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 *Apa*I (Takara) and *Nhe*I (New England Biolabs, Ipswich, Mass., USA), and the *Apa*I-*Nhe*I 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% $CO_2$ atmosphere at 37°C. After 4 days, 0.2 ml of the culture supernatant was collected and transferred to $5 \times 10^4$ 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 $\times g$ for 1 h at 4°C and washed twice with PBS(−). The pellets were suspended in 100 $\mu$l 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 $\times g$ 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 $2 \times 10^4$ 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 IRDye™ 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

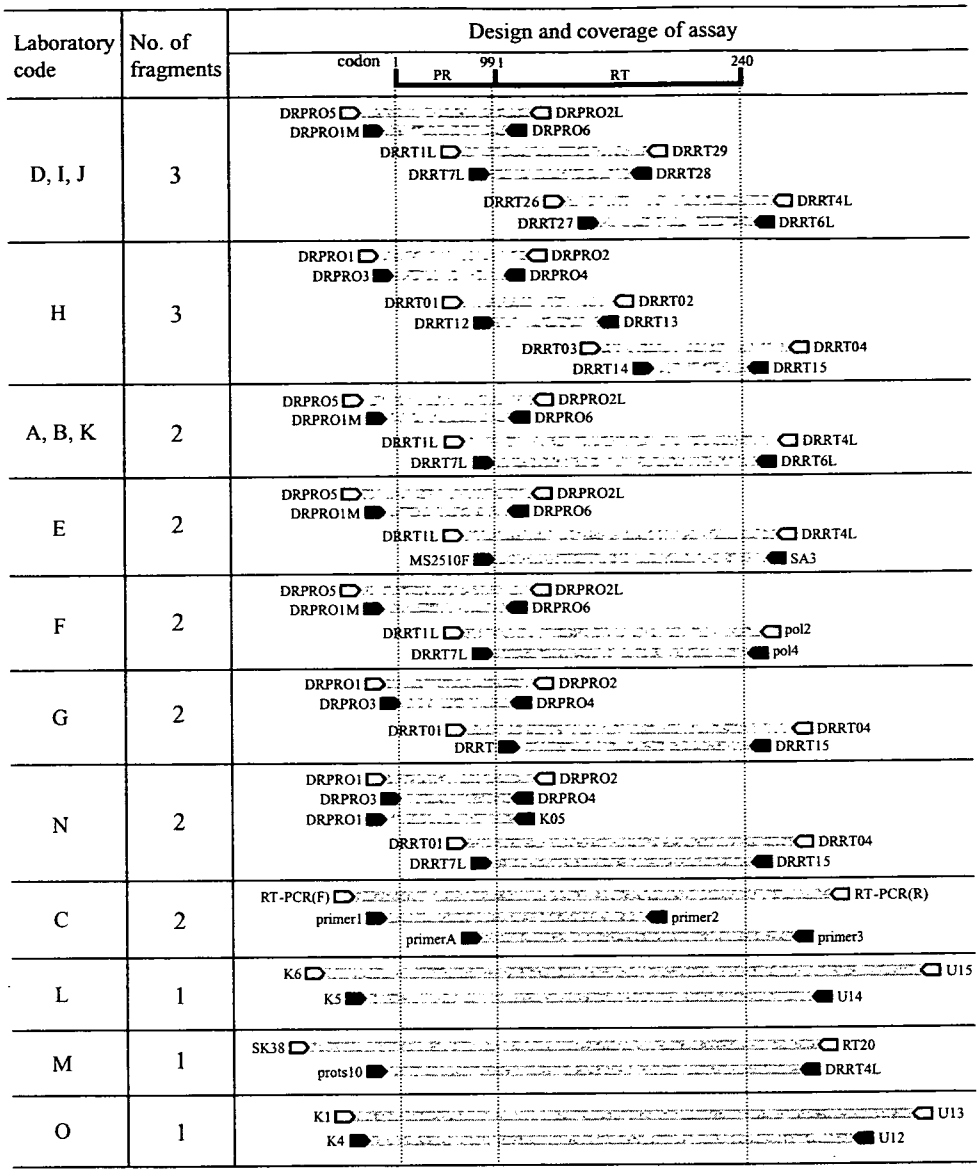


Fig. 1. Schematic pictures showing primer-binding positions on HIV-1 templates and their amplified regions. Primers for RT-PCR and nested PCR are indicated by open and closed symbols, respectively. The letters A to O indicate the 15 laboratories.

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 | – | – | – |
| E | 100 | 100 | 1 | – | – | 100 | 100 | – | – | – | 100 | 100 | 1 | – | – | 100 | 100 | – | – | – |
| F | 100 | 100 | 1 | – | – | 100 | 100 | – | – | – | 75 | 100 | 3[1)] | – | – | 100 | 100 | – | – | – |
| G | 66 | 60 | – | – | 3 | 100 | 100 | – | – | – | 100 | 100 | – | – | – | 100 | 100 | – | – | 2[2)] |
| H | 100 | 0 | – | – | 5 | 100 | 100 | – | – | – | 100 | 100 | – | – | – | 100 | 100 | – | – | 2[2)] |
| 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.
[1)]: One ghost mutation interrupted the detection of a drug-resistance mutation.
[2)]: %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×[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 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.

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<br>Peak distortions in poor electropherograms |
| Human errors 8 (1.3%) | Writing errors to the final reports 6 (1%)<br>Judgement errors in categorizing amino acid mutations 1 (0.2%)<br>Misinterpretation of codons 1 (0.2%) |
| Total problems 17 (2.7%) | |

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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原　著

# 日本におけるHIV-1遺伝子型薬剤耐性検査のコントロールサーベイ

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**目的**：日本でHIV-1遺伝子型薬剤耐性検査を実施しているほとんどの施設は，国立感染症研究所が開発した方法を参考にしたin-houseの手法を用いて検査を行っている。しかし，これらの施設が実施しているHIV-1遺伝子型薬剤耐性検査の質は，今までに評価を受けたことがない。我々は，日本で薬剤耐性検査を実施している15施設について，検査の精度と信頼性を調べることを目的としてコントロールサーベイを実施した。

**材料および方法**：HIV-1遺伝子型薬剤耐性検査を実施している15施設で，2種類のクローン化薬剤耐性HIV-1 RNAを用いて薬剤耐性検査を実施し，その検査結果を評価した。

**結果**：HIV-1遺伝子型薬剤耐性検査の質は，正解率が97.3%と，非常に高いことが明らかとなった。誤答の原因は，不適切なプライマーの使用，エレクトロフォレグラムの乱れ及び，人為的誤りなどであった。

**結論**：日本で実施されているHIV-1遺伝子型薬剤耐性検査の水準は高い事が明らかになった。しかしその水準をより一層向上させるために，今回明らかになった問題に対して解決法を提案した。

**キーワード**：AIDS, HIV-1, HIV-1遺伝子型薬剤耐性検査，コントロールサーベイ

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## 緒　言

HIV-1感染症治療において，HAART（highly active antiretroviral therapy）は，ウイルス複製を強力に抑制し，長期にわたり血中ウイルス量を検出限度以下に維持することを可能とした[1,2]。HAARTによる治療効果は通常，血中ウイルス量でモニタリングされている。血中ウイルス量が，検出限度以下に一旦抑制されたにもかかわらず，再び増加した場合には，薬剤耐性HIV-1の出現が疑われる[3-6]。この場合，遺伝子型薬剤耐性検査によって，治療薬剤の標的酵素であるプロテアーゼおよび逆転写酵素のアミノ酸配列を決定し，HIV-1が薬剤耐性を獲得しているかどうかを鑑別している。同時に，変更可能な抗HIV-1薬を選択するための情報も得ることができる[7-11]。最近，薬剤耐性HIV-1が未治療患者からも検出されることから[12,13]，HAART開始前にも本検査の実施が推奨されている。このように，遺伝子型薬剤耐性検査は治療を最適化するために必須の検査である[14,15]。

現在，日本でHIV-1遺伝子型薬剤耐性検査を実施しているほとんどの施設は，in-houseの手法を用いて検査を行っている。しかし，これらの施設が実施しているHIV-1遺伝子型薬剤耐性検査の質は，今までに評価を受けたことがない。我々は将来，HIV-1遺伝子型薬剤耐性検査を標準化することが重要と考え[16-19]，以下の検討を行っている。まず，各施設で使用している試薬・プライマー・塩基配列解析装置を調査し，第二段階として，HIV-1クローンを用いたコントロールサーベイを実施し，検査結果の解析を通して改良点を抽出する。第三段階として，改善された検査条件下で臨床検体を用いたコントロールサーベイを実施し，本検

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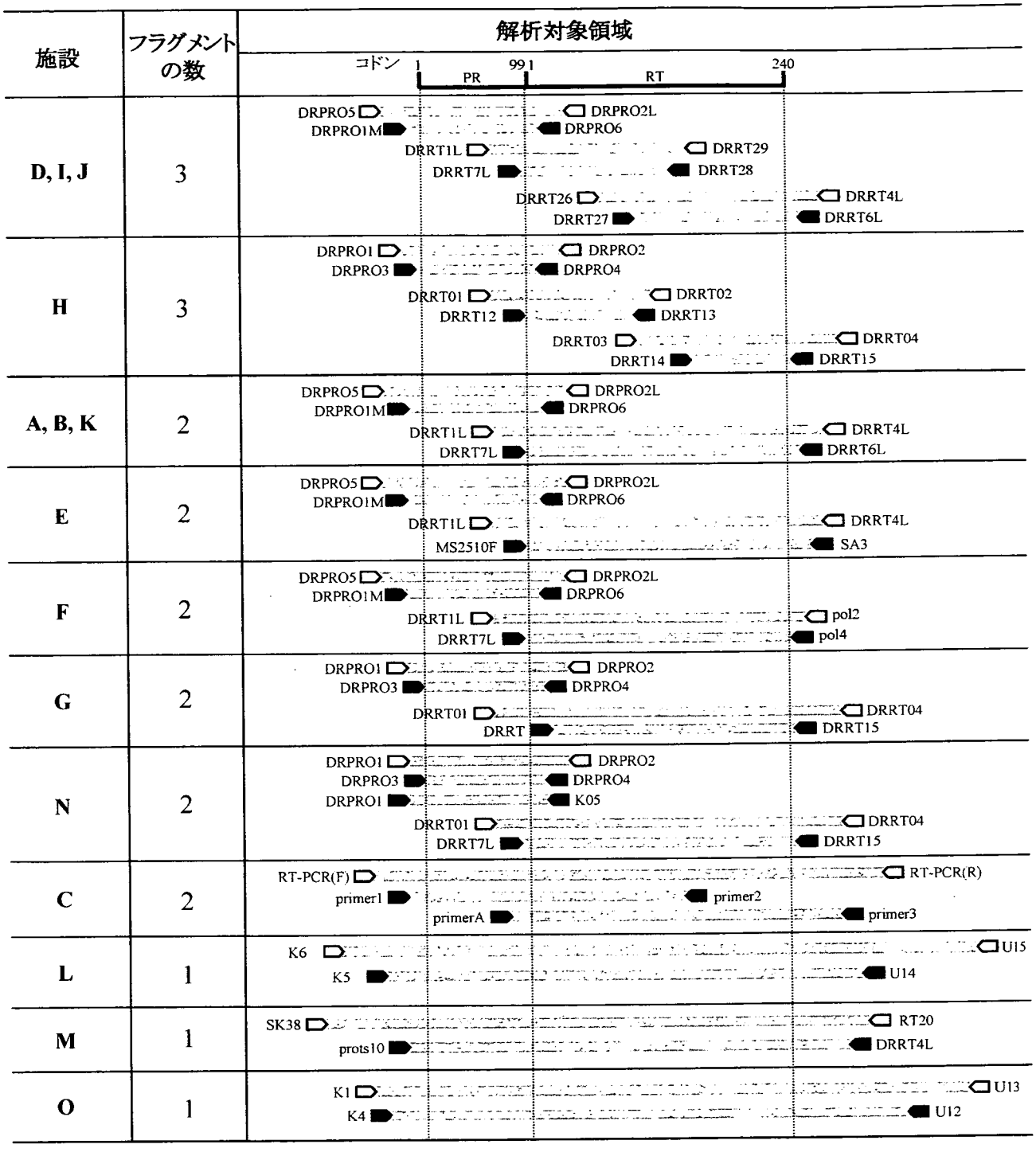



図 1 HIV-1 に対するプライマーの結合位置と，PCR による増幅領域

RT-PCR および，nested PCR に使用されたプライマーをそれぞれ，白および黒の矢印で示した。プライマー名の左に示した A から O のアルファベットは，それらのプライマーを使用した 15 施設を表している。

**表 1** 各施設で用いられた試薬および塩基配列解析装置

| RT-PCR 試薬 | 施設数 |
|---|---|
| One-step RT-PCR 法 | 12 |
| ・One Step RT-PCR (TaKaRa) | 10 |
| ・SuperScript Onestep RT-PCR for Long Template (Invitrogen) | 2 |
| Two-step RT-PCR 法 | 3 |
| ・RNA PCR Kit (AMV) ver. 3.0 (TaKaRa)/Ex Taq HS (TaKaRa) | 1 |
| ・AMV Reverse Transcriptase XL (TOYOBO)/Ampli Taq (Applied Biosystems) | 1 |
| ・M-MLV Reverse Transcriptase (Invitrogen)/Taq DNA polymerase (Greiner) | 1 |

| Nested PCR 試薬 | 施設数 |
|---|---|
| ・Ex Taq (TaKaRa) | 5 |
| ・Ampli Taq (Applied Biosystems) | 3 |
| ・LA Taq (TaKaRa) | 2 |
| ・KOD polymerase (TOYOBO) | 2 |
| ・Ex Taq HS (TaKaRa) | 1 |
| ・PCR Master Mix (Promega) | 1 |
| ・Taq DNA polymerase (Biotech International) | 1 |

| 標識試薬 | 施設数 |
|---|---|
| Big Dye Terminator V1.1 (Applied Biosystems) | 8 |
| Big Dye Terminator V3.1 (Applied Biosystems) | 5 |
| CEQ Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coulter) | 1 |
| Thermo Sequenase Cycle Sequencing Kit (USB)/IRDyeTM 800 v2 Terminator Mixes (LI-COR) | 1 |

| 標識産物の精製試薬 | 施設数 |
|---|---|
| CENTRI SEP Spin Columns (Applied Biosystems) | 5 |
| Sephadex G-50 (GE Healthcare Bio-Sciences) | 3 |
| DyeEx 2.0 Spin Kit (QIAGEN) | 1 |
| エタノール沈殿法 | 6 |

| 塩基配列解析装置 | 施設数 |
|---|---|
| ABI PRISM 310 (Applied Biosystems) | 10 |
| BECKMAN COULTER CEQ 8000 (Beckman Coulter) | 1 |
| LI-COR 4200 IR2 system (LI-COR) | 1 |
| ABI PRISM 3100 (Applied Biosystems) | 1 |
| ABI PRISM 3100 Avant (Applied Biosystems) | 1 |
| ABI PRISM 3730S (Applied Biosystems) | 1 |

査の標準化に必須の課題の整理を目指している。本論文では現在までに終了した第一段階および，第二段階の結果について報告する。

表 2 コントロールサーベイの結果

(a) クローン #1 の結果

| | アミノ酸変異 | 正解した施設数（正解率，%） | 誤答の内容 |
|---|---|---|---|
| PR | V3I | 13/15 (86.7) | 施設 B は I3V と報告。施設 H は報告無し。 |
| | E35D | 13/15 (86.7) | 施設 G と H は報告無し。 |
| | S37N | 14/15 (93.3) | 施設 H は報告無し。 |
| | R41K | 13/15 (86.7) | 施設 G と H は報告無し。 |
| | *L63P** | 15/15 (100) | |
| | K70R | 14/15 (93.3) | 施設 H は報告無し。 |
| | *A71T** | 13/15 (86.7) | 施設 A は A71V と報告。施設 G は報告無し。 |
| | *V77I** | 15/15 (100) | |
| | Ghost mutations | — | 施設 E と F は D29N および C95W をそれぞれ報告していた。これらはクローン #1 には存在していないアミノ酸変異であった。 |
| RT | *A62V** | 15/15 (100) | |
| | S68G | 14/15 (93.3) | 施設 C は報告無し。 |
| | T69V | 15/15 (100) | |
| | *V75I** | 15/15 (100) | |
| | *F77L** | 15/15 (100) | |
| | *F116Y** | 15/15 (100) | |
| | E122K | 15/15 (100) | |
| | *Q151M** | 15/15 (100) | |
| | Q197E | 14/15 (93.3) | 施設 C は報告無し。 |
| | R211K | 14/15 (93.3) | 施設 C は報告無し。 |

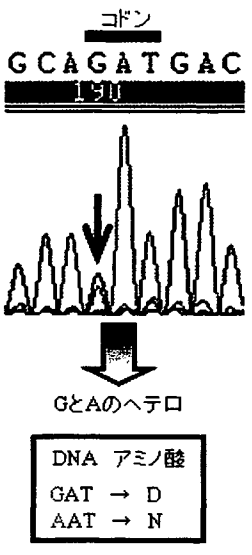
(b) クローン #2 の結果

| | アミノ酸変異 | 正解した施設数（正解率，%） | 誤答の内容 |
|---|---|---|---|
| PR | V3I | 15/15 (100) | |
| | *L10I** | 14/15 (93.3) | 施設 F は L10L/S と報告。 |
| | I15V | 15/15 (100) | |
| | S37D | 15/15 (100) | |
| | *L63P** | 15/15 (100) | |
| | *V77I** | 15/15 (100) | |
| | *L90M** | 14/15 (93.3) | 施設 D は報告無し。 |
| | I93L | 15/15 (100) | |
| | Ghost mutations | — | 施設 E は E34K を，施設 F は R41K および T96S をそれぞれ報告していた。これらはクローン #2 には存在していないアミノ酸変異であった。 |
| RT | V35T | 14/14 (100) | 施設 G は使用したプライマーの組み合わせにより V35T を含む領域が解析対象外であった。 |
| | T39A | 14/14 (100) | 施設 G は使用したプライマーの組み合わせにより T39A を含む領域が解析対象外であった。 |
| | *M41L** | 15/15 (100) | |
| | K43E | 15/15 (100) | |
| | *T69S-SG** *insertion* | 14/15 (93.3) | 施設 K は T69S-SG 挿入変異を，薬剤耐性変異ではないアミノ酸変異として報告していた。 |
| | E122K | 14/14 (100) | 施設 H は使用したプライマーの組み合わせにより E122K を含む領域が解析対象外であった。 |
| | I135T | 14/14 (100) | 施設 H は使用したプライマーの組み合わせにより I135T を含む領域が解析対象外であった。 |
| | R172K | 15/15 (100) | |
| | D177E | 15/15 (100) | |
| | *G190A** | 15/15 (100) | |
| | Q207H | 15/15 (100) | |
| | *L210W** | 15/15 (100) | |
| | R211K | 15/15 (100) | |
| | L214F | 15/15 (100) | |
| | *T215Y** | 15/15 (100) | |
| | K238S | 14/15 (93.3) | 施設 L は報告無し。 |
| | Ghost mutations | — | 施設 N は，クローン #2 には存在していないアミノ酸変異である I31T を報告していた。 |

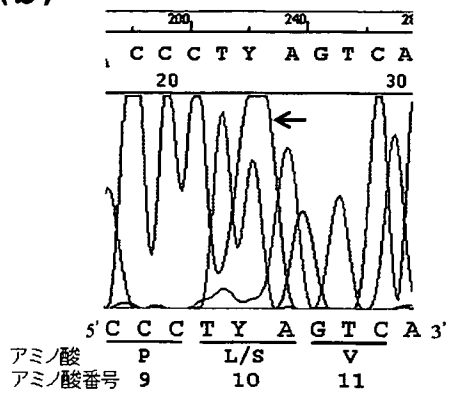
*印は薬剤耐性アミノ酸変異

(Ⅰ)

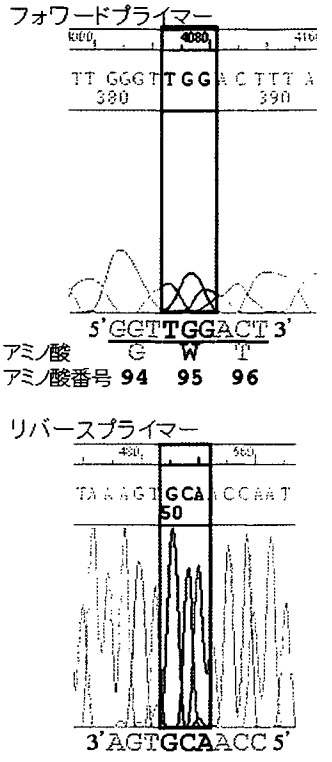
(a)


コドン
GCAGATGAC
150
GとAのヘテロ
DNA アミノ酸
GAT → D
AAT → N

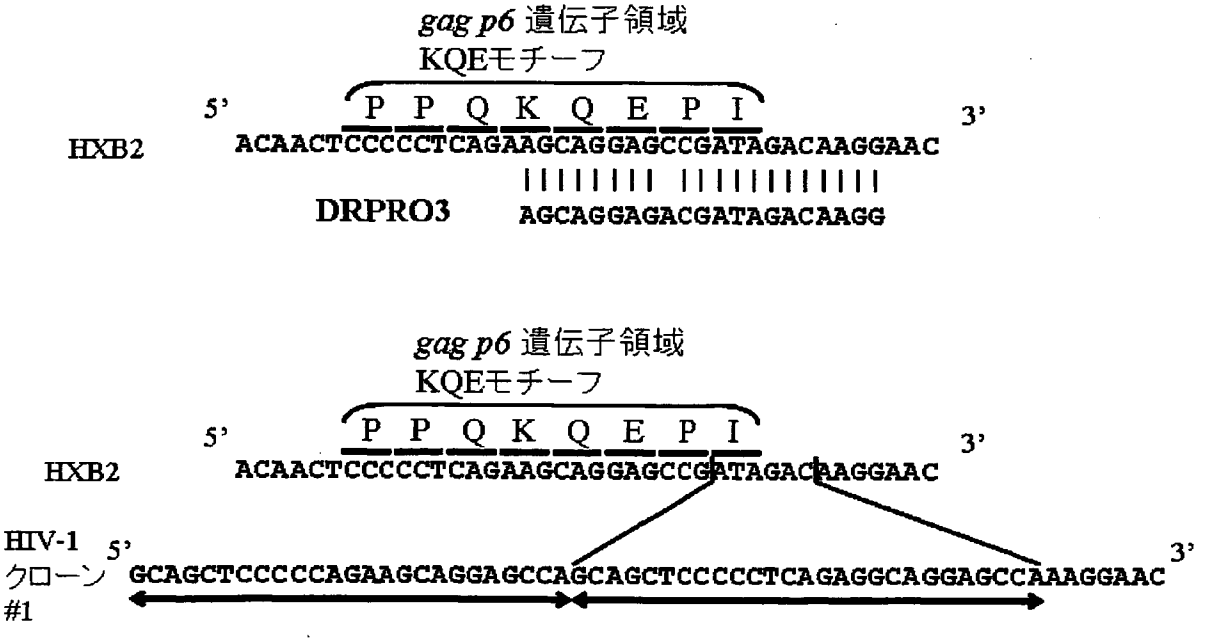
(b)


C C C T Y A G T C A
20
30
5'C C C T Y A G T C A 3'
アミノ酸 P L/S V
アミノ酸番号 9 10 11

(Ⅱ)


フォワードプライマー
5'GGTTGGACT 3'
アミノ酸 G W T
アミノ酸番号 94 95 96
リバースプライマー
3'AGTGCAACC 5'
相補鎖 5'GGTTGCACT 3'
アミノ酸 G C T
アミノ酸番号 94 95 96

(Ⅲ)


gag p6 遺伝子領域
KQEモチーフ
5'
P P Q K Q E P I
3'
HXB2
ACAACTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAAC
DRPRO3
AGCAGGAGACGATAGACAAGG
gag p6 遺伝子領域
KQEモチーフ
5'
P P Q K Q E P I
3'
HXB2
ACAACTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAAC
HIV-1
クローン
#1
5'
GCAGCTCCCCCAGAAGCAGGAGCCAGCAGCTCCCCCTCAGAGGCAGGAGCCAAAGGAAC
3'

## 材料および方法

### クローン化薬剤耐性 HIV-1 の作製と発現

各施設からの検査結果の正誤を明瞭にするために，単一な RNA ゲノムをもつクローン化薬剤耐性 HIV-1 を 2 種類作製した。まず，多剤耐性 HIV-1 を有する 2 名の患者の血漿検体から HIV-1 RNA を，QIAamp Viral RNA Kit (QIAGEN, CA, USA) を用いて精製した。次に，*gag* (アミノ酸番号 412 番から 500 番)，プロテアーゼ (PR, アミノ酸番号 1 番から 99 番)，逆転写酵素 (RT, アミノ酸番号 1 番から 260 番) の遺伝子領域を含む 1.3kb の DNA 断片を，RT-PCR にて増幅した。RT-PCR は，Superscript One-Step RT-PCR (Invitrogen, CA, USA)，K1 (5′-AAG GGC TGT TGG AAA TGT GG-3′)，U13 (5′-CCC ACT CAG GAA TCC AGG T-3′) プライマーを用いた。Nested PCR は LA Taq (Takara, Shiga, Japan)，INF-ApaI (5′-TGC TGG GCC CCT AGG AAA AAG G-3′)，INF-NheI (5′-TCT GGC TAG CCC AAT TCA ATT TTC CCA C-3′) プライマーを用いて行った。増幅した DNA は ApaI (Takara)，NheI (New England Biolabs, MA, USA) で処理し，得られた ApaI-NheI 断片を HXB2 感染性クローンである pSUM9[20] (National Cancer Institute, 熊本大学，満屋教授から供与していただいた) に組み込んだ。次いで，Lipofectamin (Invitrogen) を用いて，作製した感染性クローンを 40% コンフルエントの MT-2 細胞に遺伝子導入した。細胞は 10% 仔ウシ血清を含む RPMI1640 を用いて，5% $CO_2$ 濃度，37℃ で培養した。遺伝子導入の 4 日後，0.2 ml の培養上清を，$5\times10^4$ 個/4.8 ml の MT-4 細胞に移し，さらに 3 日間培養後，ウイルスを含む培養上清を回収し，−80℃ で保存した。

### HIV-1 RNA の調製

HIV-1 粒子を超遠心 (23,000×*g*, 1 時間，4℃) により沈降させ，PBS (−) による洗浄を 2 回行った。次に沈降物を 100 μl の PBS (−) に懸濁し，3 U のデオキシリボヌクレアーゼ (Nippon Gene, Tokyo, Japan) を添加して 37℃，15 分間 DNA 分解処理を行った。その後，23,000×*g*, 1 時間，4℃ の条件下で遠心し，沈降物を PBS (−) で洗浄した。RNA は QIAamp Viral RNA Kit を用いて精製し，$2\times10^4$ コピー数の HIV-1 RNA を 2 ml PBS (−) に懸濁して −80℃ で保存した。凍結した HIV-1 RNA 検体をドライアイスと共に梱包し，15 施設へ送付した。また，HIV-1 RNA のコピー数は Nagai ら[21] の方法を用いてリアルタイム PCR にて測定した。

### HIV-1 遺伝子型薬剤耐性検査

遺伝子型薬剤耐性検査は，国立感染症研究所，国立国際医療センター，HIV/AIDS ブロック拠点病院 8 施設，地方衛生研究所 4 施設，そして民間臨床検査会社 1 施設で実施した。本研究では，HXB2 株または NL4-3 株のアミノ酸配列と，解析したアミノ酸配列を比較し，薬剤耐性アミノ酸変異については International AIDS Society-USA (IAS-USA) panel, version March/April 2005[22] に基づいて定義した。各施設からは，薬剤耐性およびその他のアミノ酸変異を示した検査報告書，使用したプライマーの塩基配列，エレクトロフォレグラム，そして解析した塩基およびアミノ酸配列の報告を受けた。各施設から報告されたアミノ酸変異は名古屋医療センターの解析結果を基準として評価した。

**図 2** 誤判定を引き起こした 3 つの原因

(I) (a) ノイズを伴ったエレクトロフォレグラムによって誤判定が引き起こされた例

HIV-1 クローン #1 の PR 遺伝子領域にて，施設 E は本来存在していないアミノ酸 D29N を報告していた。これは，矢印で示した A (緑色) のノイズをシグナルと判定したことにより解析を誤ったためである。

(b) エレクトロフォレグラム上の巨大ノイズシグナルによって誤判定が引き起こされた例

(II) 不明瞭なエレクトロフォレグラムを解析したことによって誤判定が引き起こされた例

リバースプライマーを用いて得たエレクトロフォレグラムは鮮明であり，アミノ酸番号 95 番は TGC と正確に判断できていた。にも拘わらず，不明瞭なエレクトロフォレグラムを解析し，TGG と誤判断していた (施設 F)。

(III) 鋳型に対する結合能を欠失していた PCR プライマーを用いたことにより，誤判定が引き起こされた例

*Gag* 遺伝子の KQE モチーフ領域内に起きた重複により，DRPRO3 プライマーの 3′側結合部位が欠失していた。重複配列を矢印で示した。

## 結　　果

### 試薬，プライマー及び塩基配列解析装置の調査

今回の調査の結果，コントロールサーベイに参加した全15施設で使用されている試薬，プライマー及び塩基配列解析装置は施設によってかなり多様であった。図1にプライマーの名称および，PCRにより増幅される遺伝子領域を示した。RT-PCRおよびnested PCRに使用された試薬，標識試薬，標識産物の精製に用いられた試薬，塩基配列解析装置は表1に示した。

### アミノ酸変異検出についての評価

各施設から提出されたHIV-1アミノ酸変異検出結果を表2に示した。HIV-1クローン#1に含まれている全アミノ酸変異を正しく検出していた施設は，15施設中8施設であり，#2については15施設中7施設であった。そこで，アミノ酸変異が誤判定された事例について，使用されたプライマー，エレクトロフォレグラム，および塩基配列・アミノ酸配列のファイルを精査し，誤判定を引き起こした原因を解明した。

HIV-1クローン#1のPR領域には3つの薬剤耐性アミノ酸変異（L63P, A71T, V77I）と，その他のアミノ酸変異が5つ（V3I, E35D, S37N, R41K, K70R）存在している（表2（a））。9施設はこれら8つのアミノ酸変異を正しく報告していたが，残り6施設の報告には誤答があった。具体的には，施設AはA71TをA71Vとして，またBはV3IをI3Vとしてそれぞれ報告していた。これらの施設が提出したDNAおよびアミノ酸のファイルでは変異が正しく検出されていることから，この誤答は報告書作成時の人為的誤りと判断した。施設EとFは，本クローンには存在しないアミノ酸変異D29NとC95Wを報告していた。施設Eの誤答は，エレクトロフォレグラムに出現した高レベルのノイズシグナルが原因であった（図2（I）(a)）。施設Fは，人による編集を経ていない，自動解析による波形データの解析結果を採用し，かつ，正確に解析していたリバースプライマーを用いた解析結果を無視していた（図2（II））。施設GとHは，大きく異なるアミノ酸変異を報告した。これは，nested PCRに用いたフォワードプライマー（DRPRO-3）が不適切であったことに起因している（図2（III））。HIV-1クローン#1のgagタンパク質のアミノ酸番号470番から478番（KQEモチーフを含む）に相当する塩基配列の重複によってDRPRO3の結合部位は欠失していた。このことから，DRPRO3を用いたPCRではHIV-1クローン#1のPR領域のDNA断片を増幅することは不可能であった。

クローン#1のRT領域内には，5つの薬剤耐性アミノ酸変異（A62V, V75I, F77L, F116Y, Q151M）と，その他5つのアミノ酸変異（S68G, T69V, E122K, Q197E, R211K）が存在している（表2（a））。14施設はこれらの10アミノ酸変異を正確に報告していた。施設Cは，3つのアミノ酸変異，S68G, Q197E, R211Kについて，DNAおよびアミノ酸配列のファイルでは正しく検出していたにもかかわらず，検査の最終報告書にはこれらの変異を記載していなかった。

HIV-1クローン#2のPR領域には4つの薬剤耐性アミノ酸変異（L10I, L63P, V77I, L90M）と，その他のアミノ酸変異が4つ（V3I, I15V, S37D, I93L）存在している（表2(b)）。12施設はこれらのアミノ酸変異を正しく報告していたが，施設D, E, Fの報告には誤答が含まれていた。施設Dは，塩基配列ファイルではアミノ酸番号90番のコドン“ATG”を正確に検出していたが，翻訳後のアミノ酸ファイルではL90Mを報告していなかった。施設Eは，クローン#2に存在しないアミノ酸変異E34Kを報告していた。これはエレクトロフォレグラムに出現した高レベルのノイズシグナルによって引き起こされたものであった。施設Fは，エレクトロフォレグラムが乱れていたことにより，クローン#2に存在しないアミノ酸変異R41K, T96Sを報告していた。さらに，エレクトロフォレグラムに現れた，巨大なノイズシグナルによりアミノ酸変異L10IをL10L/Sと報告していた（図2（I）(b)）。このピークは，標識反応後の，精製過程で未反応のダイデオキシヌクレオチドの除去が不完全であったことに起因していると判断した。

クローン#2のRT領域内には，5つの薬剤耐性アミノ酸変異（M41L, T69S-SG挿入，G190A, L210W, T215Y）と，その他に11のアミノ酸変異（V35T, T39A, K43E, E122K, I135T, R172K, D177E, Q207H, R211K, L214F, K238S）が存在している（表2（b））。10施設はこれら16アミノ酸変異を正確に報告していた。一方，施設G, H, K, L, Nからの報告書に誤りがあった。まず，施設GとHの誤答についてであるが，使用したプライマーの組み合わせにより施設Gはアミノ酸番号1番から39番の領域を，施設Hはアミノ酸番号122番から135番の領域を増幅することが不可能であった。その結果，施設GはV35TとT39A，施設HはE122KとI135Tを報告していなかった。よって，施設GとHの誤答は評価対象外とした。次に，施設Kは，T69S-SG挿入変異を正しく検出していたが，薬剤耐性ではないアミノ酸変異として報告していた。施設Lは，K238Sについてアミノ酸ファイルでは正しく検出しているのにも拘わらず報告書に記載していなかった。また，施設NはクローンN#2のRT領域内に存在していないアミノ酸変異I31Tを報告しており，この誤答はクローン#1のPR領域内で施設Eが起こした誤りと同様であった。

表 3 誤答を引き起こした原因のまとめ

| 分類 | 誤答を引き起こした原因 |
|---|---|
| 技術的誤り 9（1.4%） | 不適切なプライマーの使用<br>ノイズを伴ったエレクトロフォレグラム |
| 人為的誤り 8（1.3%） | 報告書への誤記入 6（1.0%） |
| | アミノ酸変異を分類する際の誤った判断 1（0.2%） |
| | コドンの誤った翻訳 1（0.2%） |
| 全問題点 17（2.7%） | |

**誤答の分類**

全体をまとめると，17 個の誤答があった（表 3）。クローン #1 と #2 を合わせると 42 個のアミノ酸変異が存在し，また，施設 G と H については，クローン #2 の RT 領域で 4 個のアミノ酸変異を評価対象外としていることから，全 15 施設から報告されるべきアミノ酸変異の総数は 626 個である。よって，正解率は 97.3% であり，誤答率は 2.7% であった。誤答を引き起こした原因を分類すると，技術的誤りが 1.4%，人為的誤りが 1.3% であった。人為的誤りは，報告書への誤記入が 1.0%，アミノ酸変異を薬剤耐性変異もしくはその他の変異に分類する際の判断の誤りが 0.2%，コドンを翻訳する際の誤りが 0.2% であった。技術的誤りは，高いノイズを伴ったエレクトロフォレグラムに起因する不正確な解析と，不適切なプライマーの使用が原因であった。

## 考　　察

我々は今回，2 種類の HIV-1 クローンを試験標本に採用し，コントロールサーベイを実施した。その結果，全検出アミノ酸変異の正答率は 97.3%（609/626）という良好な成績を示した。しかし，改善すべき問題点も明らかになった。アミノ酸変異が誤判定された事例については，該当施設が使用したプライマー，エレクトロフォレグラム，および塩基配列・アミノ酸配列のファイルを精査することで，誤答を引き起こした原因を解明した。その結果，誤答の原因を 3 つに分類することができた。

第一の原因は，使用するプライマーの問題である。*Gag p6* 内の KQE モチーフが重複した HIV-1 に対しては結合しない，初期に使用されていたプライマー DRPRO3 が現在も 2 施設で使用されていた。この 2 施設では，クローン #1 に含まれているアミノ酸変異とは大きく異なるアミノ酸変異を報告していた。第二の原因は，ノイズを伴ったエレクトロフォレグラムを判読したことに起因している。これには 3 施設が該当した。第三の原因は，人為的誤りである。報告書作成時の誤記入とコドンの誤った翻訳が 4 施設でみられた。

これら 3 つの原因に対する解決策を以下に示す。

第一の原因は，初期のプライマーから改良したプライマーの情報について，施設間の情報伝達が徹底されていなかったことに起因している。DRPRO3 は 1996 年に国立感染症研究所が開発したプライマーであるが，KQE モチーフに挿入変異の存在が報告[23-25]されてからは使用が中止され，新しいプライマーである DRPRO3N の使用に変更されている。したがって，DRPRO3N をプライマーとして用いればこの問題は即解決できる。今後，最新のプライマー情報と塩基配列決定領域内の新規検出変異や挿入，欠失に関する HIV-1 塩基配列情報を少なくとも年に一度は全関係施設に配布する等の，検査水準をバックアップする体制を充実することによりプライマー問題は根絶できると思われる。また，注目すべきことに，施設 C, L, M, O で独自に開発されたプライマーも，国立感染症研究所で開発された最新のプライマーと同様に機能していた。

第二の原因は，明瞭なエレクトロフォレグラムを得ることで克服可能である。そのためには，精製度の高いプライマー，すなわち，HPLC 精製グレードのプライマーの使用が必須である。また，当然のことであるが，新鮮なバッファー，ゲルを用いて塩基配列解析を実施することが重要である。さらに，フォワードプライマーを用いて解析したエレクトロフォレグラムの質が悪く，解析が不正確になる場合には，リバースプライマーも用いて解析を行うことで，塩基配列決定の正確性を高めることができる。

第三の原因克服は，解析を自動化することによって可能である。例えば，我々が使用している DNA 配列解析ソフトウェア SeqScape（Applied Biosystems）では，エレクトロフォレグラムから DNA 配列およびアミノ酸配列を作成し，変異アミノ酸を検出する過程の自動化が可能である。人為的誤りを無くすソフトウェアの普及と充実がこの問題克服に必要である。

なお，異なる試薬，および塩基配列解析装置の機種の違いが誤りの原因と疑われた事例はなかった。

このように今回，HIV-1 遺伝子型薬剤耐性検査で起こり得る誤りの原因を追究し，それに対する解決法を提案した。各施設に送付した今回のコントロールサーベイの評価に基づいて種々の改善を図ることにより，HIV-1 遺伝子型薬剤耐性検査の精度向上が期待される。次回は，第三段階として臨床検体を用いたコントロールサーベイの実施を計画している。

**謝辞**：本研究は，厚生労働省エイズ対策事業「薬剤耐性HIV発生動向把握のための検査方法・調査体制確立に関する研究」班（No. H-16-エイズ-002，杉浦班）の班研究として推進した研究である。藤崎誠一郎，藤崎彩恵子はエイズ予防財団のリサーチレジデントとして本研究に参画した。

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# Control Survey of HIV-1 Genotypic Drug-resistance Testing in Japan

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***Objective*** : Most laboratories in Japan are performing the HIV-1 genotypic drug-resistance testing with their in-house methods based on the one developed by National Institute of Infectious Diseases. However, the quality of their methods has never been assessed. In this study, we aimed to investigate the accuracy and reliability of the testing performed in 15 laboratories in Japan.

***Materials and Methods*** : We assessed the accuracy and reliability of the in-house testing by sending two standard HIV-1 RNA samples to 15 voluntarily participating laboratories and by analyzing the reported results.

***Results*** : The assessment revealed that the quality of HIV-1 genotypic drug-resistance testing was very high (97.3% accuracy). But there were sources of error, including human errors, poor electrophoregrams, and use of inadequate primers.

***Conclusion*** : We proposed troubleshooting procedures to improve the quality of drug-resistance testing in Japan.

**Key words** : AIDS, HIV-1, genotypic drug-resistance testing, control survey

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# No Observable Correlation between Central Nervous System Side Effects and EFV Plasma Concentrations in Japanese HIV Type 1-Infected Patients Treated with EFV Containing HAART

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## ABSTRACT

**The present study assessed the relationship between central nervous system (CNS) side effects and plasma concentrations of efavirenz (EFV) in Japanese HIV-1-infected patients. Subjects consisted of 69 HIV-1-infected patients (57 therapy-naive and 12 therapy-experienced patients) being treated using EFV in combination with other antiretroviral agents at the outpatient HIV clinic. Successful virological treatment was achieved in 61 patients. Eight patients discontinued EFV containing therapy because CNS symptoms did not resolve (four patients), EFV-specific mutations were detected (two patients), or skin rash was observed (two patients). Mean EFV plasma concentration for 61 effectively treated patients, measured at 15 h postdosing, was 2.42 μg/ml (range: 0.78–6.82 μg/ml). This EFV concentration range contributed to suppressed viral load in these Japanese patients. Adverse CNS effects were observed in 19 patients soon after therapy onset. These effects disappeared within 1 month except for four patients who suffered severe CNS side effects. Mean EFV plasma concentrations were not significantly different between subjects with (2.45 ± 1.08 μg/ml) and without (2.42 ± 1.40 μg/ml) CNS side effects. We concluded no correlation existed between the plasma EFV concentration and the emergence of CNS side effects in Japanese HIV-1-infected patients. Further investigations, enforced with the drug concentration measurement at earlier time points and more appropriate assessment of CNS symptoms, are required.**

## INTRODUCTION

EFAVIRENZ (EFV) IS ONE OF THE NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTI) used to treat human immunodeficiency virus (HIV)-1-infected patients. This drug can be administered once daily (600 mg dose) in combination with a protease inhibitor and/or nucleoside reverse transcriptase inhibitors (NRTI), due to its long half-life, 40–55 h.[1–4] The advantage of once-daily dosing is a reduction in the patient's overall drug-taking burden. Thus, EFV use is expected to increase for many patients. Treatment success using EFV involves maintaining an effective drug concentration while avoiding both adverse effects and the occurrence of drug-resistant amino acid mutations such as K103N, Y188L, and G190S.[5] In fact, EFV plasma concentrations <1.0 μg/ml are reportedly predictive of treatment failure with the development of genotypic resistance in European populations.[6] Conversely, EFV induces effects on the central nervous system (CNS) such as dizziness, hallucinations (including frequent nightmares), abnormal dreams, and insomnia.[2,7,8] Marzolini *et al.*[9] reported central nervous system (CNS) toxicity was 3-fold more frequent in patients with EFV plasma concentrations >4.0 μg/ml than in patients with concentrations of 1.0–4.0 μg/ml. Therefore, keeping the drug concentration trough level within 1.0–4.0 μg/ml would likely lead to treatment success in the Japanese population. Measuring plasma concentrations of EFV is desirable in order to monitor

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the occurrence of adverse effects and adherence in EFV containing highly active antiretroviral therapy (HAART). However, when the focus is on outpatients, measuring exact EFV trough levels is difficult since patients take this drug in the evening. We, therefore, measured concentrations 15 h after dosing, instead of at the trough levels. This study assessed the relationship between CNS side effects and plasma concentrations of EFV in Japanese HIV-1 infected patients.

## MATERIALS AND METHODS

### *Patients*

Subjects consisted of 69 HIV-1 infected patients (57 therapy-naive patients and 12 therapy-experienced patients) recruited at the Outpatient HIV Clinic of the National Hospital Organization Nagoya Medical Center, Japan, between May 2001 and May 2006. There were 60 men and 9 women, with a mean age of 40 years (range: 24–74 years). The characteristics of these recruited patients are displayed in Table 1. Subjects were treated using EFV (600 mg/day) in combination with other antiretroviral agents. Mean therapy duration was 19 months (range: 1–59 months). Protease inhibitors were administered to five patients (lopinavir/ritonavir: four patients and atazanavir/ritonavir: one patient). Upon initiation of EFV-containing therapy, the mean CD4 cell count was 349 cells/mm$^3$ and the mean viral load was 559,009 copies/ml. Cases with CD4 cell counts <200 cells/mm$^3$ at initiation of therapy were defined as acquired immunodeficiency syndrome (AIDS), according to the guidelines.[10] Using this definition, 44 patients were AIDS cases. This study was approved by the institutional review board of the National Hospital Organization Nagoya Medical Center, and each subject provided written informed consent.

TABLE 1. CHARACTERISTICS OF 69 RECRUITED PATIENTS[a]

| *Characteristics* | n = *69* |
|---|---|
| Age (years) (mean ± SD) | 40 ± 11 |
| Sex | |
| Male (*n*) | 60 (87%) |
| Female (*n*) | 9 (13%) |
| Weight (kg) (mean ± SD) | 57 ± 12 |
| Therapy-naive (*n*) | 57 (83%) |
| Therapy-experienced (*n*) | 12 (17%) |
| Duration of EFV containing therapy (months) (mean ± SD) | 19 ± 16 |
| CD4 counts (cells/mm$^3$) (mean ± SD) | 349 ± 226 |
| HIV-RNA (copies/ml) (mean ± SD) | 559,009 ± 2,707,415 |
| AIDS diagnosis (*n*) | 44 (64%) |
| PI coadministration (*n*) | 5 (7%) |
| Viral failure: >400 copies/ml (*n*) | 2 (3%) |
| CNS side effects (*n*) | 19 (28%) |
| Discontinuation[b] (*n*) | 3 (4%) |
| Drop out[c] (*n*) | 3 (4%) |

[a]SD, standard deviation; EFV, efavirenz; AIDS, acquired immunodeficiency syndrome; PI, protease inhibitor; CNS, central nervous system.

[b]Patients refused EFV-containing therapy because CNS symptoms did not resolve after the first 1–2 months of therapy.

[c]The discontinuation was due to skin rash in two patients and hallucinations in one patient.

### *EFV plasma level and genotypic drug resistance testing*

Sampling for the determination of EFV plasma concentrations was performed in the afternoon at the outpatient HIV clinic. As EFV was administered in the evening for all patients, EFV plasma levels were measured at approximately 15 h post-dosing. We assumed EFV trough levels are similar to 15 h post-dosing plasma levels as EFV has a long half-life.[1,2] EFV plasma concentrations were determined by high-performance liquid chromatography according to our previously described methods.[11] We measured EFV plasma concentrations 10 times on average (range: 3–37 times) for therapy-continued patients and 3 times on average (range: 1–6 times) for therapy-discontinued patients. The value of EFV plasma concentration was shown as mean ± standard deviation (SD). Genotypic drug resistance testing of HIV-1 was performed according to our previously reported methods.[12,13]

### *CNS adverse effects*

CNS symptoms were assessed using a psychiatric interview and the Symptom Check List-90-Revised (SCL-90-R) questionnaire, a widely used scale in psychopathology.[14] Upon initiation of EFV containing therapy, we thoroughly explained CNS adverse effects of EFV to patients. When the patients consulted a physician on week 4 after initiating therapy, we had an interview with the patients according to the items of the SCL-90-R questionnaire, and evaluated psychological problems and psychopathological symptoms. Afterward, CNS symptom assessment was carried out every month. The SCL-90-R test contains only 90 items and can be completed in just 10–15 min. All the patients answered the 90 items of SCL-90-R completely.

## RESULTS

### *EFV plasma concentrations during therapy*

EFV concentrations were measured once a month or at least once every 3 months. The distribution of mean values is shown in Fig. 1. Mean EFV plasma concentration varied from patient to patient (range: 0.78–6.82 μg/ml) (57 therapy-naive patients: #1–57 and 12 therapy-experienced patients: #58–69). In this study 61 patients continued EFV-containing therapy and approached successful virological suppression to the detection limit of 50 copies/ml. Unfortunately, three patients had stopped EFV-containing therapy within 2 weeks after therapy was initiated. Discontinuation was due to hallucinations in one patient (#1) and skin rash in two patients (#2 and 3). Three patients discontinued EFV-containing therapy because CNS symptoms did not resolve after the first 1–2 months of therapy (#6, 39, and 47). Two patients with virological failure (>400 copies/ml) changed HAART combination because EFV-specific mutations were detected (#64: G190A and #67: L100I, G190A). Mean EFV plasma concentration for the 61 effectively treated pa-

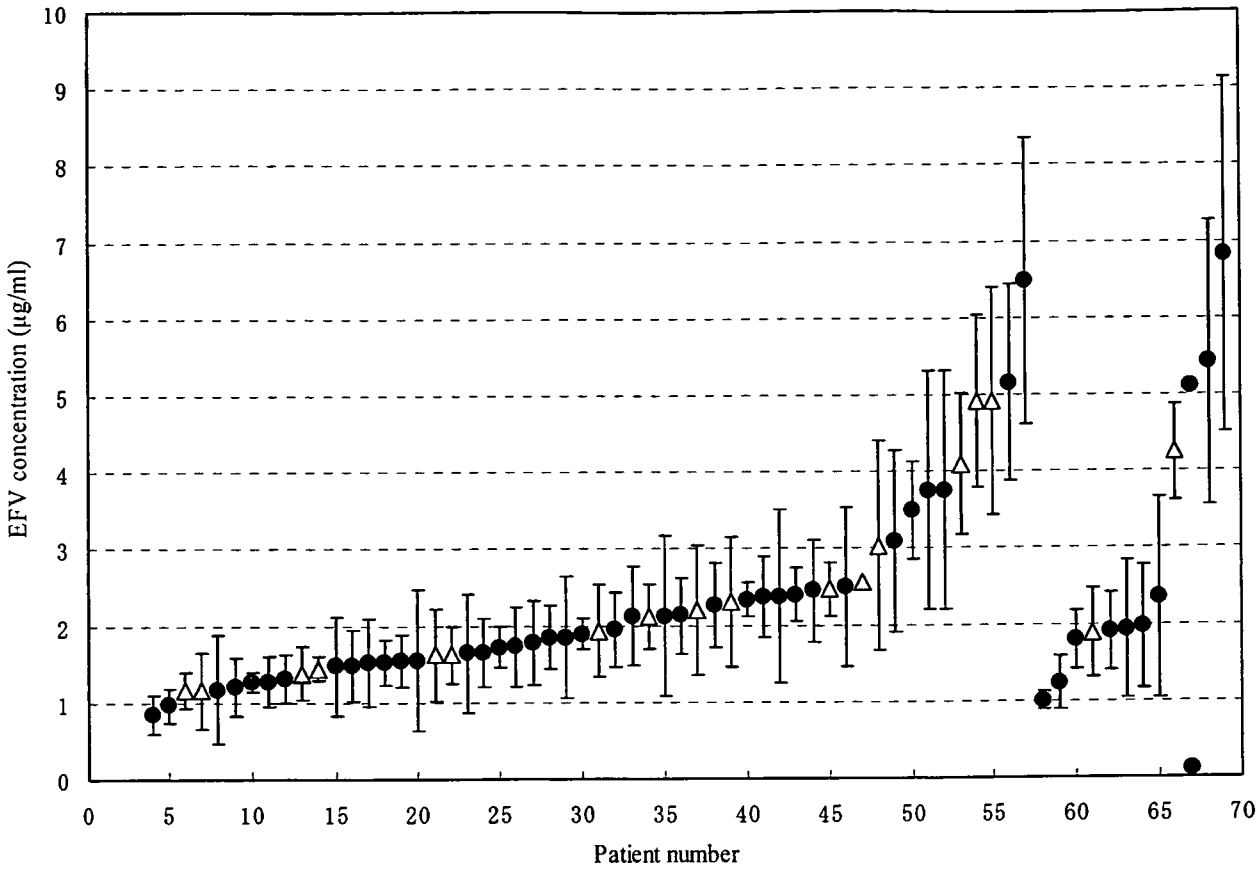


**FIG. 1.** Distribution of EFV plasma concentrations in 69 recruited patients. Patients #1–57 were therapy naive and #58–69 were therapy experienced. Closed circles, patients without CNS side effect; open triangles, patients with CNS side effect. Patients #6, 39, and 47 discontinued EFV-containing therapy because CNS symptoms did not resolve. Patients #64 and 67 changed HAART combination because EFV-specific mutations were detected. The EFV plasma concentration of patient #47 was measured only once. Measurement of EFV plasma concentration was performed twice on patient #67, as the EFV concentration was 5.1 μg/ml and undetectable (<0.1 μg/ml). The measurement could not be done in patients #1, 2, and 3 because of early drop out from this study.

tients, measured at 15 h postdosing, was 2.42 μg/ml (2.32 ± 1.14 μg/ml for 51 therapy-naive patients and 2.93 ± 1.94 μg/ml for 10 therapy-experienced patients).

### *CNS side effects*

Adverse CNS effects were observed in 19 patients soon after the onset of therapy. CNS adverse events are shown in Table 2. These adverse events were assessed by a psychiatric interview according to the items of the SCL-90-R questionnaire 4 weeks after initiating therapy. The major adverse events were abnormal dreams ($n$ = 9), dizziness ($n$ = 4), and nervousness ($n$ = 3). These effects disappeared within 1 month except for four patients with severe CNS side effects. Mean EFV plasma concentrations were not significantly different between subjects with (2.45 ± 1.08 μg/ml) and without (2.42 ± 1.40 μg/ml)

TABLE 2. CENTRAL NERVOUS SYSTEM ADVERSE EVENTS ($N$ = 69)

| *Adverse events*[a] | n *(%)* | *Patient no.* |
|---|---|---|
| Abnormal dreams | 9 (13.0) | 7, 13, 21, 22, 31, 45, 54, 55, 61 |
| Dizziness | 4 (5.8) | 6, 47, 48, 66 |
| Nervousness | 3 (4.3) | 14, 39, 53 |
| Difficulty in sleeping | 1 (1.4) | 34 |
| Loss of libido | 1 (1.4) | 37 |
| Hallucinations | 1 (1.4) | 1 |

[a]These adverse events were assessed by a psychiatric interview according to the items of the SCL-90-R questionnaire 4 weeks after initiating therapy.

TABLE 3. COMPARISON WITH PREVIOUS REPORTS IN TERMS OF CORRELATION BETWEEN EFFICACY OR SIDE EFFECT OCCURRENCE AND EFV PLASMA CONCENTRATION[a]

| | *Langmann* et al.[6] | *Marzolini* et al.[9] | *Gutierrez* et al.[15] | *Fumaz* et al.[16] | *Ours* |
|---|---|---|---|---|---|
| Patients (*n*) | 33 | 130 | 17 | 60 | 69 |
| Age (year) [mean ± SD (range)] | NA | (23–74) | 40 | 41 ± 8 | 40 ± 11 (24–74) |
| Male (*n*) | NA | 93 (72%) | 14 (82%) | 45 (75%) | 60 (87%) |
| Female (*n*) | NA | 37 (28%) | 3 (18%) | 15 (25%) | 9 (13%) |
| Duration of therapy (months) [mean ± SD (range)] | 12 (3–40) | NA | 18 | 23 ± 10 | 19 ± 16 (1–59) |
| Treatment success (*n*) | 27 (82%) | 99 (76%) | 13 (76%) | 44 (73%) | 61 (88%) |
| With CNS side effect (*n*) | NA | 13 (10%) | 10 (59%) | 32 (53%) | 19 (28%) |
| Mean EFV plasma level (μg/ml) [mean ± SD (range)] | 3.12 ± 2.50 | 2.19 (median) | 4.12 ± 2.51 | NA | 2.43 ± 1.31 |
| With CNS side effect (μg/ml) [mean ± SD (range)] | NA | NA | 5.10 ± 2.15 | 2.5 ± 1.1 | 2.45 ± 1.08 |
| Without CNS side effect (μg/ml) [mean ± SD (range)] | NA | NA | 2.79 ± 1.31 | 2.7 ± 0.7 | 2.42 ± 1.40 |

[a]NA, not available; SD, standard deviation; EFV, efavirenz; CNS, central nervous system.

CNS side effects ($p = 0.503$). Also, there was no association between CNS side effects and EFV plasma concentrations greater than 4.0 μg/ml.

### *Comparison with previously reported correlations between efficacy or side effect occurrence and EFV plasma concentration*

We compared our results with previous reports (Table 3). Langmann *et al.*[6] and Marzolini *et al.*[9] reported that treatment failure and CNS side effects were associated with low (<1.0 μg/ml) and high (>4.0 μg/ml) EFV plasma levels, respectively. Also Gutierrez *et al.*[15] reported that patients achieving higher EFV plasma levels were at increased risk of experiencing CNS adverse events. On the other hand, Fumaz *et al.*[16] reported EFV plasma levels were similar in subjects with and without CNS side effects.

In our study, 61 patients with treatment success had an EFV plasma concentration greater than 0.78 μg/ml, similar to previous European findings.[6] CNS side effects were observed in 19 of 69 patients and the rate of side effect occurrence (28%) was low in comparison with previous trials.[2,15,16] Mean EFV plasma levels were similar in subjects with (2.45 ± 1.08 μg/ml) and without (2.42 ± 1.40 μg/ml) CNS side effects. In terms of correlation between side effect occurrence and EFV plasma concentration, our results concurred with Fumaz *et al.*[16]

## DISCUSSION

EFV is an NNRTI drug used in the treatment of HIV-1-infected patients and can be administered once daily due to its long half-life. Therefore, there has been a recent increase in EFV-containing HAART regimens. However, as EFV is associated with CNS adverse effects such as dizziness, hallucinations (including frequent nightmares), abnormal dreams, and insomnia, EFV should be used carefully. This study assessed the relationship between CNS side effects and plasma EFV concentrations in Japanese HIV-1-infected patients by measuring its concentration at 15 h postdosing. Successful virological suppression was achieved in 61 of the 69 subjects, with viral load suppressed below detectable limits. Mean EFV levels showed extensive variation from 0.78 μg/ml to 6.82 μg/ml. This variation may be explained, in part, by the large interindividual variations in cytochrome P450 2B6 activity, as EFV is a substrate of this enzyme.[17–19] Actually, as has been reported, homozygous CYP2B6*6 correlates with high plasma EFV concentrations in HIV-1-infected patients.[20,21] Thus, we should study CYP2B6 genotyping in the future.

In the present study, adverse CNS effects, such as nervousness, abnormal dreams, and insomnia, were observed in 19 patients using EFV. In previous controlled trials, CNS symptoms were observed in 53% of patients receiving EFV and in 25% of patients receiving control regimens.[2] These symptoms usually appear from day 1 or 2 of therapy, resolving after the first 2–4 weeks, and are reportedly associated with high EFV plasma concentrations (>4.0 μg/ml).[9] However, the present study could not detect any such clear associations.

Finally, we conclude no correlation existed between the plasma EFV concentration and the emergence of CNS side effects in Japanese HIV-1-infected patients as reported by Fumaz *et al.*[16] in a European population. Recently, however, Clifford *et al.*[22] performed a large prospective trial on patients initiating EFV, concluding adverse CNS effect occurrence correlated to EFV plasma concentrations only in the first week after therapy was initiated and not later on. As we assessed CNS symptoms 4 weeks after initiating EFV, we cannot exclude the possibility that there is no correlation between plasma EFV concentration and CNS side effects. To clarify the clinical significance of EFV concentration determination in monitoring of CNS side effects, further investigations enforced with EFV concentration measurements at earlier time points and more appropriate assessment of CNS symptoms on larger subject population are required.

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