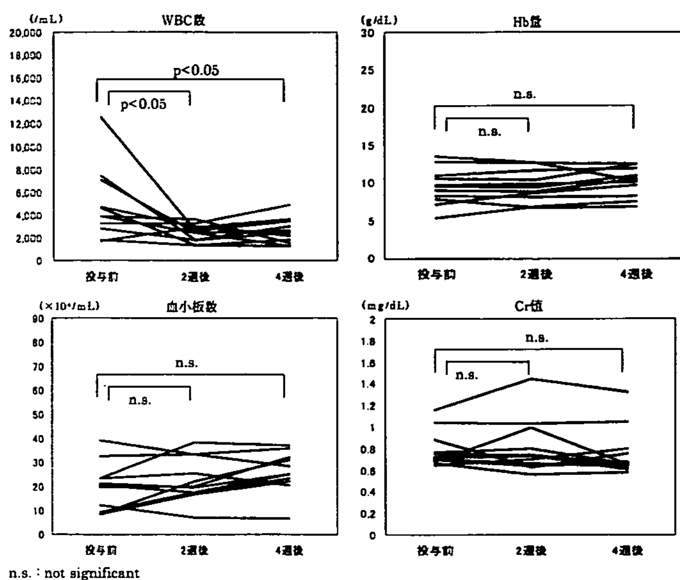

図1　バルガンシクロビル投与前後の検査値の比較

CMV網膜炎の治療剤の第一選択薬として推奨されている[4]。しかし，海外での臨床試験において下痢，悪心，嘔吐，白血球減少，貧血，骨髄抑制，腎不全等の有害事象が報告[5]されていることから，日本人エイズ患者に対して使用する場合にも十分に注意が必要である。

今回，当センターでVGCを投与された日本人エイズ患者11例を対象にVGCの有効性について検討を行った。対象患者の維持療法移行後のCMV抗原の陰性化率は100%（11例/11例）であった。このことから，日本人エイズ患者のCMV感染症に対してもVGCの効果が認められた。VGCは，ガンシクロビルに比べて投与量，投与回数を少なくできるうえ，注射剤と同等の効果が得られることから患者のquality of life（QOL）を向上させる点で有用性は高い。

次に，安全性に関する評価であるが，有害事象で投与中止となった症例が11例中5例と約半数にみられ，維持療法を継続するうえでの問題点が明らかとなった。中止理由は，WBC減少が3例，Hb減少が1例，汎血球減少が1例で，腎機能の悪化や下痢，嘔吐などはみられなかった。また，VGC投与前後の検査値でもWBC数のみが投与2，4週後で有意に減少したことから，VGCの投与に際してはWBC減少に特に注意する必要があると考える。

また，エイズ患者では数種類の抗HIV薬と併用投与されることが多いため，VGCの副作用増強が認められているジドブジン，ジダノシン，ザルシタビンとの併用投与は避けるべきである。

エイズ患者のCMV網膜炎に対するVGC維持療法の期間は，CD4細胞数の回復後6ヵ月間継続して行う必要があるといわれる[6,7]。実際，投与中止となった5例中2例で中止後7日目および22日目にCMV抗原の再燃が認められ，日本人エイズ患者においてもVGC維持療法を継続して行う必要性が示唆された。また，VGC継続群のなかの1例でCMV抗原の再燃が認められたことから，維持療法におけるVGCの投与量が少ない可能性も示唆された。しかし，すべての再燃例においてVGCの再投与あるいは増量によりCMV抗原は陰性化したことから，臨床検査値の悪化等の有害事象が発生した場合には，一時的にVGCの投与中止を検討することも必要であると考える。

日本人エイズ患者のCMV感染症に対してVGCを使用する場合には，投与患者の臨床検査値，特にWBC減少に注意を払い，患者個々の状態に応じて投与を行うことが重要である。

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Determination of Plasma Tenofovir Concentrations Using a Conventional LC-MS Method

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The quantification of tenofovir, a nucleoside reverse transcriptase inhibitor prescribed once daily, in human plasma is important due to a recent increase in its use. HPLC, however, can not easily detect and quantify tenofovir because of interfering peaks. Therefore, we developed a rapid and conventional LC-MS method, validated by estimating the precision and accuracy for inter- and intraday analysis in the concentration range of 0.019—1.567 μg/ml. The calibration curve was linear in the described concentration range. Average accuracy ranged from 95.9 to 100.7%. Relative standard deviations of both inter- and intraday assays were less than 11.6%. Recovery of tenofovir was more than 80.2%. This novel method provides a conventional, accurate and precise way to determine tenofovir in human plasma samples.

Key words human immunodeficiency virus (HIV)-1; LC-MS; therapeutic drug monitoring; tenofovir

Tenofovir is one of the nucleoside reverse transcriptase inhibitors used to treat human immunodeficiency virus (HIV)-1. Tenofovir can be administered once-daily at a dose of 300 mg in combination with other antiretroviral drugs.[1,2] The advantage of once-daily dosing is a reduction in patient drug load. Also, tenofovir appeared to be well tolerated with low rates of drug-related therapy discontinuation in several clinical trials.[3,4] Thus, tenofovir use is expected to increase. However, some drug interactions with tenofovir and coadministered drugs have been reported.[4—7] Furthermore, tenofovir has been associated with renal impairment.[8—10]

Recently, several determination methods for plasma tenofovir using high-performance liquid chromatography (HPLC)[11—13] or liquid chromatography-tandem mass spectrometry (LC-MS-MS),[14,15] have been reported. HPLC methods, however, are procedurally complicated, such as solid-phase drug extraction and derivatization with chloroacetaldehyde. Also, when using the LC-MS-MS method the MS-MS detector needs to be delicately set. In addition, LC-MS-MS equipment is very expensive. We aimed, therefore, to develop a conventional procedure for determining tenofovir using liquid chromatography coupled with mass spectrometry (LC-MS).

MATERIALS AND METHODS

Chemicals and Reagents Tenofovir was supplied by Gilead Sciences (Foster City, CA, U.S.A.). The internal standard, atenolol, was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acetonitrile (Kanto Chemical, Tokyo, Japan) was HPLC grade. Water was deionized and osmosed using a Milli-Q® system (Millipore Corp., Bedford, MA, U.S.A.). All other chemicals and solvents were of analytical grade.

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 C_{18} column (3.5 μm, 2.1×50 mm, Waters), protected by a SunFire C_{18} Guard Column.

Chromatographic and Mass Spectrometric Conditions The mobile phase was a mixture of 0.3% trifluoroacetic acid (A), 100% acetonitrile (B) and 100 mM ammonium acetate (C). An isocratic mobile phase consisting of A–B–C (95 : 0 : 5) was used during the first 3 min of the run, followed by a linear gradient elution consisting of A–B–C (45 : 50 : 5) for the next 5 min. Then, the system was reequilibrated for an additional 12 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 μl.

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 tenofovir and atenolol. 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 100—700 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 tenofovir at m/z 288, and the internal standard (IS), atenolol, at m/z 267, all in the form of ions. The quantitation calculations were performed using analytical software, MassLynx version 4.0 (Waters).

Standard Solutions Stock solutions of tenofovir and atenolol were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 188.0 μg/ml for tenofovir, and 48.1 μg/ml for atenolol. 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 tenofovir concentrations of 0.019, 0.094, 0.313, 0.940 and 1.567 μg/ml.

Sample Preparation Twenty microliters of IS solution (atenolol 48.1 μg/ml) and 200 μl of 1 N HCl were added to a 500 μl plasma sample prepared from peripheral blood antico-

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agulated with heparin. After vortexing briefly, 2 ml of 100% acetnitrile was added to the mixture. The mixture was vortexed again and then centrifuged at 3500 *g* for 5 min. The upper layer was separated and evaporated dry. The dried material was then dissolved in 100 μl of a mobile phase solution and centrifuged at 13000 *g* for 5 min. Lastly, 5 μl 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 tenofovir 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 tenofovir 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 three replicates.

RESULTS

LC-MS Chromatograms Figures 1A and B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 0.94 μg/ml of tenofovir and 1.85 μg/ml of atenolol (IS). Under the described chromatographic conditions, retention times were 4.4 min for tenofovir and 9.9 min for atenolol. Figures 1C 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 1D is the expanded figure of a circled part of Fig. 1B. These peaks did not affect on the quantification of IS. Figures 1E and F show chromatogram of a plasma sample from an HIV-1-infected patient treated with tenofovir. There were no interfering peaks affecting quantification of tenofovir in this chromatogram. Anticoagulants of heparin, EDTA and ACD did not hinder the selected-ion recording chromatograms for tenofovir and atenolol.

Validation: Linearity, Precision, Accuracy and Recovery Calibration curves of tenofovir appeared linear in the concentration range of 0.019 to 1.567 μg/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 tenofovir covers the expected plasma concentrations found in the patients. The RSDs calculated for tenofovir in the inter- and intraday assays ranged from 1.5 to 11.3%, which are similar to or much lower than previously reported values.[11—16] Accuracies ranged from 95.9 to 102.7%. Recoveries from plasma

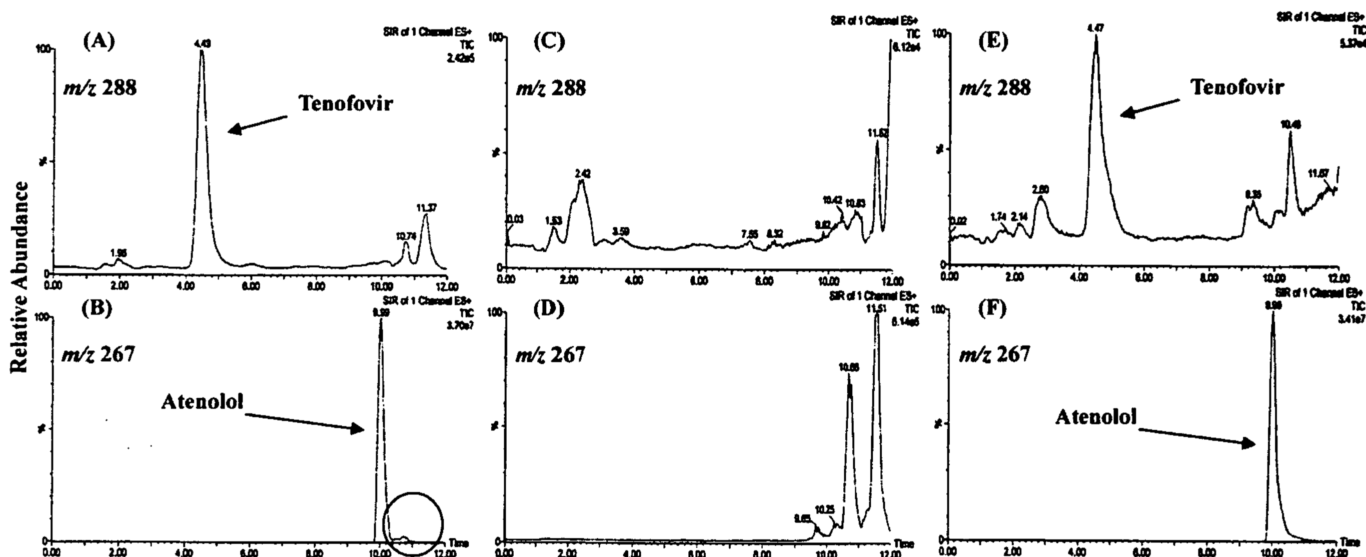

Fig. 1. Selected-Ion Recording Chromatograms for Tenofovir and Atenolol

(A) and (B) were obtained from a spiked plasma containing 0.94 μg/ml of tenofovir and 1.85 μg/ml of atenolol (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 with tenofovir. (A), (C) and (E) were monitored with *m/z* 288. (B), (D) and (F) were monitored with *m/z* 267. (D) is the expanded figure of a circled part of (B).

Table 1. Intraday and Interday Precision and Accuracy for Tenofovir

Expected (μg/ml)	Intraday (n=5)		Interday (n=3)		Accuracy (%)	Recovery (%)
	Measured (μg/ml)	RSD (%)	Measured (μg/ml)	RSD (%)		
0.019	0.019±0.002	10.0	0.020±0.002	11.3	102.7±11.6	80.2±9.4
0.094	0.095±0.007	7.4	0.092±0.005	5.7	98.2±5.7	95.2±10.4
0.313	0.313±0.012	4.0	0.300±0.016	5.2	95.9±5.0	92.2±10.4
0.940	0.921±0.039	4.3	0.931±0.054	5.8	99.1±5.7	86.1±7.4
1.567	1.570±0.024	1.5	1.578±0.045	2.8	100.7±2.9	91.1±6.2

RSD, relative standard deviation.

Table 2. Plasma Tenofovir Concentrations after the Oral Administration of 300 mg of Tenofovir to an HIV-1-Infected Patient

Time (h)	Tenofovir concentration (μg/ml)
0	0.073
4	0.316
6	0.162
12	0.106
23	0.052

ranged from 80.2 to 95.2%. These results indicate this method achieves a high degree of reproducibility and accuracy.

Tenofovir Concentrations in Plasma Plasma tenofovir concentrations in an HIV-1-infected patient are shown in Table 2. The patient received oral administration of 300 mg tenofovir daily. These concentrations were similar to those reported previously.[11,12,17)]

DISCUSSION

Prior to this study we tried to determine tenofovir plasma concentrations using a previously described HPLC method.[12)] However, we could not identify and quantify tenofovir peaks on chromatograms of plasma sample due to interfering peaks. Thus, we developed a more sensitive method. Until now there have been some methodological reports for the determination of tenofovir using LC-MS-MS.[14,15)] However, these methods have several disadvantages in terms of cost performance, time consumption and essential equipment; for example, the authors used expensive disposable cartridges at the solid-phase drug extraction and/or the setting of the LC-MS-MS equipment.

To avoid such disadvantages we decided to use a LC-MS method. Validation showed our method was successful in measuring plasma tenofovir with high precision and satisfactory RSD values. The tenofovir calibration curve was linear in the concentration range of 0.019 to 1.567 μg/ml and the average accuracy ranged from 95.9 to 100.7%. Both inter- and intraday RSDs for tenofovir were less than 11.6%, which is similar to or much lower than previously reported values.[11—16)] Recovery of tenofovir was more than 80.2%. These results indicate our newly developed method achieves a high degree of reproducibility and accuracy. As plasma concentrations of tenofovir are expected in the 0.05 to 0.40 μg/ml range when tenofovir is administered at the recommended dose,[4,17)] our method successfully covers this region with good precision and accuracy. Actually, plasma tenofovir concentrations after oral administration of 300 mg to an HIV-1-infected patient were in this range.

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

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The Validation of Plasma Darunavir Concentrations Determined by the HPLC Method for Protease Inhibitors

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Darunavir (DRV) is a new protease inhibitor used to treat human immunodeficiency virus (HIV) type-1. The aim of this study was to validate the determination of plasma DRV concentrations using the HPLC method, a simple procedure for simultaneous determination of seven HIV protease inhibitors and efavirenz. The calibration curve was linear (range of 0.13 to 10.36 μg/ml). The average accuracy ranged from 100.7 to 105.6%. Both the interday and intraday coefficients of variation were less than 6.7%, which was similar to or much lower than previously reported values by the LC/MS/MS method. It is concluded that HPLC can be used to determine plasma DRV concentrations and routinely in the clinical setting; thus, this HPLC method enables further study of DRV pharmacokinetics in conventional hospital laboratories.

Key words darunavir; human immunodeficiency virus type-1; HPLC; therapeutic drug monitoring

Darunavir (DRV), a new protease inhibitor (PI), is used to treat human immunodeficiency virus (HIV) type-1. According to *in vitro* experiments, DRV was active against HIV-1 with PI resistance mutations and against PI resistant clinical isolates.[1—4] This drug is expected to be effective in antiretroviral treatment-experienced patients, such as those possessing HIV-1 strains which are resistant to more than one PI.[5—8]

Bouche *et al.* recently determined plasma DRV concentrations using liquid chromatography-tandem mass spectrometry (LC/MS/MS).[9] However, as LC/MS/MS equipment is very expensive and unavailable in conventional hospital laboratories, development of alternate methods is necessary.

We have already developed a simple HPLC method for simultaneous quantitative determination of seven HIV protease inhibitors and efavirenz.[10] We expect DRV can be measured using this method because amprenavir, whose chemical structure is quite similar to DRV, was successfully measured.

In this study we aimed to validate the measurement of plasma DRV concentrations using the HPLC method. This is the first report where plasma DRV concentration has been measured using this HPLC method.

MATERIALS AND METHODS

Standard Solutions and Chemicals DRV was supplied by Tibotec Pharmaceuticals Ltd. (Eastgate Village, Eastgate, Little Island, Co Cork, Ireland). The internal standard (IS), 6,7-dimethyl-2,3-di(2-pyridyl)-quinoxaline, was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Stock solutions of DRV and IS were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 259 μg/ml for DRV, and 588 μg/ml for IS. These stock solutions were stored at −80 °C and thawed until the day of analysis. The stock solution was diluted in drug-free plasma to yield concentrations of 0.13, 1.30, 2.59, 5.18 and 10.36 μg/ml for DRV. All other chemicals and solvents were of analytical grade and have been described in our previous report.[10]

Chromatography The HPLC system consisted of a Waters pump (model 515), a 717 plus autosampler, and a 2487 dual λ absorbance detector coupled to the Empower™ software (Waters, Milford MA, U.S.A.). The analytical column was a Radial-Pak Nova-Pak C_{18} column (4 μm, 8×100 mm, Waters) protected by Guard-Pak Inserts Nova-Pak C_{18} precolumn. Absorbance was measured at 205 nm and separations were performed at 30 °C. The mobile phase consisted of 39% 50 mM phosphate buffer (pH 5.9), 22% methanol and 39% acetonitrile. The assay run time was 30 min with a flow rate of 1.8 ml/min. Drugs were quantified by measuring the peak areas under the chromatograms. The other equipment and methodology used in this study have been described in our previous report.[10]

Sample Preparation Two milliliters of ethyl acetate/*n*-hexane (50 : 50, v/v) containing the IS (3.55 μg/ml) and 1 ml of 0.5 M sodium carbonate were added to a 500 μl plasma sample. The mixture was vortexed and then centrifuged at 3500×*g* for 5 min. The organic layer was separated and evaporated dry. The dried material was then dissolved in 100 μl of a mobile phase solution and centrifuged at 13000×*g* for 5 min. Lastly, 25 μl of the upper solution was injected into the HPLC column.

The institutional review board of National Hospital Organization Nagoya Medical Center approved this study and plasma samples were prepared from patients after obtaining written informed consent.

Validation Intraday and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of DRV five times on the same day and on three separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of the nominal concentration. Drug recovery from plasma was evaluated by analyzing triplicate samples with or without extraction.

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RESULTS

Chromatograms of Plasma Sample Figure 1A shows the chromatogram of a spiked plasma sample containing 5.18 μg/ml of DRV. Under the described chromatographic conditions, retention times were 3.8 min for DRV and 5.0 min for IS. Because the chemical structure of DRV is closely related to amprenavir (Fig. 1A), DRV retention time was similar to that of amprenavir in our previous report.[10] At a detection wavelength of 205 nm, the assay performed on drug-free human plasma succeeded to show no interfering peaks during the interested retention time intervals (Fig. 1B).

Figure 2A shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with atazanavir, ritonavir, abacavir and lamivudine. Figure 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with lopinavir, ritonavir, tenofovir and zidovudine. There are no interfering peaks affecting on the quantification of DRV in these chromatograms.

Validation: Linearity, Precision, Accuracy and Recovery Calibration curve of DRV appeared linear in the concentration range of 0.13 to 10.36 μg/ml with a correlation of 1.000.

Precision, accuracy and recovery for DRV are shown in Table 1. The selected concentration of DRV covers the expected plasma concentrations found in patients. The CVs calculated for DRV in the inter- and intraday assays ranged from

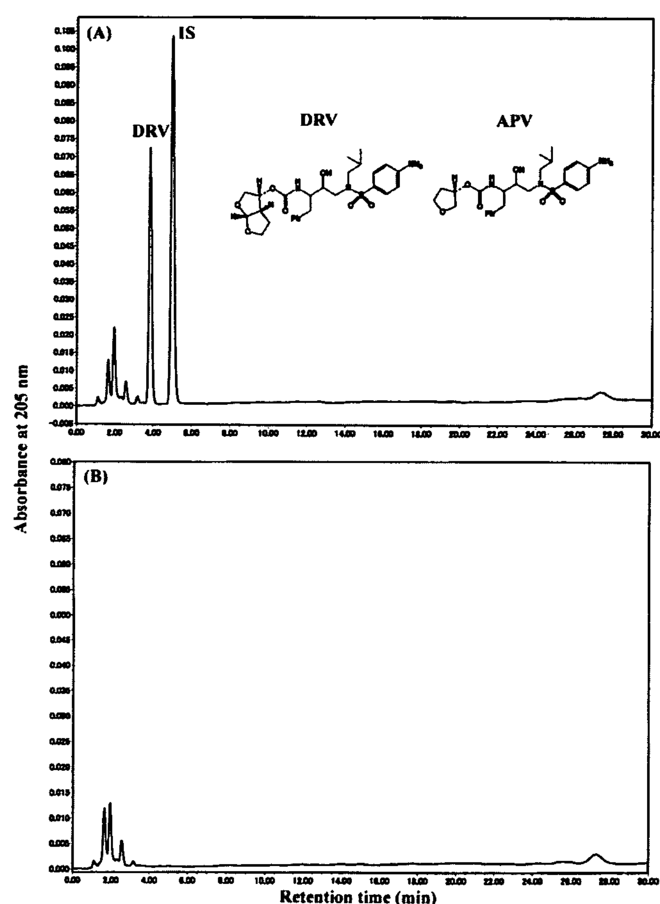

Fig. 1. Chromatograms Obtained after Extraction of (A) Spiked Plasma Sample Containing 5.18 μg/ml of DRV and (B) Drug-Free Human Plasma Sample from a Healthy Volunteer

DRV, darunavir; APV, amprenavir; IS, internal standard.

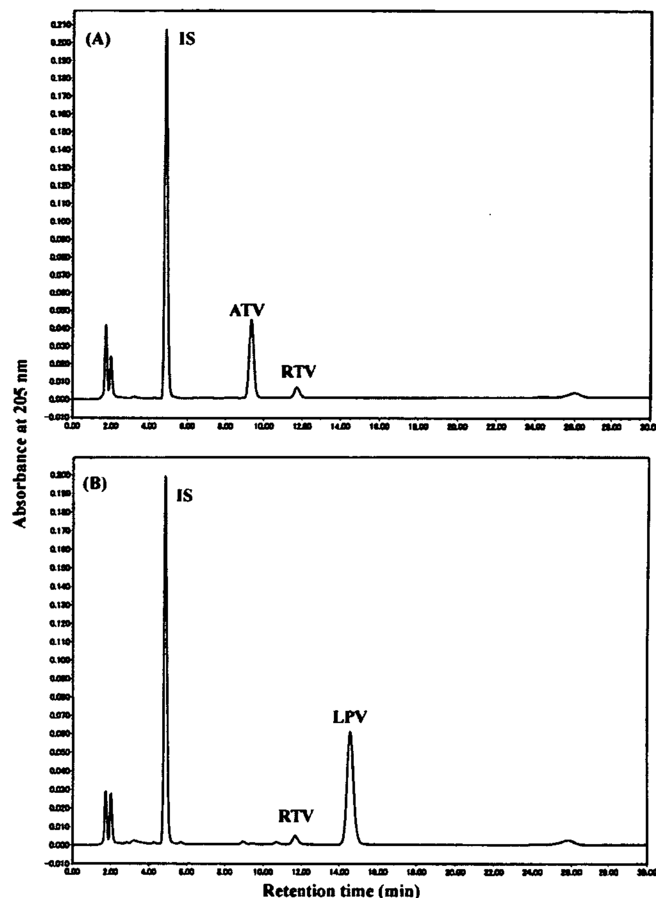

Fig. 2. Chromatograms Obtained after Extraction of a Plasma Sample from an HIV-1-Infected Patient (A) Treated with Atazanavir, Abacavir, Lamivudine and Ritonavir, and (B) Treated with Lopinavir, Ritonavir, Tenofovir and Zidovudine

IS, internal standard; ATV, atazanavir; RTV, ritonavir; LPV, lopinavir.

Table 1. Intraday and Interday Precision, Accuracy and Recovery for Darunavir

Expected (μg/ml)	Intraday (n=5)		Interday (n=15)		Accuracy (%)	Recovery (%)
	Measured (μg/ml)	CV (%)	Measured (μg/ml)	CV (%)		
0.13	0.13±0.01	6.7	0.14±0.01	4.3	105.6±4.6	90.7±3.6
1.30	1.36±0.08	6.2	1.35±0.07	4.9	103.5±5.0	88.0±4.6
2.59	2.67±0.12	4.5	2.62±0.08	3.1	101.1±3.2	95.8±2.2
5.18	5.18±0.10	2.0	5.22±0.28	5.4	100.8±5.5	88.9±7.0
10.36	10.41±0.13	1.3	10.43±0.15	1.4	100.7±1.4	100.9±2.1

CV, coefficient of variation.

1.3 to 6.7%, which were similar to or much lower than previously reported values.[11,12] Accuracies ranged from 100.7 to 105.6%. Recoveries from plasma ranged from 88.0 to 100.9%. Mean extraction recovery of the IS was 80.4%.

DISCUSSION

In this study we demonstrated the determination of plasma DRV concentrations was possible using the HPLC method previously established in our laboratory for simultaneous detection of seven PIs. We validated the determination method using a concentration range between 0.13 and 10.36 μg/ml. The resulting HPLC method achieved reproducibility and accuracy for DRV detection.

Because the chemical structure of DRV is closely related to amprenavir, DRV retention time was similar to that of amprenavir in this study. DRV is expected to use in "salvage" therapy because this drug possesses low cross-resistance rates in patients who failed to respond to treatment with other PIs containing amprenavir. In another word, co-administration of DRV and amprenavir is not generally expected. Therefore, the similar retention times do not cause any problems in a practical use of the HPLC system.

The plasma DRV concentration was expected in the 2.8 to 5.8 μg/ml range when DRV was administered at the recommended dose with low-dose ritonavir.[11] Our HPLC method successfully covered this region with both precision and accuracy.

In conclusion, our HPLC method can be routinely applied in the clinical setting and enables the study of DRV pharmacokinetics in conventional hospital laboratories.

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原著

リアルタイム PCR 法による *Pneumocystis jirovecii* 迅速定量法の確立

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Diagnostic utility of real-time PCR assay of *Pneumocystis jirovecii*

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Summary

Pneumocystis jirovecii formerly designated as *Pneumocystis carinii f. sp. Hominis* causes pneumonia in immunocompromised patients leading to fatal outcomes in some cases. Pneumocystis pneumonia (PCP) is well-known as AIDS defining disease constituting about percent of AIDS cases. Typical PCP cases are readily diagnosed by conventional staining methods such as Grocott's methenamine silver staining or immunofluorescence staining. However, less severe cases of PCP which harbor relatively small numbers of the pathogens escape detections by means of these microscopic enumerations of the microbes at times. Usefulness of qualitative PCR for detection of this atypical fungal pathogen is well documented. On the other hand, it is reported that immunocompromised patient with apparent absence of PCP or immunocompetent subjects with chronic pulmonary diseases are colonized with this pathogen compromising its specificity as diagnostic utility. Staining methods require expertise ; therefore unskilled technicians tend to fail spotting the pathogens which is not the case with PCR. Various species of Pneumocystis organism are known with each species being host specific. Detection of *Pneumocystis jirovecii* by PCR has been shown valuable and real-time PCR assay for *Pneumocystis carinii f. sp. carinii* has been developed to facilitate animal model experiments. Using real-time PCR, we successfully performed qualitative measurement of DNA for *Pneumocystis jirovecii* in the respiratory samples providing a rapid and qualitative method without constraints of individual variation in order to detect of the human fungal pathogen based on a multiple major surface glycoprotein gene family.

キーワード
リアルタイム PCR 法　HIV　日和見感染　ニューモシスチス肺炎　経時的定量法

以前までニューモシスチス肺炎の病原体は *Pneumocystis carinii* (*P. carinii*) と呼ばれ，この病原体が引き起こす肺炎はカリニ肺炎と総称されていた．しかし，最近になって遺伝子解析によりヒト寄生型と，ヒト以外のサルやマウスなどの動物寄生型に区別されてきている．ヒトに寄生する菌名は *Pneumocystis jirovecii* (*P. jirovecii*)，ヒト以外の動物に寄生する菌名は *P. carinii* と改名された．また，分類も以前までは治療薬や生活史などから原虫類とされていたが，遺伝子塩基配列や細胞壁の構造が真菌に近いことが明らかになり，最近になって真菌に再分類されたが，人工培養ができないなど未だに不明な点が多い菌種である[1~4)]．

P. jirovecii は acquired immunodeficiency syndrome (AIDS) における日和見感染の代表的な病原体で重篤なニューモシスチス肺炎を発症させる．実際，ニューモシスチス肺炎は AIDS 拠点病院である当院においても AIDS 死因の第1位であり，今後 AIDS

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患者の増加に伴い，臨床の現場においてその対策の重要性がさらに高まってくると予測される．従来のニューモシスチス肺炎の確定診断は患者呼吸器材料から病原体を証明することであり，グロコット染色，トルイジンブルーO染色，ギムザ染色，蛍光抗体染色等の特殊染色にて顕微鏡法で肉眼的に病原体を検出することであった[1,5,6]．しかし，顕微鏡による病原体の証明は検体中に菌量が多い場合では有効だが，菌量が少ない材料では検出に限界がある．特にギムザ染色は特異的な染色法ではないため，かなり経験を積まないと容易に見落としてしまう可能性がある[5]．また，われわれ検査技師は検査科内配置替えがあり担当技師の異動がある．熟練者が異動した後，次の担当者が習熟するまでには相当の時間を要するため，その期間，診断率が低下することが十分に考えられる．加えて，顕微鏡による病原体の証明は感度が低いため検査者によって結果が異なることがあり，診断性に欠ける検査法である．そこで，確実にニューモシスチス肺炎を診断できる検査法の開発が待たれている．

I 目　的

顕微鏡による病原体の検出法は感度が低いため，近年，感度の高いPCR法が利用されてきている[5,6]．しかし，従来のPCR法は定性的判定であり，HIV感染患者ではニューモシスチス肺炎と考えにくい症例においても陽性となる場合がある[6]．それは従来のPCR法ではその病原体量を測定できないため，*P. jirovecii*が活動状態(感染症の発症)であるか否かを明確にすることは困難であった．そこで，筆者らは*P. jirovecii*量を定量化することにより臨床状態の鑑別化を試みてきた．本研究ではmajor surface glycoprotein(MSG)遺伝子を定量領域に用いたリアルタイムPCR法による*P. jirovecii*迅速定量法を確立し，迅速確定診断法を開発することを目的とした．また，重篤なニューモシスチス肺炎では標準的な21日間の治療では改善しない症例もあり，治療薬による副作用を考慮しつつ，いつまで治療をすべきかの指標が重要になる．このような場合には*P. jirovecii* MSGコピー数のモニターが治療上有効と考えられ，経時的に用いる定量リアルタイムPCR診断法の臨床的有用性についても報告する．

II 材料・方法および対象

1. 材料・方法

1) 材料からのDNA抽出

ニューモシスチス肺炎が疑われた患者から得た呼吸器材料を喀痰溶解剤で約30分間室温にて攪拌し完全に溶解した．ここで，溶解不十分の場合は溶解時間を延長し，完全に粘性を溶解することが重要である[5]．溶解後，沈査をphosphate buffed saline(PBS)で2回洗浄し，洗浄後，沈査を1,670×gで15分間遠心し，沈査を回収した．その沈査200 μlからDNA抽出を行った．筆者らは市販されている商業キット(QIAamp DNA Mini Kit：キアゲン)を用いた．抽出したDNAは総量50 μlになるように蒸留水で溶解した．

2) 標準物質の作成

ニューモシスチス肺炎確定患者(蛍光染色法強陽性)の気管支洗浄液から，DNAを抽出し，PCRにより*P. jirovecii* MSG遺伝子領域(1024 bp)を増幅した．PCR反応液の組成は500 nM F-Pc-Stdプライマー(フォワード，5'-CTT CGA GGA AAG CTG AGT GA-3')，500 nM R-Pc-Stdプライマー(リバース，5'-ACT CTT TCC TGT CCT ACC CT-3')，1×LA Taq Buffer，各2.5 mM dNTP，1 U LA Taq，2.5 mM $MgCl_2$，蒸留水，サンプルDNA溶液で全量は50 μlに調整した．温度条件はまず96℃で1分間加温し，95℃1分：65℃1分：72℃1分を42サイクル行い，最終伸長反応を72℃で10分間行った．その後，大腸菌用クローニングベクターであるpUC 118へ組み込んで環状DNAを作製し，それを標準DNA物質(スタンダード)とした．

3) リアルタイムPCRのプライマーおよびプローブ

プライマーはHuangらが*P. jirovecii*特異性が高いと報告したMSGプライマー(JKK 14, 15, 17)を参考にし[7~10]，新たに設計した．各プライマーはF-PC-ogaプライマー(フォワード，5'-TGG GTT ACA CAG ACA TCG ACA CA-3')，R-PC-ogaプライマー(リバース，5'-TGC ACC GCC TCG TTG AT-3')を用いた．プローブは汎用度が高く，標的配列の特異的検出に優れたTaqManプローブを用いた．TaqMan

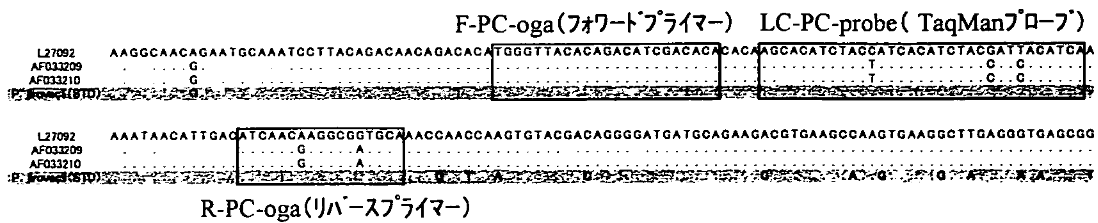

図1　プライマー・プローブ設定箇所

プライマーは，*P. jirovecii* 特異性が高いMSGプライマーを参考にし新たに設計した．マザーシーケンスとして多用されているL 27092，AF 033209，AF 033210と比べ比較的変異の少ない箇所にTaqManプローブを設定した．

プローブ(LC-PC-probe：5′-AGC ACA TCT ACC ATC ACA TCT ACG ATT ACA TCA-3′)はマザーシーケンスとして多用されているL 27092, AF 033209, AF 033210と比較し，変異が比較的少ない箇所に独自に設定した(図1).

4) リアルタイムPCR

リアルタイムPCRの基本的条件はわれわれが以前に報告した条件を用いた[11,12]．調整したスタンダードの希釈系列を作製し，定量の標準物質とした．反応液の組成は1×LightCycler-FastStart酵素液(ロシュ)，400 nM TaqManプローブ，500 nMフォワードプライマー，500 nMリバースプライマー，4 mM $MgCl_2$，サンプルDNA溶液は2 μlを使用し，全量は20 μlに調整した．DNAの増幅・定量にはLight Cycler(ロシュ)を用いた．温度条件は，まず95℃で10分間予備加温し，その後，95℃10秒間：60℃30秒間を45サイクル行った．60℃の伸長反応時にTaqManプローブが分解されることにより発生する蛍光シグナルをリアルタイムに検出していき，最後に40℃で30秒間冷却した．全行程は約1時間であった(図2).

反応液組成	容量	終濃度
DNA溶液（サンプル）	2 μl	
10pmol/μl フォワードプライマー	1 μl	500nM
10pmol/μl リバースプライマー	1 μl	500nM
4pmol/μl TaqMan プローブ	2 μl	400nM
10×Buffer 酵素液	2 μl	1×
25mM $MgCl_2$	3.2 μl	4mM
PCR グレード滅菌処理水	8.8 μl	
計	20 μl	

PCR条件
- 95℃：10分
- 95℃：10秒 ↔ 60℃：30秒（45サイクル）
- 40℃：30秒

計：55分

図2　リアルタイムPCRの反応液組成とPCR条件

2. 対　象

肺炎が疑われたHIV感染患者14例，基礎疾患を持ち日和見感染肺炎が疑われた非HIV感染患者13例，および比較対照として健常者(当院職員)3例の計30例の各呼吸器材料(喀痰，気管支洗浄液，口腔うがい液，気管支内チューブ)からDNAを抽出し，リアルタイムPCR法により *P. jirovecii* のMSG遺伝子を増幅・定量した．同時に蛍光染色法での *P. jirovecii* の検出も行った(表1)．また，1例のHIV感染患者についてはニューモシスチス肺炎発症から終焉までの約2か月間経時的に喀痰を材料に用いてリアルタイムPCR法による定量測定を実施した．

Ⅲ 結　果

1. 検量線

図3に今回得られた蛍光強度曲線と検量線を示した．蛍光強度曲線のグラフは縦軸が蛍光強度，横軸がサイクル数を示している．コピー数の高い順に蛍光強度曲線が早く立ち上がり，10^1～10^{10}コピーの範囲で良好な検量線を得ることができた．濃度既知の標準DNA物質の希釈系列を用いて作製した検量線(Crossing Point vs log濃度)を利用し，サンプルのCrossing Point(CP)からコピー数を算出する．ここでのCPとは各検体を用いて測定した際に一定の蛍光強度を発するまでにかかったPCRサイクル数である．

表1　対象症例

症例	材料	HIV 感染	蛍光染色法陽性	背景
1	気管支洗浄液	●	○	HIV　ニューモシスチス肺炎疑い
2	気管支洗浄液	●	○	HIV　ニューモシスチス肺炎疑い
3	喀痰	●	○	HIV　ニューモシスチス肺炎疑い
4	喀痰	●		HIV　ニューモシスチス肺炎疑い
5	喀痰	●		HIV　ニューモシスチス肺炎疑いなし
6	喀痰	●	○	HIV　ニューモシスチス肺炎疑い
7	喀痰	●		HIV　ニューモシスチス肺炎疑いなし
8	喀痰	●		HIV　ニューモシスチス肺炎疑いなし
9	喀痰	●		HIV　ニューモシスチス肺炎疑いなし
10	喀痰	●		HIV　ニューモシスチス肺炎疑い
11	喀痰	●		HIV　ニューモシスチス肺炎疑いなし
12	喀痰	●		HIV　ニューモシスチス肺炎疑い
13	喀痰	●		HIV　ニューモシスチス肺炎疑い
14	喀痰	●	○	HIV　ニューモシスチス肺炎疑い
15	喀痰		○	糖尿病，肺線維症，間質性肺炎
16	気管支洗浄液			血管腫，ステロイド長期使用　間質性肺炎
17	気管支洗浄液			悪性リンパ腫，間質性肺炎
18	気管支洗浄液		○	肺癌，糖尿病
19	気管支洗浄液			全身性エリテマトーデス
20	気管支洗浄液			前立腺癌，重症肺炎
21	喀痰		○	成人T細胞性白血病，骨髄移植
22	気管支洗浄液			悪性リンパ腫
23	うがい液			全身性エリテマトーデス：長期ステロイド投与
24	気管支内チューブ			急性リンパ性白血病
25	気管支洗浄液		○	強皮症，血管炎：長期ステロイド投与
26	気管支洗浄液			糖尿病，間質性肺炎(ニューモシスチス肺炎除外のため)
27	喀痰			糖尿病，間質性肺炎(ニューモシスチス肺炎除外のため)
28	喀痰			健常人(対照検体1)
29	喀痰			健常人(対照検体2)
30	喀痰			健常人(対照検体3)

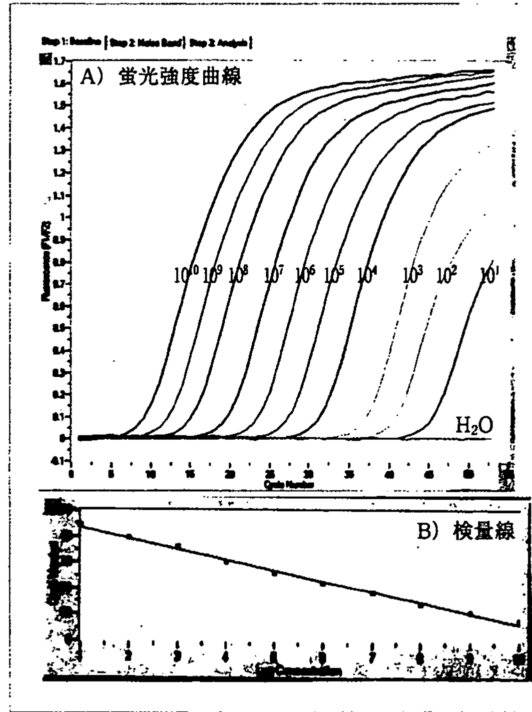

図3　蛍光強度曲線および検量線

A)増幅中の蛍光強度曲線：縦軸が蛍光強度，横軸がサイクル数である．コピー数の高い順に蛍光強度が上昇し始め，陰性コントロール(H_2O)では50サイクルを超えても蛍光強度は上昇しなかった．
B)検量線：10^1～10^{10}コピーの範囲で良好な検量線が得られた．

表2　定量再現性

n=15	STD 100,000,000	STD 10,000,000	STD 1,000,000	STD 100,000	STD 10,000	STD 1,000	STD 100	STD 10
1	98,130,000	9,034,000	960,300	95,370	10,330	961	92	19
2	107,100,000	9,493,000	958,000	104,200	10,720	1,039	113	13
3	97,420,000	9,442,000	1,017,000	94,160	10,110	891	95	15
4	99,970,000	9,275,000	927,500	96,690	9,669	967	103	18
5	100,500,000	9,269,000	1,018,000	103,800	9,715	928	108	16
6	106,700,000	10,420,000	1,145,000	97,280	9,858	936	109	15
7	108,700,000	8,871,000	1,069,000	101,900	10,160	945	111	13
8	111,700,000	9,544,000	977,200	98,990	9,455	1,006	110	9
9	105,400,000	10,020,000	1,036,000	109,100	10,180	962	105	8
10	111,900,000	9,397,000	1,031,000	102,700	9,499	943	94	11
11	125,800,000	9,911,000	1,010,000	100,000	9,025	938	89	11
12	115,550,000	10,070,000	1,036,000	98,090	11,900	1,054	95	19
13	124,500,000	9,468,000	934,600	92,300	9,055	932	116	12
14	130,300,000	9,522,000	965,800	97,130	8,932	959	103	12
15	107,900,000	8,795,000	1,045,000	101,500	8,942	919	107	15
Mean	110,104,667	9,502,067	1,008,693	99,547	9,837	959	103	14
SD	10161842	450542	57013	4400	791	44	8	3
CV(%)	9.2	4.7	5.6	4.4	8.0	4.6	7.8	21.4
Accueacy(%)	110	95	101	100	98	96	103	140

STD：標準 DNA 物質，SD：標準偏差，CV：変動係数．

2. 再現性

スタンダードを用いて再現性・正確性の検討(N=15)を行った結果を表2に示した．スタンダード10^8 MSGコピーではMean：110,104,667 MSGコピー(回収率110%)，10^7MSGコピーではMean：9,502,067 MSGコピー(95%)，10^6MSGコピーではMean：1,008,693 MSGコピー(101%)，10^5 MSGコピーではMean：99,547 MSGコピー(100%)，10^4 MSGコピーではMean：9,837 MSGコピー(98%)，10^3 MSGコピーでは：Mean 959 MSGコピー(96%)，10^2 MSGコピーではMean：103 MSGコピー(103%)，10^1 MSGコピーではMean：14 MSGコピー(140%)と良好な結果を得ることができ，十分な精度での定量が可能であった．変動係数(CV%)は4.4～21.4%であり，低濃度(10^1 MSGコピー)にて高い傾向を認め，10コピーが検出限界と思われた．

3. 症例および対照検体

表3，図4に示したように対象とした全30症例中，蛍光染色法陽性が8例(HIV感染4例，非HIV感染4例)，リアルタイムPCR法陽性が14例(HIV感染7例，非HIV感染7例)，蛍光染色法陰性でリアルタイムPCR法のみ陽性となった症例が6例(HIV感染3例，非HIV感染3例)であった．今回定量測定した臨床的に日和見感染肺炎が疑われた27例においてHIV感染患者5例と非HIV感染患者7例でニューモシスチス肺炎の確定診断を得ることができた．蛍光染色法で陽性の症例はすべてニューモシスチス肺炎の確定診断という結果であったが，病原体量が少ない傾向にある非HIV感染患者[6]では蛍光染色法陰性でリアルタイムPCR法のみで検出可能であった症例があり，低コピーでもリアルタイムPCR法で陽性であればほぼニューモシスチス肺炎と考えられる結果であった．染色法のみでの診断には限界があると思われた．

一方，HIV感染患者では臨床上，ニューモシスチス肺炎と考えにくい症例においても，従来の定性PCR法では陽性となる場合があり[6]，診断において鑑別が困難であったが，リアルタイムPCR法ではニューモシスチス肺炎を発症したHIV感染患者では病原体量が格段に多く高コピー(S 1，2，3，6，14：3.7×10^4～1.8×10^8コピー/μg DNA)となり，リアルタイムPCR法陽性でも低コピー(S 7，8：2.6×10^3，2.4×10^3コピー/μg DNA)は陰性と判定することができるようになった．対照として定量測定した健常者3例はともに感度以下であった(S 28～30)．

表3 測定結果

症例	蛍光染色法陽性	リアルタイムPCR法	MSGコピー/μg DNA	HIV感染	ニューモシスチス肺炎診断
1	○	○	2.3×10^7	●	◎
2	○	○	7.6×10^7	●	◎
3	○	○	1.2×10^5	●	◎
4		nd		●	
5		nd		●	
6		○	3.7×10^4	●	◎
7		○	2.6×10^3	●	
8		○	2.4×10^3	●	
9		nd		●	
10		nd		●	
11		nd		●	
12		nd		●	
13		nd		●	
14	○	○	1.8×10^8	●	◎
15	○	○	1.0×10^4		◎
16		nd			
17		○	2.0×10^3		◎
18	○	○	8.3×10		◎
19		○	3.8×10^3		◎
20		nd			
21	○	○	3.0×10		◎
22		○	3.2×10^3		◎
23		nd			
24		nd			
25	○	○	2.3×10^4		◎
26		nd			
27		nd			
28		nd			
29		nd			
30		nd			

nd：感度以下．

4. 経時的定量測定

治療マーカーとしてリアルタイムPCR法が有効であった症例を図5に示した．7月28日，1例のHIV感染患者が重症肺炎を発症，臨床所見よりニューモシスチス肺炎が疑われ喀痰を採取後ただちに定量リアルタイムPCR(day 1)を実施した．その結果1.4×10^4コピー/μg DNAと高コピーであったためニューモシスチス肺炎と確定診断，治療を開始した(day 1)．投薬後，肺炎症状は改善傾向に向かったが1か月後に肺炎症状が悪化した．細菌性肺炎を疑うもニューモシスチス肺炎の再発が否定できず，再び喀痰を採取し定量リアルタイムPCRを実施した．その結果，

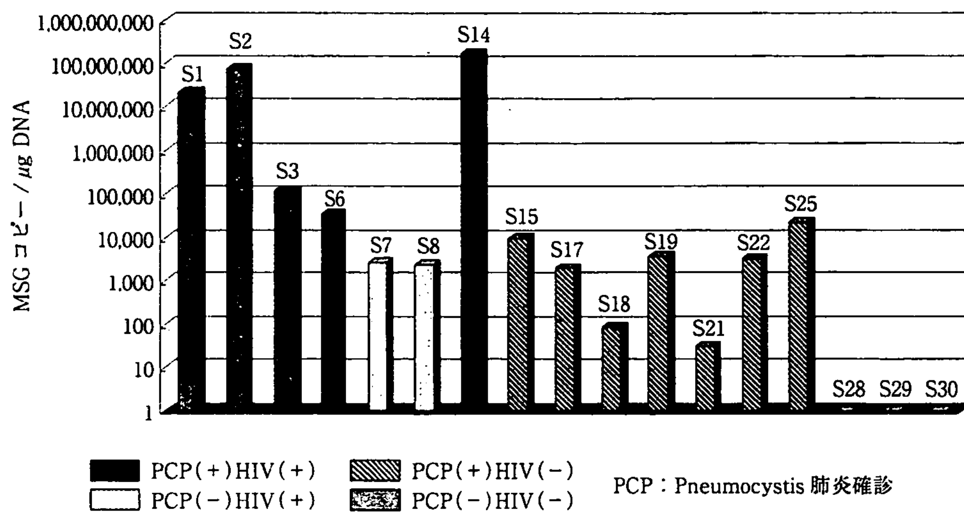

図4　リアルタイム PCR 法陽性症例の比較

HIV 感染患者5例(S 1, 2, 3, 6, 14)，非 HIV 感染患者7例(S 15, 17, 18, 19, 21, 22, 25)でニューモシスチス肺炎の確定診断が得られた．HIV 感染患者においては，ニューモシスチス肺炎を発症した場合，3.7×10^4～1.8×10^8 コピー/μg DNA と高値となり，一方，ニューモシスチス肺炎と考えにくい症例(S 7, 8)では 2.6×10^3, 2.4×10^3 コピー/μg DNA と比較的低値を示した．従来の定性 PCR 法で陽性を示し診断が困難であった HIV 感染患者では，リアルタイム PCR 法で定量することにより陽性であっても 10^4 コピー/μg DNA 以下はニューモシスチス肺炎を否定できると思われた．非 HIV 感染患者の場合ニューモシスチス肺炎発症例では 3.0×10^1～2.3×10^4 コピー/μg DNA と病原体量が少ない傾向にあり，リアルタイム PCR 法で陽性であれば，ニューモシスチス肺炎が発症したと考えられる結果であった．健常者3例(S 28, 29, 30)ではともに感度以下であった．

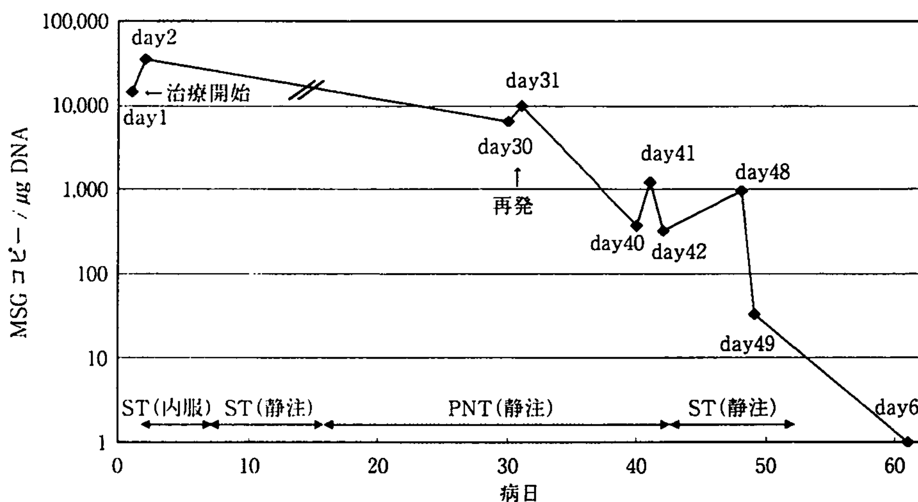

図5　経時的測定結果

1例の HIV 感染患者におけるニューモシスチス肺炎発症から終焉までの喀痰中の *P. jirovecii* MSG コピー数を経時的に定量し，その変動を示した．Day 1(14,000 コピー/μg DNA)後に治療を開始したが，day 2 ではコピー数の増加(36,000 コピー/μg DNA)を認めた．治療開始1か月後に再発(day 30, 31：6,300 コピー, 9,800 コピー/μg DNA)し，投薬の延長を行った．その後，肺炎症状の軽減とともにコピー数は減少傾向(Day 40, 41, 42, 48, 49：380, 1,200, 330, 960, 34 コピー/μg DNA)を示し，day 61 では感度以下となった．

ST：Sulfamethoxazole/Trimethoprim，PNT：Pentamidine.

9.8×10^3 コピー/μg DNAと高コピーであった(day 31)のでニューモシスチス肺炎の再発と診断し，投薬の延長を行った．その後，症状の改善とともに定量値も減少した．ニューモシスチス肺炎が終焉に向かうにつれて*P. jirovecii* 量の減少傾向が認められ，最終的には感度以下(day 61)となった．抗*P. jirovecii* 薬投与後(発症後)，経時的に喀痰を採取し，リアルタイムPCR法で定量することによって病原体量の消長を確認することが可能であった．

このことから，今回の症例のように重篤なニューモシスチス肺炎では標準的な21日間での治療では改善しない例もあり，臨床経過中に経時的に菌量を定量することによって再発，薬剤耐性菌発生の場合に薬剤の変更，投薬の延長，投与量の増加などの迅速対応が可能であると考えられた．

Ⅳ 考　察

本研究で確立した定量PCR法はDNA抽出後，約1時間で結果が得られる迅速性に優れた検査法であり，*P. jirovecii* MSGコピー数はニューモシスチス肺炎の成立の有無を判断するための重要なパラメーターとして有用であると思われる．経時的に病原体量を定量測定することによって，再発した場合の迅速診断への有効性が期待でき，薬剤耐性化など治療効果の判定の重要な指標になると思われる．

HIV感染症例ではニューモシスチス肺炎を発症していれば病原体量が多量に存在することが今回の研究で証明され，良質な喀痰からほぼ確実に診断できることが確認できた．肺胞内*P. jirovecii* 菌量が少ない傾向にある非HIV感染症例[6]では低コピーでもリアルタイムPCR法で陽性であればほぼニューモシスチス肺炎と考えられたが，その閾値設定を今後検討していく必要性がある．本研究により，HIV感染の有無によって層別化したうえで測定値を解釈することによりかなり確実にニューモシスチス肺炎を診断することができることがわかった．また，今回はHIV感染の有無に着目して研究を行ったが，各検体種別での結果の解釈とカットオフ値をどの値に設定するかは今後，国立病院機構政策医療共同研究施設を含め全国から検体を集めて症例数を増やすことにより解決できると筆者らは考えている．また，今回の研究では経験しなかったが，蛍光染色法で陽性かつリアルタイムPCR法で陰性(感度以下)となる場合が考えられ，そのような時はプライマーやプローブが検体に合っていない，PCR阻害物質の存在，DNA抽出時の操作ミス等も考慮する必要性があると思われた．

以上，筆者らが確立したリアルタイムPCR法による検査法がニューモシスチス肺炎において診断，治療に有効であることを述べてきた．今後，検体数を増やすことでさらに精度を増していくことが期待でき，診断を飛躍的に向上させていくことが可能かと思われる．

Ⅴ 今後の課題

重症例では患者侵襲の大きい生検，気管支洗浄などの採取が懸念され低侵襲的な喀痰に頼らざるをえない．しかし，喀痰が採れない患者や良質な喀痰が採取できない場合もあり，今後，口腔洗浄液，誘導痰を材料に用いた診断の可能性を検討する必要性がある[13]．非侵襲的方法での診断の可能性とその限界を知ることは適正な診断手順を確立するのに不可欠であると思われる．

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Letter to the Editor

Pharmacokinetic Parameters of Lopinavir Determined by Moment Analysis in Japanese HIV Type 1-Infected Patients

EDITOR: We determined pharmacokinetic (PK) parameters of lopinavir (LPV) in Japanese HIV-1-infected patients, as PK data for LPV have not yet been reported from Japanese multiple-dose studies. Subjects comprised 65 HIV-1-infected patients (57 men, 8 women) with a mean age of 38 years (range: 22–62 years) and a mean weight of 63.8 kg (range: 50.0–80.8 kg), recruited at the Outpatient HIV Clinic of the National Hospital Organization Nagoya Medical Center, Japan, between March 2002 and January 2007. Patients were treated for >1 month with LPV/ritonavir (400/100 mg twice daily: Kaletra capsule) in combination with other antiretroviral agents. The combination of coadministered nucleoside reverse transcriptase inhibitors was ZDV-3TC in 37 patients, 3TC-TDF in 8 patients, 3TC-d4T in 8 patients, 3TC-ABC in 5 patients, ABC-TDF in 3 patients, TDF-FTC in 2 patients, ddI-TDF in 1 patient, and ddI-ABC in 1 patient. The drug adherence of each patient was confirmed by interview and viral load (<50 copies/ml). Also patients had not been administered any drugs metabolized through the CYP3A pathway. The hepatic and renal functions of all patients were normal.

Plasma sampling was performed in the afternoon at an outpatient HIV clinic. All samples were prospectively obtained for the purpose of routine therapeutic drug monitoring. LPV plasma concentrations were determined by high-performance liquid chromatography according to our previously described methods.[1] We measured LPV plasma concentrations six times on average (range: 1–32 times) for recruited patients. The distribution of LPV plasma concentrations ($n = 419$) is shown in Fig. 1. PK parameters (mean ± SD) for LPV obtained using moment analysis were as follows: total area under the plasma concentration curve (AUC), 94.4 ± 8.6 μg · h/ml: mean residence time (MRT), 10.0 ± 1.2 h; elimination half-life ($t_{1/2}$), 5.1 ± 0.9 h; appearance oral clearance (CL/F, F = oral bioavailability), 4.23 ± 0.3 liters/h; and volume of distribution (V/F), 42.6 ± 1.6 l. AUC, $t_{1/2}$, CL/F, and V/F values obtained in this study were similar to those reported in American subjects.[2–5] We have previously reported that the $t_{1/2}$ of LPV tended to be prolonged after food intake in a single-dose study of healthy Japanese individuals,[6] but no such tendency was detected in this multidose study of Japanese HIV-1-infected patients. Our present data suggest that Japanese patients are similar to American patients in terms of PK findings for LPV at steady state. The recommended dose of LPV in the guideline[7] will thus also be suitable for Japanese HIV-1-infected patients.

The institutional review board of the National Hospital Organization Nagoya Medical Center approved this study and plasma samples were prepared from patients after obtaining written informed consent.

Moment analysis was performed using a PK analysis program developed by Tabata *et al.*[8] Details of the method were previously reported by Yamaoka *et al.*[9]

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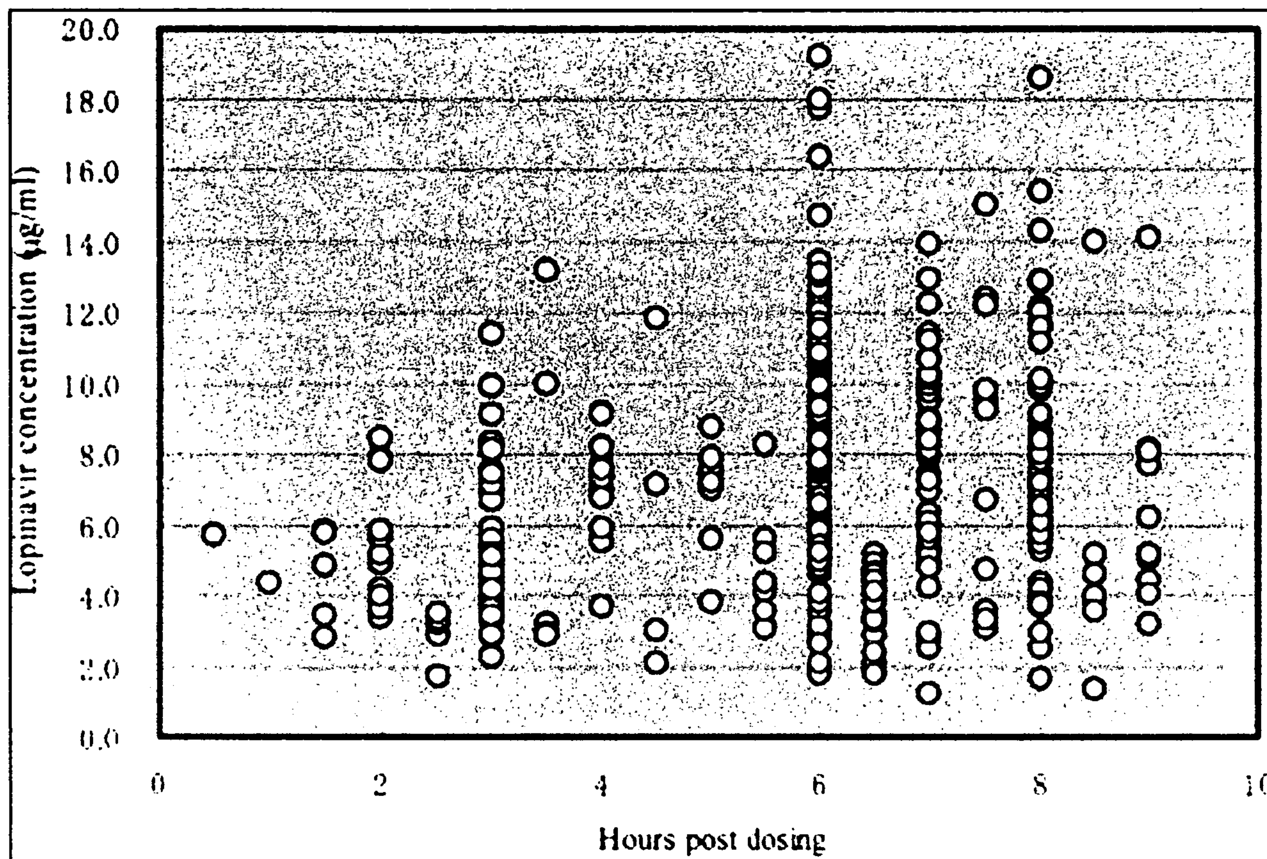

FIG. 1. Distribution of lopinavir plasma concentrations in 65 Japanese HIV-1-infected patients (n = 419). Measured concentrations are plotted versus hours postdosing.

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Trend of Drug-Resistant HIV Type 1 Emergence among Therapy-Naive Patients in Nagoya, Japan: An 8-Year Surveillance from 1999 to 2006

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ABSTRACT

We studied the emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) with major amino acid mutations in 402 therapy-naive patients at Nagoya Medical Center, Japan, between 1999 and 2006. The mean prevalence of drug-resistant HIV-1 was 6.7% (range, 2.3–10.0%; *n* = 27). HIV-1 variants with protease inhibitor (PI)-resistant mutations alone were most frequently found (3.5%, *n* = 14), followed by those with nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant mutations alone (1.7%, *n* = 7). Variants with nucleoside reverse transcriptase inhibitor (NRTI)-resistant mutations alone were sporadically found (1.0%, *n* = 4). A variant possessing both NRTI- and PI-resistant mutations was detected in one patient (0.2%) and a variant possessing both NNRTI- and PI-resistant mutations was identified in another patient (0.2%). In addition, another 17 variants (4.2%, *n* = 17) with only 215-revertant mutations (T215C/D/G/L/S) that can easily reconvert to the nucleoside analogue-associated mutation of T215Y/F were found. The 402 viruses were phylogenetically analyzed, revealing three independent clusters comprising PI-resistant variants with the M46I or L90M mutation, NNRTI-resistant variants with the K103N mutation, and 215-revertant variants. The PI-resistant and 215-revertant strains have been spreading since 2000, and the NNRTI-resistant strain has started spreading since 2003. The nature of the epidemic and information for successfully blocking the spread of drug-resistant HIV-1 were clarified in this study.

INTRODUCTION

Combination therapy with three or more antiretroviral drugs (highly active antiretroviral therapy, HAART) can strongly suppress the replication of human immunodeficiency virus type 1 (HIV-1) and maintain the amount of HIV-1 RNA in plasma (viral load) under detectable levels in many cases.[1–5] However, HIV-1 variants with decreased susceptibility to antiretroviral drugs are sometimes found under conditions in which the drug concentration is insufficient to suppress viral replication following poor adherence to treatment regimens.[4–9] Such variants might become an origin for HIV-1 transmission, resulting in the finding of drug-resistant HIV-1 in therapy-naive individuals. This represents a serious problem in therapy, as such variants hinder antiretroviral therapy from the first trial.[10–16] Determining whether therapy-naive patients are infected by drug-resistant HIV-1 before starting HAART is thus important. The present study studied emergence trends for drug-resistant HIV-1 with major mutations among therapy-naive patients in the Nagoya Medical Center, Japan, between 1999 and 2006. We also studied the emergence of HIV-1 with 215-revertant amino acid mutations in the reverse transcriptase (RT), as 215-revertant variants can easily change to nucleoside RT inhibitor (NRTI)-resistant variants.[17–20] The final aim of the study was to understand the epidemiological nature of drug-resistant variants and obtain information to successfully block their spread.

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MATERIALS AND METHODS

Patients

A total of 441 therapy-naive HIV-1-infected patients underwent their initial consultation at Nagoya Medical Center in Nagoya, Japan, between January 1999 and December 2006. Genotypic drug-resistance testing for HIV-1 was performed on 402 of the 441 patients (91%) after obtaining patient consent. The characteristics of the 402 patients are shown in Table 1.

Genotypic drug-resistance testing for HIV-1

Genotypic drug-resistance testing for HIV-1 was performed as previously reported.[21,22] HIV-1 RNA was purified from a plasma sample using a QIAamp viral RNA mini kit (QIAGEN, Tokyo, Japan). A single DNA fragment containing both protease (PR) and reverse transcriptase (RT) genes was amplified by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using the Superscript one-step RT-PCR for long templates kit (Invitrogen, Tokyo, Japan) and LA Taq polymerase (Takara, Shiga, Japan). A labeling reaction for DNA sequencing was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan), and DNA sequences were determined by the direct sequencing method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA sequences were converted to amino acid sequences, and then amino acid mutations were extracted through comparison with amino acid sequences of the HIV-1 HXB2 strain. Judgment of drug-resistant amino acid mutations was performed according to the latest version of the International AIDS Society USA panel, Fall 2006.[23]

TABLE 1. CHARACTERISTICS OF 402 THERAPY-NAÏVE HIV-1-INFECTED PATIENTS

Age, years		
Median (IQR[a])	33 (28–41)	
Sex		
Male	362	90.0%
Female	40	10.0%
Nationality		
Japanese	335	83.3%
Foreign	67	16.7%
Risk factor for infection		
Homosexual	237	59.0%
Heterosexual	87	21.6%
Bisexual	32	8.0%
Unknown	46	11.4%
CD4 cell count, cells/μl		
Median (IQR[a])	270 (94–400)	
Viral load, $\log_{10}$ copies/ml		
Median (IQR[a])	4.77 (4.26–5.26)	
HIV-1 subtype		
B	346	86.1%
Non-B[b]	56	13.9%

[a]IQR, interquartile range.
[b]CRF01_AE, 30; A, 9; C, 8; D, 4; F, 2; G, 2; unclassified, 1.

Phylogenetic analysis

Phylogenetic analysis was performed using the nucleotide sequences of HIV-1 obtained from all 402 therapy-naive patients. Nucleotide sequences (1005 bases) containing both PR (codons 1–99) and RT (codons 1–236) genes were used. Multiple sequence alignment was performed using CLUSTAL W, and evolutionary distances were calculated using the Kimura two-parameter model. A phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates. These analyses were performed using MEGA software version 3.1.[24] Nucleotide sequences of 32 reference HIV-1 strains were obtained from the HIV sequence database in the Los Alamos National Laboratory. Subtyping of HIV-1 was also performed using the phylogenetic tree.

Measurement of viral load and CD4 cell count

Viral load was measured using an Amplicor HIV-1 monitor v1.5 system (Roche Diagnostics, Tokyo, Japan). CD4 cell counts were measured using a FACSCalibur flow cytometry system (Becton Dickinson, Tokyo, Japan).

Statistics

Multiple logistic regression analysis was performed to assess associations between patient characteristics and infection with drug-resistant or 215-revertant HIV-1 variants. Values of $p < 0.05$ were considered statistically significant. Analyses were performed using SYSTAT version 10.2 software (SYSTAT Software, California, USA).

RESULTS

Emergence trend of drug-resistant HIV-1 in therapy-naive patients

The prevalence of drug-resistant HIV-1 fluctuated between 2.3% and 10.0% through the period from 1999 to 2006 (Fig. 1). The first wave was observed from 2001 to 2003, with prevalence increasing from a trough of 2.3% in 2001 and peaking at 10.0% in 2003. After that, the prevalence dropped to 4.2% in 2004, but increased again to reach 8.8% by 2006. The mean prevalence for the past 8 years was 6.7% (27/402).

Variants with NRTI-resistant mutations were sporadically found (Fig. 2A). Concerning nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant variants, none was found from 1999 to 2002 (Fig. 2B). However, two variants with the K103N mutation first emerged in 2003, and this type of variant was continuously detected thereafter. Variants with the V108I and P225H mutations first emerged in 2004 and 2006, respectively. Variants with protease inhibitor (PI)-resistant mutations appeared continuously from 2000 (Fig. 2C). The most abundant variant was that with the M46I mutation alone, found in a total of 12 cases (2000, $n = 1$; 2002, $n = 2$; 2003 and 2004, $n = 1$ each; 2005, $n = 2$; and 2006, $n = 5$). In contrast, variants with the L90M, L33F, or M46L mutation alone appeared once each in 2001, 2003, and 2006, respectively. A variant possessing

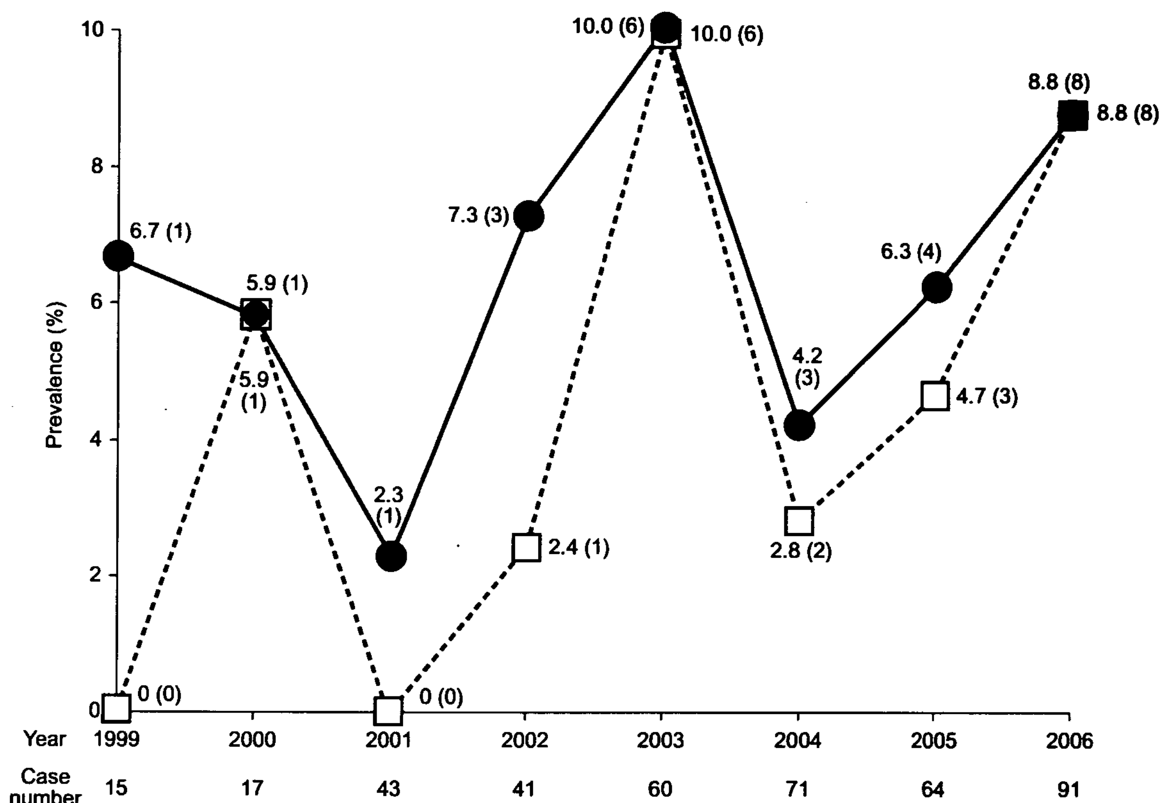

FIG. 1. Changes in prevalence of drug-resistant HIV-1 with major amino acid mutations (●—●) and 215-revertant variants (□—□) in therapy-naive patients. Genotypic drug-resistance testing was performed for 402 therapy-naive patients from 1999 to 2006. Detected numbers are shown in parentheses.

multiple mutations of V32I, M46I, I47V, and L90M was found very recently.

Characteristics of drug-resistant HIV-1

Characteristics of drug-resistant HIV-1 found in our surveillance are shown in Table 2. The most frequently found variant was a PI-resistant virus with the M46I mutation alone (n = 12), followed by an NNRTI-resistant virus with the K103N mutation alone (n = 4). Variants with two-class resistance were found in two cases, one possessing both PR- and NNRTI-resistant mutations, and the other with both PI- and NRTI-resistant mutations. Of note is the fact that no virus with resistance against all three classes was found in our surveillance.

Emergence trends for HIV-1 variants possessing the 215-revertant amino acid mutation in the reverse transcriptase

T215A/C/D/E/G/H/I/L/N/S/V amino acid substitutions in the RT represent revertant mutations of the T215Y/F NRTI-resistant mutation.[23] The 215-revertant mutations do not exhibit NRTI resistance by themselves, but most can reconvert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation. In other word, most 215-revertant variants can much more easily change to NRTI-resistant variants under the pressure of NRTIs than wild-type HIV-1.[17–20] We feel drug-resistant variants with the T215Y/F mutation are difficult to survive in the drug-free condition, as only one variant with the T215Y mutation has been found during an 8-year surveillance. The results of other researchers support our feelings.[17,18] Examination of the emergence of the 215-revertant variant in addition to the T215Y/F-possessing resistant variant is thus important. In our surveillance, variants possessing the T215A/C/D/E/G/L/S mutation were found in 21 cases; since T215G/D was found in 2000, such variants have been increasing (Fig. 2D). Among these, 17 cases (81%) can reconvert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation.

Phylogenetic analysis

This study identified 27 drug-resistant variants from 402 therapy-naive patients. We next performed phylogenetic analysis to clarify whether specific drug-resistant strains were spreading. Three different clusters were identified from 20 of 27 drug-resistant variants (#1–13, #14–18, and #19–20) on a phylogenetic tree (Fig. 3A). All the clusters were consisted of subtype B viruses. The remaining seven variants were dispersed over the tree (Fig. 3A, #21–27). Two out of the seven were non-B viruses, subtype D and CRF01_AE. Detailed divergence of