GBV-C genotypes: genotype 1 (accession #U36380), genotype 2 (U44402), genotype 3 (D90601), genotype 4 (AB013188), and genotype 5 (AF131112) in the GenBank of NCBI. The genotype for each GBV-C-positive sample was determined with an UPGMA method using Genetyx-Mac (version 10.1) system (Software Development, Tokyo).

Quantification of plasma GBV-C load. Total RNA from each GBV-C-positive patient was purified from 200 μl plasma samples using Trizol LS Reagent (Gibco BRL Life Technologies, Md., U.S.A.) according to the manufacturer's instructions. The obtained RNA was dissolved in 10 μl DEPC-treated water, and a half of the volume was reverse transcribed by Superscript II RNase H- Reverse Transcriptase (Invitrogen) at 42°C for 50 min. Subsequently, 2 μl of the 20 μl RT reaction mixture was applied to quantify the GBV-C copy numbers in triplicate by real-time PCR using a TaqMan probe. The same primer/probe set was used as in our previous report (6).

Determination of plasma RANTES concentrations. Concentrations of regulated upon activation, normal T-cell expressed and secreted (RANTES) in the plasma samples were measured using the Human RANTES enzyme-linked immunosorbent assay (ELISA) Kit (Biosource, Calif., U.S.A.) according to the manufacturer's protocol.

Measurement of plasma HIV-1 viral loads and CD4 cell counts. Plasma HIV-1 viral loads were measured using an Amplicor HIV-1 monitor version 1.5 system (Roche, Tokyo). CD4 cell counts were flow-cytometrically measured by FACSCalibur (Becton Dickinson, Tokyo).

Statistical analysis. The means of GBV-C VL among 3 GBV-C genotypes were compared by the Kruskal-Wallis test. The Mann-Whitney U test was used to compare the means of HIV-1 VL, or RANTES concentration between GBV-C co-infected group and uninfected group. Linear regression analysis was used to evaluate the relationship between GBV-C VL and HIV-1 VL. The significance level was set at 0.05, and all P values were two-sided.

Results

Prevalence of GBV-C Infection among HIV-1-Infected Patients

RT/nested PCR was performed to detect GBV-C RNA from the plasma samples of HIV-1-infected

patients. PCR products of approximately 250 bp were detected in 37 of the 182 samples (20.3%) by agarose gel electrophoresis. The characteristics of these patients are summarized in Table 1.

GBV-C Genotypes

Following the determination of the GBV-C prevalence, the nucleotide sequences of the PCR products were analyzed to classify the 37 samples into the 5 GBV-C genotypes. Of the 37 samples, 22 samples were GBV-C genotype 3, which is abundant in Asia. Twelve were classified as genotype 2 found in Europe and the U.S., and the remaining 3 were genotype 1 found in Africa. None were classified as either genotype 4 or 5 (Fig. 1).

Plasma GBV-C RNA Load

The GBV-C copy number in each of the 37 plasma samples was quantified with a real-time PCR method using a Roche LightCycler. Viral loads ranged from 7.8×10^3 to 3.3×10^6 copies/ml. The distribution of the viral loads among GBV-C genotypes 1, 2 and 3 were 7.3×10^5 – 3.3×10^6 copies/ml (2.4×10^6 copies/ml av.), 7.8×10^3 – 1.4×10^6 copies/ml (3.4×10^5 copies/ml av.),

and 2.1×10^4 – 2.3×10^6 copies/ml (3.0×10^5 copies/ml av.), respectively (Fig. 2). The average viral loads of the 3 genotypes were compared, and it was found that the viral loads among genotype 1 were significantly higher than for the remaining genotypes ($P=0.04$). However, in the follow-up study, the GBV-C viral loads, which ranged from 2.4×10^4 to 5.1×10^6 copies/ml, did not differ among the 3 genotypes ($P=0.57$) (data not shown). Thus, a difference in viral load among genotypes may not exist.

HIV-1 VL among HAART-Naïve Patients and Its Correlation with GBV-C VL

To evaluate the effect of GBV-C co-infection on HIV-1-infected patients, we analyzed the HIV-1 viral load of highly active antiretroviral therapy (HAART)-naïve patients. The HIV-1 viral load for 19 GBV-C-infected and 65 GBV-C-uninfected patients ranged from 7.8×10^3 to 3.2×10^6 copies/ml, and from 4.7×10^2 to 1.1×10^6 copies/ml, respectively, and there was a statistically significant difference between the two groups ($P=0.01$) (Fig. 3). In addition, a weakly inverse correlation was observed between the GBV-C viral load and HIV-1 viral load in 19 HAART-naïve GBV-C-co-infected patients (Fig. 4).

Plasma RANTES Concentrations

Plasma RANTES concentrations of the HAART-naïve HIV-1-infected patients with or without GBV-C viremia were measured using a commercially available ELISA kit. The RANTES concentrations of those who showed a GBV-C viremia ranged from 190 to 9,959 pg/ml (3,856 pg/ml av.). Unexpectedly, in those without GBV-C viremia, the RANTES concentration ranged from 264 to 31,038 pg/ml (9,233 pg/ml av.), which was significantly higher than in those with viremia ($P=0.004$) (Fig. 5A).

In the follow-up study, the number of the patients decreased to 47 (11 GBV-C-co-infected, and 36 GBV-C-uninfected patients) since approximately half of the patients had started receiving HAART. The RANTES concentration in those with GBV-C viremia ranged from 72 to 14,007 pg/ml (5,369 pg/ml av.), and in those without viremia ranged from 241 to 22,670 pg/ml (5,973 pg/ml av.). The RANTES levels of the 2 groups were not significantly different ($P=0.86$) (Fig. 5B).

Discussion

We have performed RT/nested PCR to detect GBV-C RNA in the plasma samples of 182 HIV-1-infected patients, who were seen at the Nagoya Medical Center. The prevalence rate was determined to be 20.3%, and

Table 1. Characteristics of 182 patients with HIV-1 infection, grouped by GBV-C status

GBV-C status	n=182	
	+	-
Mean CD4+ cell count (cells/ml)	376.8	412.3
HAART received	18	80
Mode of HIV-1 transmission		
Sexual contact		
Homosexual	31	112
Heterosexual	22	56
Bisexual	7	46
Bisexual	2	10
Blood or blood products	3	10
IVDU	0	1
Needle-stick	0	1
Unknown	3	21
Sex		
Male	34	117
Female	3	28
Nationality		
Japan	33	114
South America	2	19
East Asia	0	7
South/Southeast Asia	2	1
North America	0	2
Africa	0	1
Eastern Europe	0	1

HAART, highly active antiretroviral therapy; IVDU, intravenous drug use.

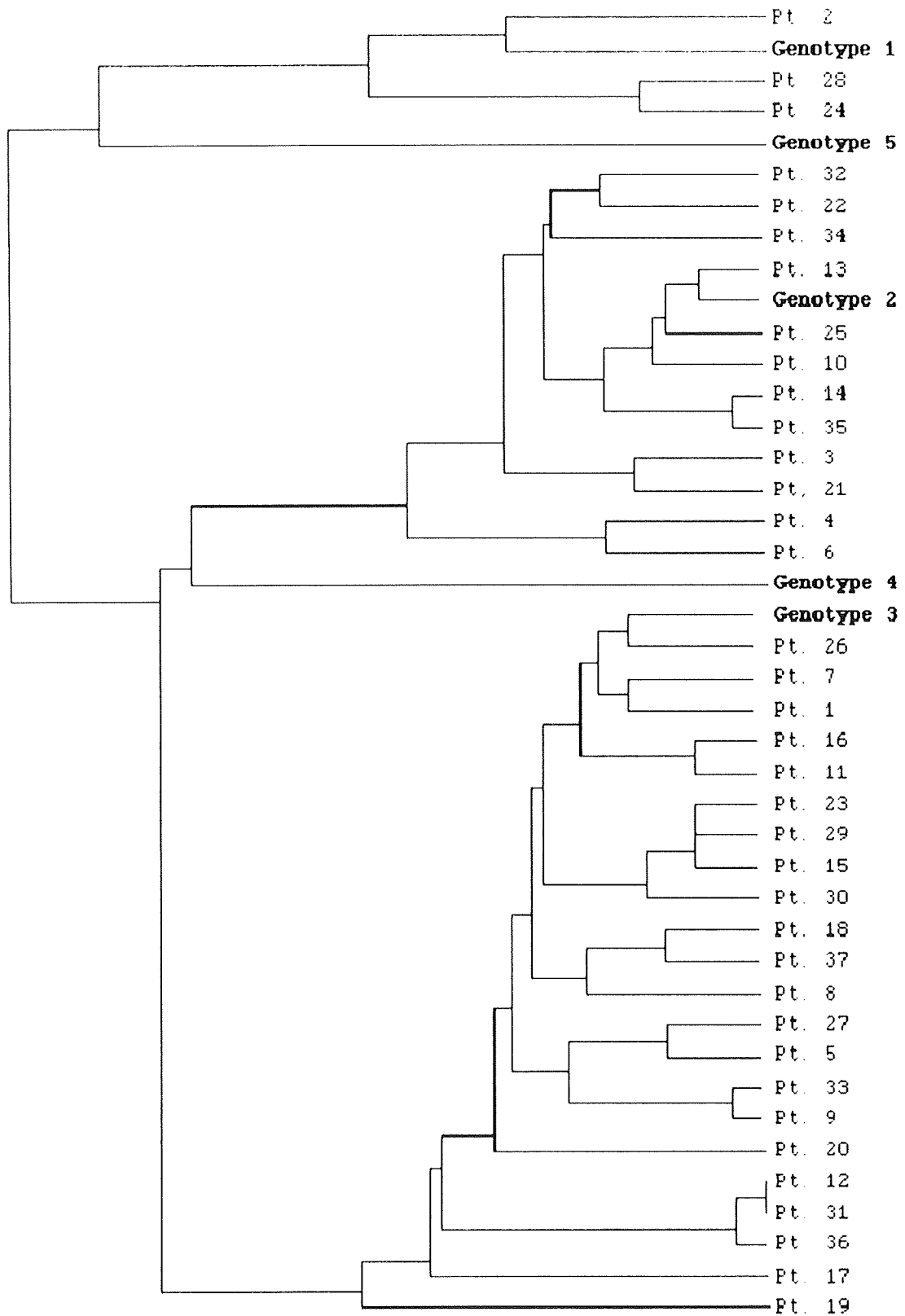


Fig. 1. Phylogenetic tree of GBV-C co-infected patients. The 37 GBV-C-infected patients (Pt.) are numbered 1 through 37. The reference sequences of the 5 GBV-C genotypes are shown in bold letters.

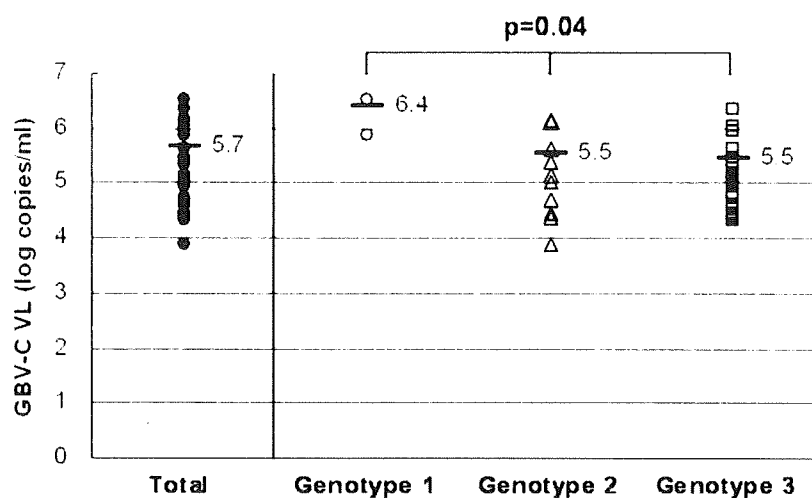


Fig. 2. Distribution of GBV-C viral load among 37 patients (●), and according to the genotypes 1 (○), 2 (△), and 3 (□). The bars indicate the mean viral load.

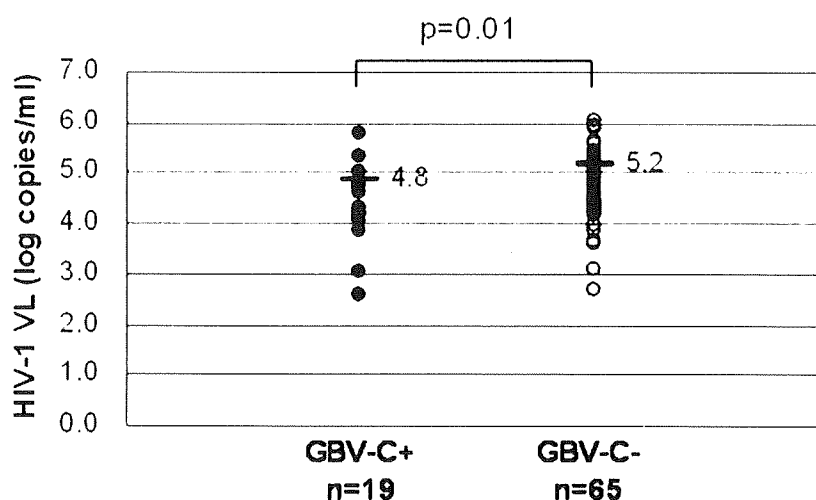


Fig. 3. Distribution of HIV-1 viral load among HAART-naïve GBV-C-co-infected (●) and GBV-C-uninfected (○) patients. The bars indicate the mean viral load.

was similar to the results of HIV-1-infected patients obtained by others (3, 21, 22). This fairly high value seems reasonable as the prevalence seen among homosexual men (12.5%) in our previous report was higher than that of healthy blood donors, and the majority of subjects in this study were infected with HIV-1 through homosexual contacts.

GBV-C genotypic analysis showed that 59.5% (22/37) of the GBV-C-infected individuals were classified as genotype 3, 32.4% (12/37) as genotype 2, and 8.1% (3/37) as genotype 1. None were classified as genotype 4 or 5. Our results agreed with reports by

others in that genotype 3 is prevalent in Japan (1, 14). Two of the 3 individuals with GBV-C genotype 1 were hemophiliacs, agreeing with a previous report by Liu et al. (13).

To study the effect of GBV-C co-infection on HIV-1-infected patients, plasma HIV-1 viral loads of antiretroviral therapy-naïve GBV-C-infected and -uninfected groups were compared. Statistical analysis showed that the HIV-1 viral loads of the GBV-C co-infected group were significantly lower than those of the singly-infected group ($P=0.01$). Consequently, we looked into the relationship between GBV-C viral load and HIV-1 viral

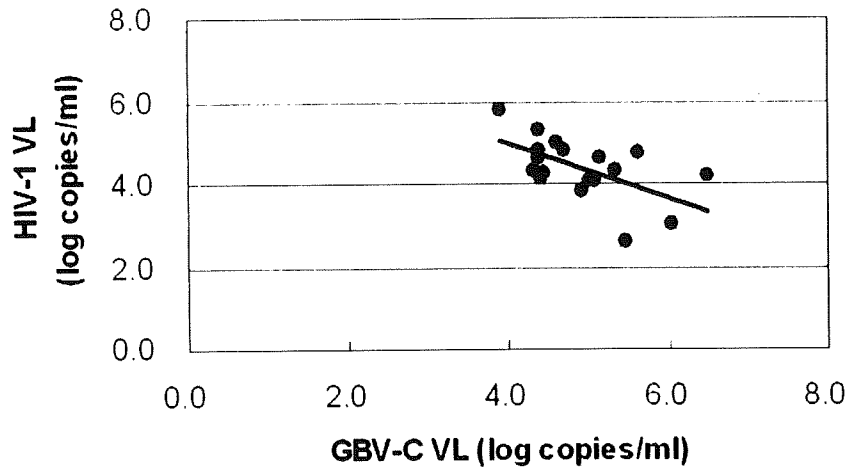


Fig. 4. Correlation between the GBV-C viral load and HIV-1 viral load ($R^2=0.34$).

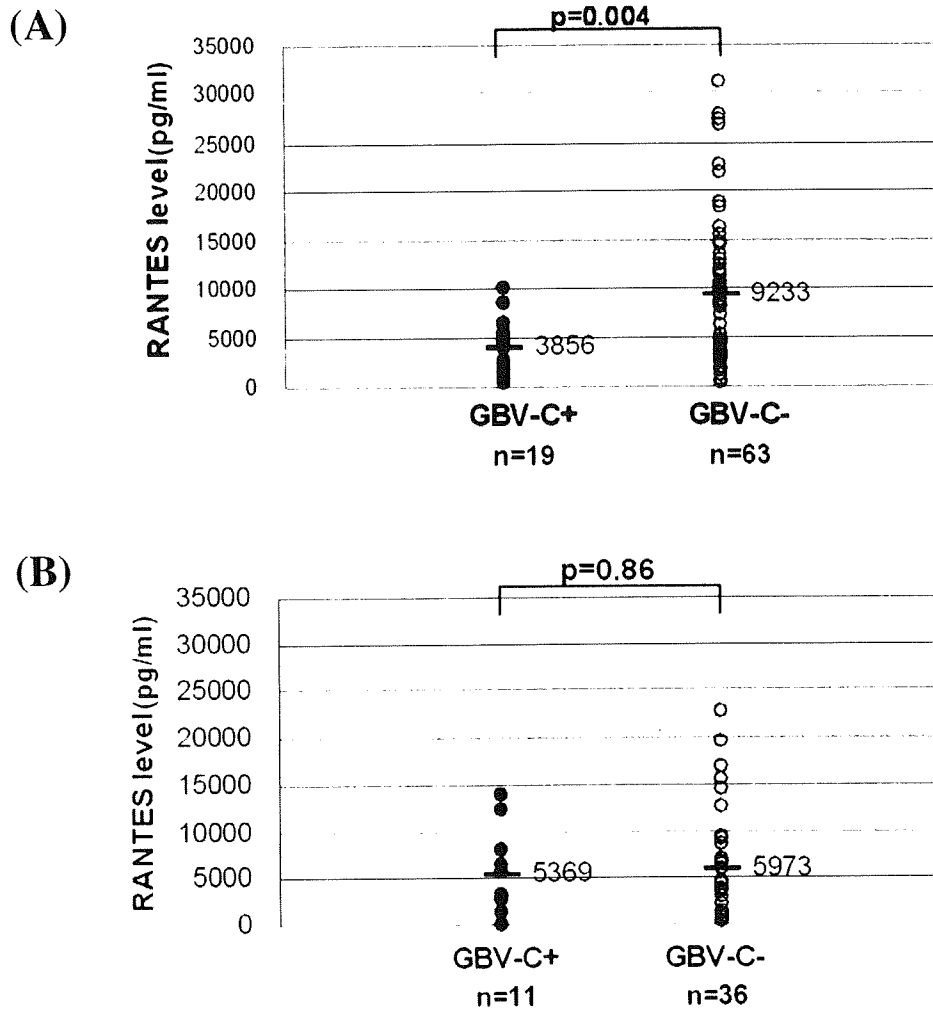


Fig. 5. Distribution of RANTES concentrations among HAART-naïve GBV-C-co-infected (●) and GBV-C-uninfected (○) patients in (A) the initial study, and (B) the follow-up study. The bars represent the mean RANTES concentrations.

load of the same study groups, and observed an inverse correlation in that individuals with higher GBV-C viral loads tended to have lower HIV-1 viral loads ($R^2=0.34$). Although the suppression level shown here was weak and partial, it is clear that the GBV-C infection is associated with the suppression of HIV-1 replication. Our results do not directly support the findings by Xiang et al. (26) or Tillmann et al. (22), but we believe that the HIV-1 replication suppressive effect by GBV-C possibly leads to a better prognosis and a low mortality rate. The question of why some research groups found a beneficial effect of GBV-C co-infection while others did not still remains. The discrepancy may occur from the differences in race, HIV-1 clade, or GBV-C genotype. However, divergent results were observed even when the studies were composed of mostly Caucasian subjects who were presumably infected with HIV-1 subtype B (18, 19, 22, 26). In addition, the differences in GBV-C genotype do not seem to account for the contrasting findings since the HIV-1 VLs between GBV-C genotypes 2 and 3 were similar as observed in the present study. Thus, other reasons may exist for the conflicting observations.

One possible mechanism of the suppression of HIV-1 replication is the inhibition of HIV-1 entry into target cells. As Xiang et al. reported that the co-infection of GBV-C suppressed the HIV-1 replication through up-regulation of RANTES, a natural CCR5 ligand, and also known as CCL5, in an *in vitro* study (25), we assessed the scenario by measuring the RANTES concentration in patients' plasma. In contrast to the *in vitro* study, however, much higher RANTES concentrations were observed in GBV-C-uninfected individuals. In addition, a mean RANTES level of 3,856 pg/ml in HAART-naïve GBV-C-infected patients at the first medical examination increased to 5,369 pg/ml after 1 year, whereas that of 9,233 pg/ml in HAART-naïve GBV-C-uninfected patients decreased to 5,973 pg/ml. This increase in the RANTES level in the GBV-C co-infected group, and in contrast, the decrease in the uninfected group, was unexpected and is inexplicable. Kwofie et al. reported a transient decrease in RANTES level at the early stage of HIV-1 infection in macaques (9). A similar fluctuation in RANTES level might occur in humans as well. Regrettably, we were unable to conclude that the HIV-1 replication was suppressed by up-regulation of RANTES by GBV-C, and the inhibition of HIV-1 replication might be supported through other mechanisms induced by GBV-C co-infection.

We can conclude that GBV-C co-infection affects the clinical status of HIV-1-infected patients, and that the co-infection confers a beneficial effect on the patient.

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<症例報告>

HIV・HCV重複感染に対する リバビリン併用ペグ・インターフェロン療法により CD4, CD8陽性リンパ球数が減少した1症例

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はじめに

I 症例提示

多剤併用療法 (highly active antiretroviral therapy: HAART) 導入後のHIV感染者の死亡原因は、AIDS関連と非AIDS関連が半々で、非AIDS関連死の約9割が慢性HCV感染症によるといわれている¹⁾。HIVとHCVの主な感染経路は血液を媒介するもので、静注薬物使用者や血液製剤による感染例で重複感染が多いのが実状である。HIV・HCV重複感染例におけるHCV治療は、HCV単独感染の標準ガイドラインに従いインターフェロンを基本とし、近年ではリバビリンを併用することが多い。

今回、HAART導入によりHIV-RNA量が検出限界 (50copies/mL) 以下、CD4陽性リンパ球数が200/ μ L以上と良好なコントロールを得たHIV・HCV重複感染患者に対してリバビリン併用ペグ・インターフェロン療法を行ったところ、CD4, CD8陽性リンパ球数が200/ μ L以下まで減少した症例を経験したので報告する。

【症例】49歳, 男性

既往歴: 血友病B, C型慢性肝炎

臨床経過: 1985年にHIV感染が判明し、91年1月よりジドブジン (AZT, レトロビル[®]) の内服を開始、93年12月からジダノシン (ddI, ヴァイデックス) の内服に変更した。その後、97年4月よりAZT+ラミブジン (3TC, エピビル[®]) + インジナビル (IDV, クリキシバン[®]) の3剤の内服 (HAART) を開始した。2004年9月からAZT+3TC+ロピナビル・リトナビル配合剤 (LPV/r, カレトラ[®]) の組み合わせに変更となった。この間、HIV-RNA量は検出限界以下を維持しており、CD4陽性リンパ球数は平均397/ μ L、CD8陽性リンパ球数は平均910/ μ Lと安定していた。

一方でHCV-RNA量は、04年11月時点で 190×10^4 IU/mL、05年7月時点で 86×10^4 IU/mLと高値を示した。そのため、抗HCV療法と

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して、05年8月から06年8月までの1年間、リバビリン併用ペグ・インターフェロン療法を行った。その結果HCV-RNA量は05年10月から陰性となったが、 $68 \times 10^2/\mu\text{L}$ あった白血球数は、最も少ない時で $29 \times 10^2/\mu\text{L}$ まで減少した。なかでもCD4陽性リンパ球数は、05年9月で $315/\mu\text{L}$ 、12月で $194/\mu\text{L}$ 、06年2月で $116/\mu\text{L}$ 、4月で $134/\mu\text{L}$ 、8月で $141/\mu\text{L}$ と、免疫不全状態の指標である $200/\mu\text{L}$ 以下まで減少し、その状態は8カ月にわたり継続した。またCD8陽性リンパ球数も05年9月で $656/\mu\text{L}$ 、12月で $334/\mu\text{L}$ 、06年2月で $171/\mu\text{L}$ 、4月で $176/\mu\text{L}$ 、8月で $137/\mu\text{L}$ まで減少した。なおこの間、赤血球数は $23 \times 10^5/\mu\text{L}$ 以上、血小板数は $9 \times 10^4/\mu\text{L}$ 以上を維持していたが、ヘモグロビン量については 13g/dL から徐々に減少し、06年4月には 10g/dL 以下となった。そのため、エポエチンアルファ（エスポー[®]）12000IUの皮下注射を予防投与も含めて4回行い造血の活性化を促した。その効果は残念ながら判然としなかったが、抗HCV療法終了後、赤血球数、ヘモグロビン量および白血球数は増加し、CD4陽性リンパ球数は06年9月で $389/\mu\text{L}$ 、10月で $291/\mu\text{L}$ に、CD8陽性リンパ球数もそれぞれ $613/\mu\text{L}$ 、 $442/\mu\text{L}$ と増加した（図1）。

なお抗HCV療法を行うにあたり、リバビリンとAZTの併用による副作用と考えられる貧血を防止するため、HAARTの組み合わせを05年8月からテノホビル（TDF、ビリアード[®]）+3TC+LPV/rに変更した。TDF変更後もHIV-RNA量は検出限界以下を維持していた。

一方でTDFには、副作用として重度の腎機能障害が報告¹⁾されている。そこでTDF内服開始後のTDF血中濃度（内服3時間後）と血中クレアチニンの推移について検討した。TDF内服前（05年7月）の血中クレアチニンは 1.1mg/dL であったが、内服後徐々に上昇し、06年4月以降は 1.4mg/dL を超えていた。同様にTDF血中濃度も05年9月での $0.45\mu\text{g/mL}$ から徐々に高くなり、内服1年後の06年9月では

$0.67\mu\text{g/mL}$ となった。通常TDFの最高血中濃度は $0.30\sim 0.38\mu\text{g/mL}$ とされており、本症例では2倍近い高い値を示した（図2）。

II 考 察

HIV・HCV重複感染患者に対する抗HCV療法は、HIV-RNA量が良好にコントロールされ、CD4陽性リンパ球数が $200/\mu\text{L}$ を超える安定した患者を対象に行われている¹⁾。しかしHIV非感染者に比べてHIV・HCV重複感染患者に対しては、インターフェロン投与に伴う白血球数減少やリバビリン投与に伴う貧血などの副作用について特に注意が必要である。なかでもAZTとリバビリンの併用は重度の貧血を起こすことが知られているので²⁾、本症例もリバビリン投与開始時にHAARTの組み合わせをAZTからTDFに変更した。またHIV・HCV重複感染患者では、インターフェロンによるCD4陽性リンパ球数の一時的減少が報告^{3)~5)}されている。本症例においてもリバビリン併用ペグ・インターフェロン療法を開始した直後から白血球数の減少が認められた。なかでもCD4、CD8陽性リンパ球数は免疫不全状態の指標である $200/\mu\text{L}$ 以下まで減少した。本症例ではCD4陽性リンパ球数の減少が緩やかであり日和見感染もみられなかったため、抗HCV療法を継続したが、CD4陽性リンパ球数の減少が重度である場合にはインターフェロンの中止が必要と考える。

一方、リバビリン投与開始時にAZTから変更したTDFについても好中球減少等の副作用が知られており、本症例でもその副作用は顕著に観察された。また本症例では徐々に腎機能が悪化しており、血中クレアチニンの上昇と共にTDF血中濃度も上昇していた。TDFは腎排泄型の薬剤であり、腎機能が悪化すればその代謝も遅延し、血中濃度が上昇すると考えられる。本症例における高いTDF血中濃度と白血球数等の減少との関連は否定できないが、抗HCV療法終了後に再び白血球数が増加

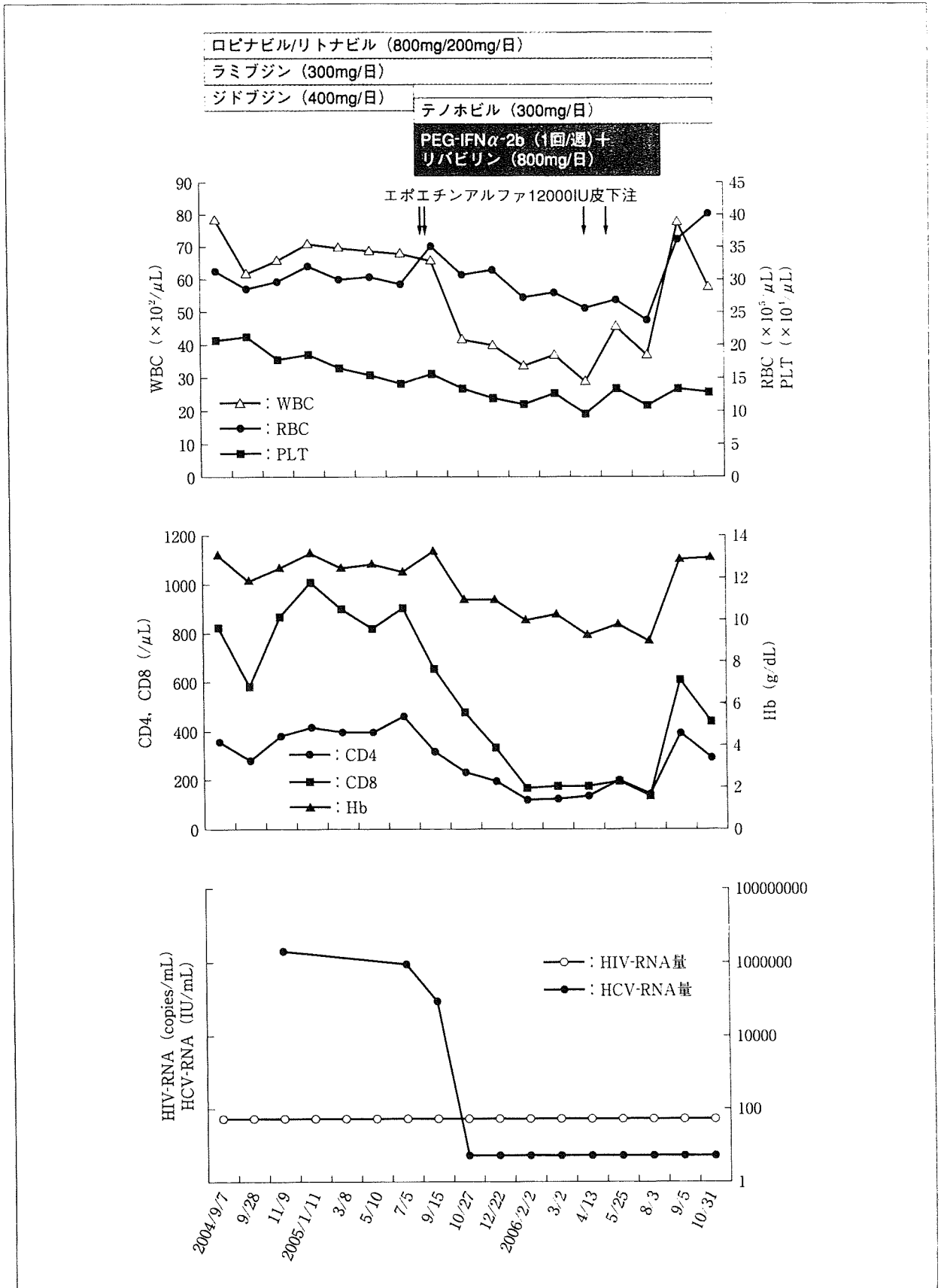


図1 臨床経過

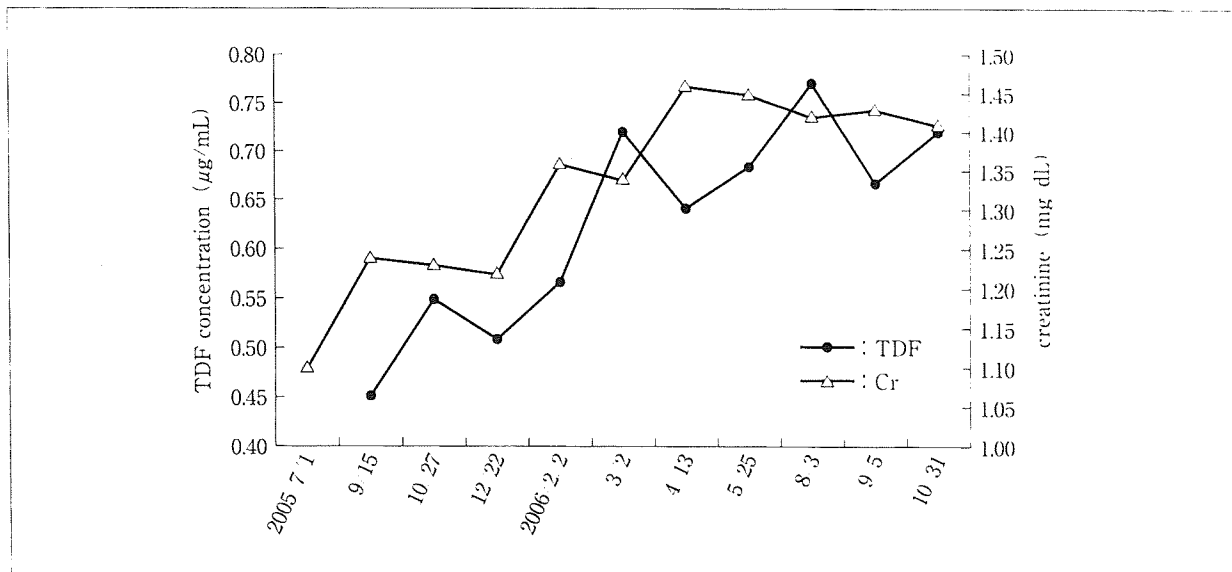


図2 内服3時間後のテノホビル (TDF) 血中濃度と血中クレアチニン (Cr) の推移

したことからTDFの関与は小さいと考える。

HIV・HCV重複感染患者に対して抗HCV療法を行う場合には、日和見感染を予防する上でCD4陽性リンパ球数の減少に十分に注意を払う必要がある。加えてHAARTにおいてTDFを選択した場合には腎機能低下に注意し、腎障害時には投与間隔の調節を考慮することが望ましい。

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Original Article

Performance and Quality Assurance of Genotypic Drug-Resistance Testing for Human Immunodeficiency Virus Type 1 in Japan

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SUMMARY: Highly active antiretroviral therapy (HAART) can suppress human immunodeficiency virus type 1 (HIV-1) replication and plasma HIV-1 to below detectable levels. However, HAART becomes ineffective when drug-resistant viruses emerge during HAART. Monitoring drug-resistance mutations in viruses is necessary for selecting new drugs or therapies effective at inhibiting such HIV-1 variants. Most laboratories in Japan perform the tests using in-house protocols. However, the quality of these tests has never been assessed. Our study assessing the accuracy and reliability of HIV-1 genotypic drug-resistance testing in 15 laboratories in Japan revealed that the quality was very high (97.3% accurate). The errors, though rare, were caused by human errors, poor electropherograms, and the use of inadequate primers. Here, we propose troubleshooting procedures to improve testing accuracy and reliability in Japan.

INTRODUCTION

Genotypic drug-resistance testing for human immunodeficiency virus type 1 (HIV-1) is clinically useful for successful antiretroviral treatment (1-5). In Japan, the test, which could initially be performed at only a few virological laboratories in 1996, is now conducted at more than 15 laboratories. The cost of testing is almost entirely covered by government research funds. The laboratories use in-house protocols, the main advantage of which is their low cost relative to that of commercial kits. As the quality of these protocols had not previously been evaluated, we conducted an assessment of in-house protocol reliability by sending HIV-1 RNA samples to the relevant laboratories.

MATERIALS AND METHODS

Laboratories participating in the quality assurance assessment of genotypic drug-resistance testing protocols: Eight HIV/AIDS clinical centers, five local government institutes of public health, one commercial laboratory, and the National Institute of Infectious Diseases (NIID) were enrolled in this study on voluntary basis.

Construction of drug-resistant HIV-1 clones: We chose two plasma specimens, i.e., viruses well-characterized for harboring multidrug-resistance mutations. The HIV-1 of one specimen (case 1) had eight drug-resistance mutations: L63P, A71T, and V77I in the protease (PR), and A62V, V75I, F77L, F116Y, and Q151M in the reverse transcriptase (RT); and 10 mutations unrelated to drug-resistance: V3I, E35D, S37N, R41K, and K70R in the PR, and S68G, T69V, E122K, Q197E, and R211K in the RT. The HIV-1 of the other specimen (case 2) had nine drug-resistance mutations: L10I, L63P, V77I, and L90M in the PR, and M41L, T69S-SG insertion, G190A, L210W, and T215Y in the RT; and 15 mutations unrelated to drug-resistance: V3I, I15V, S37D, and I93L in the PR, and

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V35T, T39A, K43E, E122K, I135T, R172K, D177E, Q207H, R211K, L214F, and K238S in RT.

HIV-1 RNA was extracted from the plasma using the QIAamp Viral RNA kit (QIAGEN, Valencia, Calif., USA). DNA fragments sized 1.3 kb containing *gag* (codons #412-500), *protease* (codons #1-99), and *reverse transcriptase* (codons #1-260) were amplified by RT-PCR using SuperScript One-Step RT-PCR (Invitrogen, Carlsbad, Calif., USA) and the primer pair K1 (5'-AAG GGC TGT TGG AAA TGT GG-3') and U13 (5'-CCC ACT CAG GAA TCC AGG T-3'), followed by a second-round PCR using LA Taq (Takara, Shiga, Japan) with the primer pair INF-ApaI (5'-TGC TGG GCC CCT AGG AAA AAG G-3') and INF-NheI (5'-TCT GGC TAG CCC AAT TCA ATT TTC CCA C-3'). The amplified fragments were sequenced and the presence of the target mutations was confirmed. The amplicon was then digested with *ApaI* (Takara) and *NheI* (New England Biolabs, Ipswich, Mass., USA), and the *ApaI-NheI* fragment was inserted into the corresponding site of a pSUM9 HXB2 expression vector (kindly provided by Dr. H. Mitsuya, National Cancer Institute, Bethesda, Md., USA) (6). Each clone was transfected into MT-2 cells (approximately 40% confluence in 1 ml of RPMI 1640) with Lipofectamine (Invitrogen). The cells were cultured with RPMI 1640 containing 10% FBS under a 5% CO₂ atmosphere at 37°C. After 4 days, 0.2 ml of the culture supernatant was collected and transferred to 5 × 10⁴ MT-4 cells in 4.8 ml of culture medium. Three days after infection, the culture supernatants were harvested and stored at -80°C until use.

Preparation and shipping of HIV-1 RNA samples: HIV-1 virions were precipitated by ultracentrifugation at 23,000 × g for 1 h at 4°C and washed twice with PBS(-). The pellets were suspended in 100 μl of PBS(-) and incubated with 3 U of deoxyribonuclease (RT Grade) (Nippon Gene, Tokyo, Japan) at 37°C for 15 min. Virions were precipitated again by ultracentrifugation at 23,000 × g for 1 h at 4°C and washed twice with PBS(-) to remove the deoxyribonuclease. RNA was extracted using the QIAamp Viral RNA kit. The number of HIV copies was determined by real-time PCR according to the method reported by Nagai et al. (7), and aliquots containing 2 × 10⁴ copies of HIV-1 RNA in 2 ml of PBS(-) were stored at -80°C. The samples were packed on dry ice and shipped to the participating laboratories.

Data collection and evaluation: Each laboratory was requested to provide a detailed protocol for genotypic testing, including the primer sequences and the enzymes used for reverse transcription and PCR conditions. Laboratories were asked to submit electropherograms and a list of drug-resistance mutations determined according to International AIDS Society-USA panel criteria, version March/April 2005 (8).

The rate of successful detection of mutations was expressed as follows: %DR (or NDR) = detected number of drug-resistance mutations (non-drug-resistance mutations)/total number of drug-resistance mutations (non-drug-resistance mutations). Reported mutations absent in the test samples (ghost mutations) were noted as "E1" errors, erroneous categorization as an "E2" error, and errors in preparing the reports as "E3" errors.

RESULTS

Variations in protocols for genotypic drug-resistance testing in 15 laboratories: As shown in Figure 1, the enrolled

laboratories used different protocols with respect to primers, sizes of amplified fragments, and the number of amplified fragments. Eight laboratories (A, B, D, E, F, I, J, and K) used the latest NIID protocol with minor modifications, and three laboratories (G, H, and N) used the 1996 version of the NIID protocol with or without modifications. Four laboratories (C, L, M, and O) used their own protocols.

Regarding the reverse transcription and amplification procedures, 12 laboratories used one-step RT-PCR, and the other three laboratories used two-step RT-PCR. In the reverse-transcription process, AMV RT was used in two laboratories, and M-MLV RT was used in one laboratory. As regards the DNA polymerase used for the first and second PCR, most laboratories used Taq polymerase but other few laboratories used KOD polymerase.

For sequencing, Big Dye Terminator V1.1 (Applied Biosystems, Foster City, Calif., USA), Big Dye Terminator V3.1 (Applied Biosystems), CEQ Dye Terminator Cycle Sequencing with a Quick Start kit (Beckman Coulter, Fullerton, Calif., USA), and Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, Ohio, USA) with IRDyeTM 800 v2 Terminator Mixes (LI-COR, Lincoln, Nebr., USA) were used in eight, five, one, and one of the laboratories, respectively. For the purification of labeled products, CENTRI SEP Spin Columns (Applied Biosystems), Sephadex G-50 (GE Healthcare Bio-Sciences, Piscataway, N.J., USA), and DyeEx 2.0 Spin kit (QIAGEN) were used in five, three, and one of the laboratories, respectively. The other six laboratories used the ethanol precipitation method.

For electrophoresis, 14 laboratories used a capillary-type auto-sequencer and the remaining laboratory used a plate-type auto-sequencer. Ten laboratories used the ABI PRISM 310 (Applied Biosystems) auto-sequencer and five laboratories used other sequencers, i.e., the CEQ 8000 (Beckman Coulter), LI-COR 4200 IR2 System (LI-COR), ABI PRISM 3100 (Applied Biosystems), ABI PRISM 3100 Avant (Applied Biosystems), and ABI PRISM 3730S (Applied Biosystems) auto-sequencers.

Results of case 1 testing: As shown in Table 1, in the PR mutation analyses, all laboratories except G detected 100% of the drug-resistance mutations. On the other hand, when detecting the non-drug-resistance mutations, the amino acid mutations reported by laboratories G and H were totally different from those reported by the other laboratories. Laboratory G missed one drug-resistance mutation, A71T, and two non-drug-resistance mutations, E35D and R41K. Laboratory H reported all of the three drug-resistance mutations correctly, but not all of the five non-drug-resistance mutations. Laboratories G and H appear to have amplified the wrong samples, suggesting that contamination may have occurred. Checking their protocols, we noticed that laboratories G and H used DRPRO3 primer in their second round of PCR (Figure 1). As the case 1 sample had an insertion mutation in the DRPRO3 annealing region, a mismatch with this primer in the region caused these errors.

Laboratories E and F reported ghost mutations D29N and C95W, respectively. In the case of laboratory E, the error appeared to be due to the high background noise in the electropherogram. In the case of laboratory F, the data were analyzed only by automatic base sequence analysis, and no manual editing was performed to eliminate the error.

Two laboratories (A and B) made errors in their final reports. We confirmed the raw data from these two laboratories, and found that their electropherograms, nucleotide

Laboratory code	No. of fragments	Design and coverage of assay			
		codon 1	PR	99 1	RT 240
D, I, J	3	DRPRO5 (open), DRPRO1M (closed)	DRRT1L (open), DRRT7L (closed)	DRPRO2L (open), DRPRO6 (closed)	DRRT29 (open), DRRT28 (closed), DRRT4L (open), DRRT6L (closed)
H	3	DRPRO1 (open), DRPRO3 (closed)	DRRT01 (open), DRRT12 (closed)	DRPRO2 (open), DRPRO4 (closed), DRRT02 (open), DRRT13 (closed), DRRT03 (open), DRRT14 (closed)	DRRT04 (open), DRRT15 (closed)
A, B, K	2	DRPRO5 (open), DRPRO1M (closed)	DRRT1L (open), DRRT7L (closed)	DRPRO2L (open), DRPRO6 (closed)	DRRT4L (open), DRRT6L (closed)
E	2	DRPRO5 (open), DRPRO1M (closed)	DRRT1L (open)	DRPRO2L (open), DRPRO6 (closed), MS2510F (closed)	DRRT4L (open), SA3 (closed)
F	2	DRPRO5 (open), DRPRO1M (closed)	DRRT1L (open), DRRT7L (closed)	DRPRO2L (open), DRPRO6 (closed)	pol2 (open), pol4 (closed)
G	2	DRPRO1 (open), DRPRO3 (closed)	DRRT01 (open), DRRT (closed)	DRPRO2 (open), DRPRO4 (closed)	DRRT04 (open), DRRT15 (closed)
N	2	DRPRO1 (open), DRPRO3 (closed), DRPRO1 (closed)	DRRT01 (open), DRRT7L (closed)	DRPRO2 (open), DRPRO4 (closed), K05 (closed)	DRRT04 (open), DRRT15 (closed)
C	2	RT-PCR(F) (open), primer1 (closed)	primerA (closed)	primer2 (closed)	RT-PCR(R) (open), primer3 (closed)
L	1	K6 (open), K5 (closed)			U15 (open), U14 (closed)
M	1	SK38 (open), prots10 (closed)			RT20 (open), DRRT4L (closed)
O	1	K1 (open), K4 (closed)			U13 (open), U12 (closed)

Fig. 1. Schematic pictures showing primer-binding positions on HIV-1 templates and their amplified regions. Primers for RT-PCR and nested PCR are indicated by open and closed symbols, respectively. The letters A to O indicate the 15 laboratories.

sequences, and amino acid sequences were correct, but that they misrecorded the amino acid mutations during the preparation of their final reports.

As regards the RT results, 14 laboratories correctly reported all of the drug-resistance and non-drug-resistance mutations. Laboratory C did not report three non-drug-resistance mutations (S68G, Q197E, and R211K), although these were correctly displayed in the nucleotide and amino acid sequence files, thus indicating an error in the preparation of the final report.

Results of case 2 testing: In the PR mutation analysis, 12 laboratories correctly reported all of the drug-resistance and non-drug-resistance mutations. Laboratory D failed to report the L90M drug-resistance mutation owing to a translation error, although "ATG" was clearly seen in the electropherogram. Laboratory E reported ghost mutation E34K due to the high background noise in the electropherogram. Peak distortion on the electropherograms caused Laboratory F to report ghost mutations R41K and T96S, and an extra large peak on the electropherogram caused the misdetection of L10L/S instead of L10I. This extra large peak may be due to the insufficient removal of residual free dideoxynucleotides after the sequence

reaction.

As regards the RT region, 14 laboratories, but not laboratory K, correctly reported all drug-resistance mutations. Laboratory K detected the T69S-SG insertion, but misidentified it as a non-drug-resistance mutation. As for the non-drug-resistance mutations, four laboratories (G, H, L, and N) prepared incorrect reports. Laboratory G did not report V35T and T39A because they were unable to analyze the region between codon #1 and #39. Laboratory H did not report E122K and I135T, because they did not amplify the RT region from codon #122 to #135. These errors reported from laboratories G and H were excluded in the calculation of the rate of detection of non-drug-resistance mutations (%NDR). Laboratory L failed to report K238S, although it was detected in the amino acid sequence file. Laboratory N reported ghost mutation I31T.

DISCUSSION

To assess the quality of in-house genotypic drug-resistance testing, we used two HIV-1 RNA specimens to conduct profi-

Table 1. Detection rates and number of errors for the 15 in-house genotypic drug-resistance testings

	Case 1										Case 2									
	Protease					Reverse transcriptase					Protease					Reverse transcriptase				
	%DR	%NDR	E1	E2	E3	%DR	%NDR	E1	E2	E3	%DR	%NDR	E1	E2	E3	%DR	%NDR	E1	E2	E3
A	100	80			1	100	100				100	100				100	100			
B	100	80			1	100	100				100	100				100	100			
C	100	100				100	40			3	100	100				100	100			
D	100	100				100	100				75	100			1	100	100			
F	100	100	1			100	100				100	100	1		100	100				
F	100	100	1			100	100				75	100	3 ^{b)}		100	100				
G	66	60			3	100	100				100	100			100	100			2 ^{b)}	
H	100	0			5	100	100				100	100			100	100			2 ^{b)}	
I	100	100				100	100				100	100			100	100				
J	100	100				100	100				100	100			100	100				
K	100	100				100	100				100	100			80	100			1	
L	100	100				100	100				100	100			100	91			1	
M	100	100				100	100				100	100			100	100				
N	100	100				100	100				100	100			100	100	1			
O	100	100				100	100				100	100			100	100				

%DR, successful detection rate of drug-resistance mutations; %NDR, successful detection rate of non-drug-resistance mutations; E1, mutations which did not exist in the test samples (ghost mutations); E2, errors in defining drug-resistance mutations; E3, errors in writing or copying to the reports.

^{b)} One ghost mutation interrupted the detection of a drug-resistance mutation.

^{c)} %NDR were estimated without these errors because laboratories G and H could not analyse or amplify the region from codon #1 to #39 and from #122 to #135, respectively.

ciency tests for 15 voluntarily participating laboratories. Since there were 42 amino acid mutations within the sequences of the two test samples, 13 of the laboratories should have reported 546 amino acid mutations and the remaining two (G and H) should have reported 80. Accordingly, the total number of amino acid mutations reported from 15 laboratories should have been 626. There were a total of 17 errors in their reports. The calculated overall detection percentage was 97.3% (= $100 \times [626 - 17] / [626]$), and the error rate was 2.7%. The average correction rate of eight laboratories (A, B, D, E, F, I, J, and K) adopting the NIID protocol with the latest primers, and that of four laboratories (C, L, M, and O) that had developed in-house protocols were 97.0 and 97.6%, respectively. Thus, there was no obvious benefit of in-house protocols in terms of accuracy. However, the in-house protocols of L, M, and O possessed one superior point where the single DNA fragment including PR and RT genes were amplified. Based on these results, we can propose that the NIID protocol be considered as the standard protocol. As the primers developed by laboratories L, M, and O functioned well, we can recommend the usage of these primers in the standard protocol.

The errors observed in this study were categorized as either technical or human errors, and the frequency of these errors was 1.4 and 1.3%, respectively. As regards the technical errors, we found two problems that need to be addressed. First, the use of mismatched primers led to errors in the detection of case 1 HIV-1 PR mutations. The case 1 sample had an insertion at the annealing site of the DRPRO3 primer, and the laboratories using that primer (G and H) failed to detect the mutations. A new primer, DRPRO1M, replaced DRPRO3 when the insertion mutation at *gag p6* was reported (9-11). However, laboratories G and H did not know about this new primer, in spite of the fact that the NIID (the developer of DRPRO3) had sent them several announcements regarding this new primer. This episode highlights the importance of constructing a nationwide network of laboratories working on drug-resistance testing. Second, the quality of the primers appeared to be important. Using impure primers may

Table 2. Summary of sources of error and problems leading to errors

Category	Cause of misjudgement
Technical errors 9 (1.4%)	Use of inadequate primers Peak distortions in poor electropherograms
Human errors 8 (1.3%)	Writing errors to the final reports 6 (1%) Judgement errors in categorizing amino acid mutations 1 (0.2%) Misinterpretation of codons 1 (0.2%)
Total problems 17 (2.7%)	

result in high background noise on the electropherogram, which may lead to the misinterpretation of results. Therefore, using highly purified primers is recommended for better sequence reliability. Needless to say, the buffers, matrix, and capillaries of the sequencers should always be in good condition.

Several types of human error were observed, including errors in writing or copying the final reports (1%), errors in categorizing mutations as resistance or non-resistance mutations (0.2%), and misinterpretation of codons (0.2%) (Table 2). The ideal way to prevent such human errors would be the development of a computation program that would automatically analyze electropherograms, identify drug-resistance mutations, and create the final report. Until such resources are available, several manual validation steps will be necessary to check the data and the content of the reports.

As regards the detection of different HIV-1 subtypes, both the latest NIID protocol and the in-house protocol of laboratory O have successfully amplified the PR and RT genes of HIV-1 subtypes A, B, C, D, AE, and F.

In conclusion, we are pleased to report that the reliability of genotypic drug-resistance testing in Japan is excellent (97.3% correct results). However, as noted above, there are a few points related to implementation which still need to be improved upon in order to achieve even better results. We have sent out the annotated results of our evaluation to each laboratory, and hope that this report will help to improve the

quality of testing. Furthermore, as the test samples used here were extracted viral RNAs and not the virus itself, the technical error expected with the RNA extraction procedure was not considered in the present assessment. Thus, the error rate of 2.7% may be an underestimation; to clarify the exact rate, we are currently planning a second round of assessment using patient plasma samples.

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